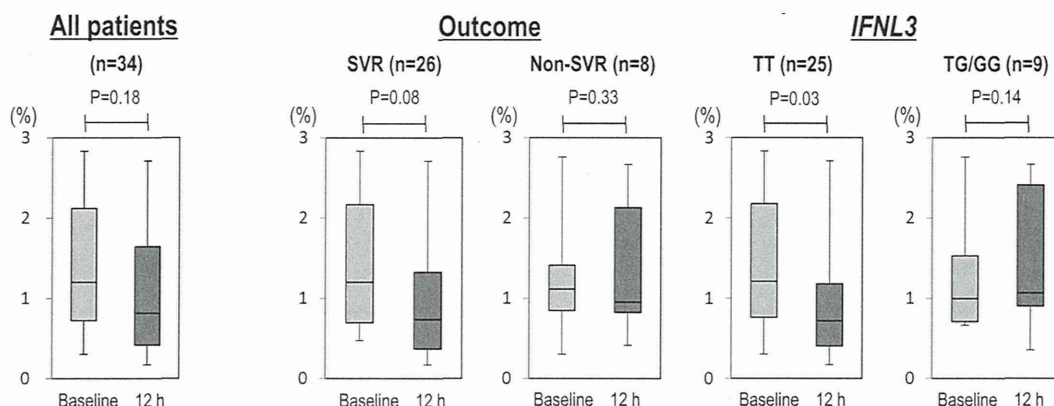

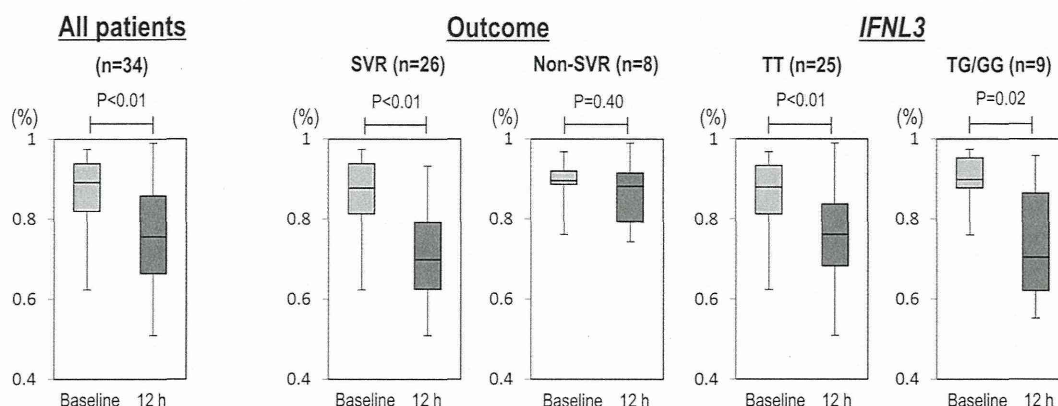

FIG 2 Changes in the genetic complexity of the NS3 region 12 h after the introduction of TVR/PEG-IFN/RBV triple therapy are shown for all patients, patients divided by treatment outcome, and patients divided by *IFNL3* SNPs. Changes were analyzed by mutation frequency (A) and by normalized Shannon entropy (B). Boxes represent the 25th to 75th percentiles, and horizontal lines within the boxes show the median values. Whisker ends show the minimum and maximum values of all data. *P* values were obtained using the Wilcoxon signed-rank test.

patient, the total number of nucleotides that differed from the consensus nucleotide was counted in each genome. Then that number was divided by the total number of genomes that were sequenced. The normalized Sn is the proportion of different genomes in a distribution of mutants, calculated as follows:

$$Sn = -\frac{\sum_{i=1}^{N} (Pi \times \ln Pi)}{\ln N}$$

in which *Pi* is the frequency of each sequence in the population and *N* is the total number of sequences analyzed.

Phylogenetic trees were constructed with BioEdit and MEGA6.05 using the neighbor-joining method, and bootstrapping was performed with 1,000 replicates (27). To calculate the genetic distance, the top 10 most frequent isolates at each time point were selected for each patient. By calculating all the genetic distances between any 2 isolates belonging to 2 different time points, the average genetic distance between 2 different time points was determined for each patient. Likewise, all of these average genetic distances were determined in the 8 non-SVR patients, and the distance data were subjected to statistical analysis.

Detection of amino acid substitutions in core and NS5A regions of HCV-1b. With the use of HCV-J (GenBank accession number D90208) as a reference, substitutions at amino acid (aa) 70 of arginine (70R) or glutamine (70Q) in the core of HCV-1b (28) were determined as described previously (29). Likewise, the sequence of aa 2,209 to 2,248 in the NS5A of

TABLE 2 HCV RNA titers at baseline and 12 h

Time after introduction of triple therapy	Comparison of HCV RNA titer (range) for SVR vs non-SVR			Comparison of HCV RNA titer (range) for *IFNL3* SNP		
	SVR (n = 26)	Non-SVR (n = 8)	*P* value	TT (n = 25)	TG/GG (n = 9)	*P* value
Baseline	6.4 (4.7–7.4)	6.9 (6.2–7.4)	0.08	6.7 (4.7–7.4)	6.7 (5.5–7.4)	0.74
12 h	4.6 (2.6–5.8)	5.2 (4.4–6.4)	0.06	4.6 (2.6–5.8)	5.1 (3.9–6.4)	0.41
Δ (baseline − 12 h)	1.7 (1.2–3.4)	1.7 (1.0–2.4)	0.54	1.7 (1.2–3.4)	1.7 (1.0–2.0)	0.30

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TABLE 3 Telaprevir-resistant variants responsible for treatment failure in non-SVR patients and their time-dependent changes

Patient no.	Outcome	Previous response	*IFNL3*	Resistant variant				% with variants at:		
				V36	T54	R155	A156	Baseline	Reelevation	Last observation (no. of wks after treatment)
1	Discontinuation	Relapse	T/G							
2	Discontinuation	Relapse	T/T							
3	Discontinuation	Naive	T/G		A			0.36	99.79	0.08 (26)
4	Breakthrough	No response	T/T				F	0	98.19	0 (45)
5	Relapse	Naive	T/G							
6	Relapse	No response	T/T				S	0	99.48	1.27 (47)
7	No response	Naive	T/G		A			0.28	99.84	0.04 (34)
8	No response	Naive	T/G	C				0	98.11	96.05 (25)

HCV-1b (IFN sensitivity-determining region [ISDR]) (30) was determined by direct sequencing, and the numbers of amino acid substitutions in ISDR were counted in comparison with HCV-J (29). Furthermore, the sequence of aa 2,334 to 2,379 in the NS5A of HCV-1b (IFN-RBV resistance-determining region [IRRDR]) (31) was determined by direct sequencing, after which the numbers of amino acid substitutions in IRRDR were counted in comparison with the consensus sequence constructed in the previous study (29). From those studies, it was reported that HCV-1b with the core aa 70Q with ISDR of ≥1 or IRRDR of ≥4 was IFN resistant, while that with core aa 70R and ISDR of ≤2 or IRRDR of ≤5 was considered IFN sensitive.

***IFNL3* SNP analysis.** Human genomic DNA was extracted from peripheral blood by using a blood DNA extraction kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. The allele typing of each DNA sample was performed by real-time PCR with a model 7500 sequencer (ABI, Tokyo, Japan) using a 6-carboxyfluorescein-labeled single nucleotide polymorphism (SNP) primer for the locus rs8099917 at *IFNL3* (interferon lambda 3) (ABI). For this analysis, TT was the major variant while TG and GG were minor variants.

Statistical analysis. Data on the patients' backgrounds are expressed as mean or median values with standard deviations. Statistical differences in the parameters between the 2 groups (SVR and non-SVR) were determined by the Mann-Whitney U test and Fisher's exact test. Time-dependent changes in genomic complexity and genetic distance were analyzed statistically with the Wilcoxon rank sum test. All *P* values of <0.05 (two-tailed test) were considered significant.

RESULTS

Clinical characteristics of patients receiving TVR/PEG-IFN/RBV triple therapy. Clinical backgrounds of 34 patients who received TVR/PEG-IFN/RBV triple therapy are reported separately for the SVR and non-SVR groups (Table 1). SVR was achieved in 26 patients (76.5%) and was not achieved in 8 (23.5%). The only

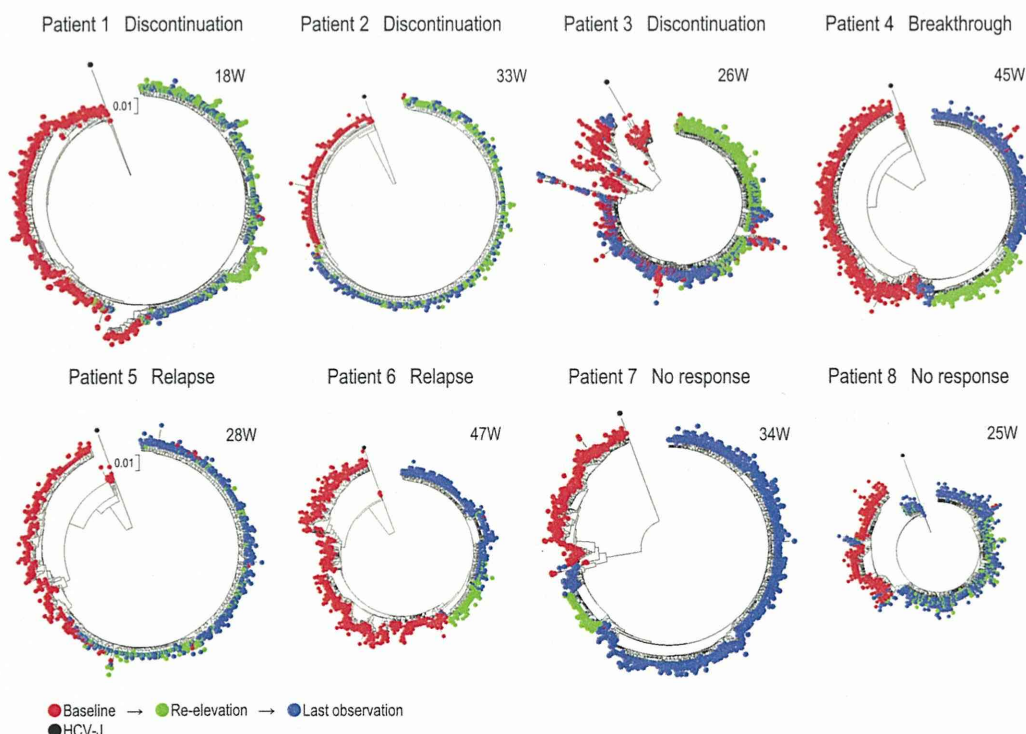

FIG 3 Phylogenetic trees were constructed using all isolates at baseline, at reelevation, and at the last observation. Numbers at the top right of each phylogenic tree indicate the number of weeks after the end of treatment.

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A Patient 1 Discontinuation

Nucleotide position	Rank	Read count	84	87	90	96	102	103	104	128	132	156	162	174	211	212	225	234	280	297	312	316	336	349	396	435	487	498	501	532	534
Reference			A	T	G	G	A	G	T	T	A	T	T	C	A	T	C	A	T	C	T	C	C	C	T	T	G	G	G	A	C
HCV-J			G	C	T	-	G	-	-	-	G	-	C	T	-	-	-	-	A	-	-	T	T	-	C	C	-	-	-	-	-
Baseline	1	360	-	C	-	-	-	-	-	-	G	-	-	-	-	-	-	G	-	-	-	-	T	-	-	C	-	-	A	-	-
	2	211	-	C	C	-	-	-	-	-	G	-	-	-	-	-	-	G	-	-	-	-	T	-	-	C	-	-	A	-	-
	3	80	-	C	-	-	-	-	-	-	G	-	-	-	-	-	-	G	-	-	-	-	T	-	-	C	-	-	A	-	T
	4	57	-	C	C	-	-	-	-	-	G	-	-	-	-	-	-	G	-	-	-	-	T	-	-	C	-	-	A	-	T
	◎5	47	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-
	6	41	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	G	-	-	-	T	T	-	-	C	-	-	-	-	-
	7	35	-	C	-	-	-	-	-	-	G	-	-	-	-	-	-	G	-	-	-	-	T	-	C	C	-	-	A	-	-
	8	30	-	C	-	-	-	-	-	-	G	-	-	-	-	-	-	G	-	-	-	-	T	-	-	C	-	-	-	-	-
	9	26	-	C	C	-	G	T	C	-	G	-	-	-	-	-	-	G	-	-	-	-	T	-	-	C	-	-	A	-	-
	10	26	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	G	-	-	-	-	T	-	-	C	-	-	-	-	-
12 h	◎1	164	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-
	2	97	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-
	3	53	-	C	-	-	-	-	-	-	G	-	-	-	-	-	-	G	-	-	-	-	T	-	-	C	-	-	A	-	-
	4	47	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	T
	5	42	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-
	6	39	-	-	-	-	-	-	-	-	G	C	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-
	7	37	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	G	-	-	-	-	T	-	-	C	-	-	A	-	-
	8	36	-	-	-	-	-	-	-	-	-	-	C	T	-	-	-	G	C	-	-	-	-	-	-	-	-	-	-	-	-
	9	31	-	C	C	-	-	-	-	-	G	-	-	-	-	-	-	G	-	-	-	-	T	-	-	C	-	-	A	-	-
	10	30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	T	-	-	-	-	-	-	-	-	-	-	-
Re-elevation (Post 4W)	◎1	1335	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2	360	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T
	3	164	-	-	-	-	-	-	-	G	G	-	-	-	-	-	-	G	-	-	C	-	-	-	-	-	-	-	-	-	-
	4	111	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-
	5	57	-	-	-	-	-	-	-	G	G	-	-	-	-	-	-	G	-	-	C	-	-	-	-	-	-	-	-	-	T
	6	54	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	7	28	G	C	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	8	27	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	T
	9	26	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T
	10	24	-	-	-	-	-	-	-	G	G	-	-	-	-	-	-	G	-	-	C	-	-	-	-	-	-	A	-	-	-
Last observation (Post 18W)	◎1	912	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2	386	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	3	87	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-
	4	39	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T
	5	20	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T
	6	18	-	-	-	A	-	-	-	-	-	-	-	-	-	-	T	G	-	T	-	-	-	-	-	-	-	-	-	-	-
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	8	15	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	9	13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-
	10	12	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

B Patient 2 Discontinuation

Nucleotide position	Rank	Read count	88	90	104	130	156	160	174	203	207	213	216	225	234	257	261	267	306	336	342	372	438	462	474	531	532	534
Reference			G	G	T	T	T	A	C	A	T	C	T	C	A	A	G	C	G	T	T	A	T	C	A	T	A	C
HCV-J			-	T	-	C	-	-	T	-	-	-	C	-	-	C	-	-	-	-	-	G	-	-	G	-	-	-
Baseline	1	799	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-
	2	79	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	T	-	C	-	-
	3	32	-	-	-	-	-	-	T	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-
	4	25	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	T	-	-	C	-	-	T	-	C	-	-
	5	24	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	C	-	-	-	-	-	C	-	-
	6	18	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	G	-	-	-	C	-	-
	7	18	-	-	-	-	-	-	-	-	-	T	C	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-
	8	17	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	C	-	-	C	-	-
	9	16	-	-	-	C	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-
	10	16	-	-	-	-	-	-	-	-	C	-	C	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-
	◎78	4	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12 h	◎1	2150	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-
	2	386	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	C	-	T
	3	332	-	C	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-
	4	238	-	-	-	-	-	-	-	-	C	-	C	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-
	5	217	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	G	C	-	-
	6	168	-	-	-	-	C	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-
	7	62	-	-	-	-	-	-	-	C	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-
	8	55	-	C	-	-	-	-	-	-	C	-	C	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-
	9	47	-	C	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	C	-	T
	10	43	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	G	-	-	-	C	-	-
Re-elevation (Post 20W)	◎1	1376	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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	3	46	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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	5	23	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	6	20	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	7	19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	G
	8	15	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	9	14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-
	10	10	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T
Last observation (Post 33W)	◎1	1465	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2	651	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	3	56	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	4	47	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-
	5	41	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	6	38	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	G
	7	37	-	C	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-
	8	34	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	9	26	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-
	10	25	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-

FIG 4 Time-dependent changes in the top 10 most populated isolates at baseline, 12 h, reelevation, and last observation in each patient. To characterize viral sequences specific for each time point, only nucleotide positions with time-dependent nucleotide changes are shown. The most frequent isolate at reelevation was used as the reference sequence. Nucleotide positions in the figure indicate the nucleotide positions from the start nucleotide in genotype 1b NS3. An isolate closest to the most frequent isolate found at reelevation is indicated by the double ring for each time point. In patients with the appearance of resistance mutations, time courses in the changes of hot spot nucleotides and resultant changes in amino acids are demonstrated at the right (C, D, F, G, and H).

factor that differed between the SVR and non-SVR groups was an *IFNL3* SNP. The *IFNL3* TG/GG SNP was observed in 15.4% of the SVR group patients, which was significantly lower than that in the non-SVR group (62.5%).

Early changes in genetic complexity after introduction of triple therapy. 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) to examine early changes in the genetic complexity of viral quasispecies (Mf and Sn) after introduction of triple therapy (12 h) (Fig. 1). In terms of Mf, there was no apparent difference in genetic complexity between baseline and 12 h after introduction of the therapy in the 34 patients as a whole ($P = 0.18$) (Fig. 2A). However, when the patients were categorized into 2 groups according to treatment response, genetic complexity tended to decrease in the SVR group but not in the non-SVR group ($P = 0.08$ and $P = 0.33$, respectively). Furthermore, when the change in genetic complexity was compared according to the *IFNL3* SNP, it was significantly decreased in the *IFNL3* TT group but not in the *IFNL3* TG/GG group ($P = 0.03$ and $P = 0.14$, respectively).

Similarly, changes in genetic complexity 12 h after the introduction of therapy were examined in terms of Sn (Fig. 2B). In this analysis, genetic complexity was significantly decreased 12 h after the introduction of therapy in the 34 patients as a whole ($P < 0.01$). In comparisons of treatment response, genetic complexity was significantly decreased in the SVR group but not in the non-SVR group ($P < 0.01$ and $P = 0.4$, respectively). In addition, with regard to the *IFNL3* SNP, genetic complexity was significantly decreased in both the *IFNL3* TT and TG/GG groups, with the phenomenon being more remarkable in the TT group than in the TG/GG group ($P < 0.01$ and $P = 0.02$, respectively).

Technically, comparison of viral genomic complexity in different samples should be done carefully when a PCR procedure is used and when viral titers are significantly different: a sample with a low viral titer with small amounts of PCR template might be erroneously interpreted as having lower genomic complexity (32). In this sense, it is possible that the decreased viral titer at 12 h might have influenced the Sn and Mf values. However, since the HCV RNA titers at each time point (at baseline and at 12 h) were not significantly different between SVR and non-SVR or between *IFNL3* TT and TG/GG (Table 2), it is evident that patterns of changes in viral complexity are different between SVR versus non-SVR and between *IFNL3* TT versus TG/GG.

Clinical courses in non-SVR patients. Next, changes in the genetic composition of viral populations (quasispecies) as well as the origin of TVR-resistant mutations of HCV were further analyzed with a particular focus on the 8 non-SVR patients. The causes of non-SVR were defined as follows: termination of drugs due to adverse effects (discontinuation); reappearance of HCV after it was undetected once and drug administration was com-

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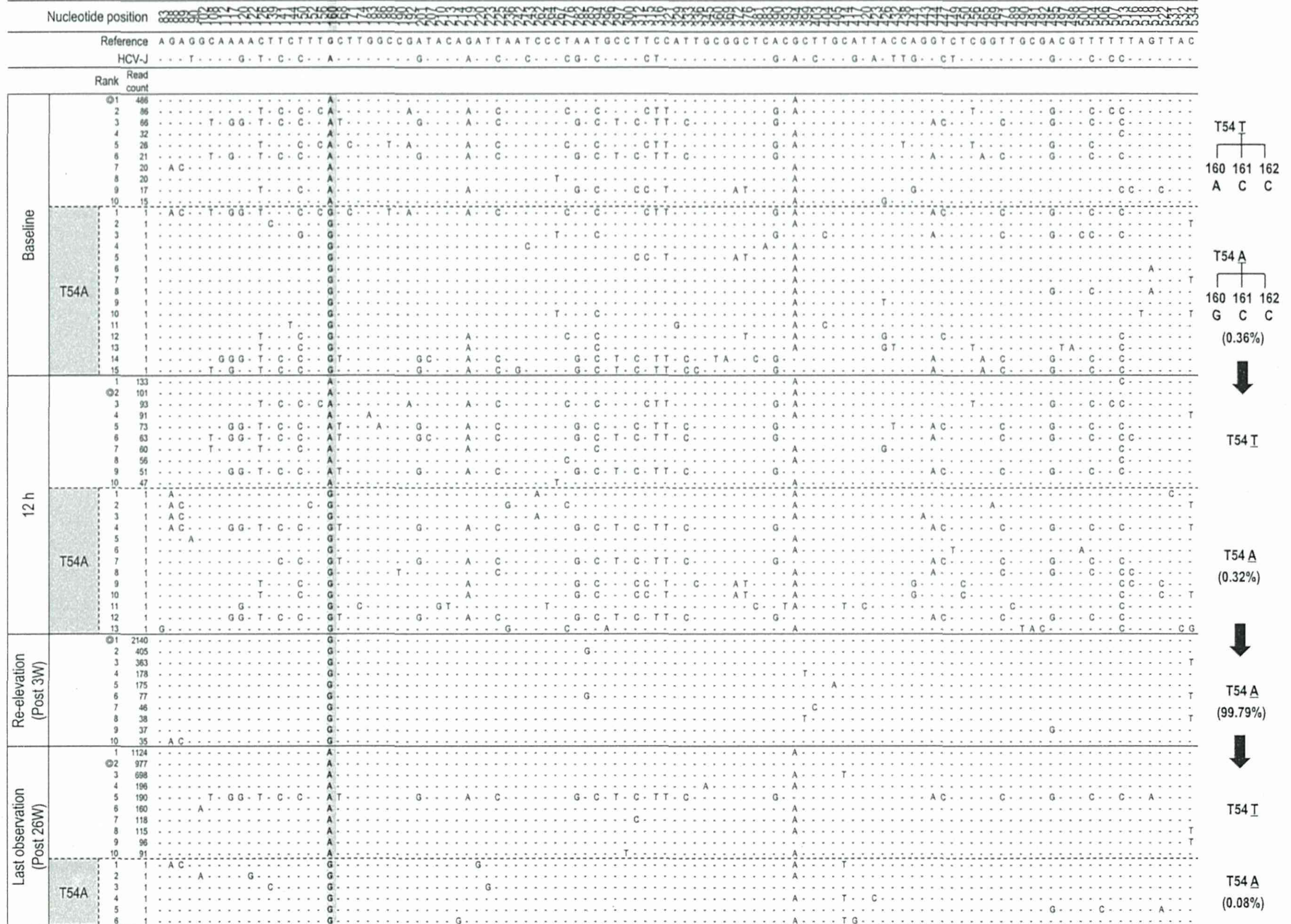

FIG 4 continued

pleted (relapse); failure of elimination of HCV during drug administration (no response); reappearance of HCV during drug administration although HCV was once undetected (breakthrough). The distribution of the non-SVR patients according to cause was as follows: discontinuation, 3; relapse, 2; no response, 2; breakthrough, 1. Table 3 summarizes the viral profiles and treatment course for the 8 patients. The exact time points for the deep sequence analysis are shown in Fig. S1 in the supplemental material.

A clinically resistant mutation was observed in 5 of the 8 patients (62.5%) during treatment and during the follow-up after completion of treatment (V36C, 1; T54A, 2; A156F, 1; A156S, 1). The same mutation as the clinically resistant mutation was not observed at baseline in 3 of the 5 patients, even via deep sequencing, but a resistant HCV variant (T54A) was recognized at baseline as a minor population in 2 patients (patients 3 and 7). TVR-resistant HCV variants accounted for 98% or more when the viral titer was again elevated in all 5 patients, but the rate of a resistance mutation decreased in 4 patients (patients 3, 4, 6, and 7), and the wild type became the dominant form during the follow-up after the end of treatment.

Changes in the compositions of viral populations over time in non-SVR patients. Next, changes in the compositions of HCV populations over time were investigated in all of the non-SVR patients by constructing phylogenetic trees from all isolates obtained at 3 time points, i.e., baseline, reelevation of the viral titer, and the last observation. The isolates obtained at the reelevation of the viral titer had clusters different from those at baseline (Fig. 3). Moreover, the isolates at the last observation were distinct from those at baseline but seemed to be close to the isolates obtained at reelevation except in patient 3 (Fig. 3). Since it was difficult to demonstrate bootstrap values in these large trees, small-scale trees using approximately the 10 most populated isolates at each point in each non-SVR patient were also constructed to demonstrate the interrelationship among isolates at different time points; bootstrap values of 50 or more were determined (see Fig. S2 in the supplemental material). As shown in these trees, isolates at the same time point tended to be separated from isolates at different time points by the branching nodes with high bootstrap values.

To clarify the changes in major viral populations, approximately 10 dominant populations were determined at 4 points (baseline, 12 h, reelevation of the viral titer, and the last observation) in each patient, and such changes were demonstrated. As shown in Fig. 4A, to H the composition of the dominant population at reelevation greatly changed compared with that at baseline

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D Patient 4 Breakthrough

Nucleotide positions: 90 124 126 132 168 171 174 177 190 207 225 234 240 267 316 318 337 357 364 372 373 375 393 396 408 427 450 **466** **467** 486 513 521 534

Sample	Rank	Read count	Sequence
Reference			G T T G T C T C C C C A G G T G G A G G A T C C G C T T T A C C C
HCV-J			T - - - C T - - - T - - - C - - - G A - - C - - - - - G C G - - -
Baseline	1	1110	- A - - C T C T - - - - A A - A - G - - - - - T - - - G C G - - -
Baseline	2	64	- A - - C T C T - - - - - A - A - G - - - - - T - - - G C G - - -
Baseline	3	63	- A - - C T - T - - - - A A - A - G - - - - - T - - - G C G - - -
Baseline	4	53	- A - - C T C T - - - - A A - A - G - - - - - - - - - G C G - - -
Baseline	5	47	- A - - C T C T - - - - A A - - - G - - - - - T - - - G C G - - -
Baseline	6	46	- A - - C T C T T - - - A A - A - G - - - - - T - - - G C G - - -
Baseline	7	45	- A C - C T C T - - - - A A - A - G - - - - - T - - - G C G - - -
Baseline	8	43	- A - - C T C T - - - - A A - A - G - - - - - T - - - G C G - - T
Baseline	9	42	- A - - C T C T - - - - A A C A - G - - - - - T - - - G C G - - -
Baseline	10	35	- A - - C T C T - - - G A A - A - G - - - - - T - - - G C G - - -
Baseline	◎127	7	- - - - C - G C - - - -
12 h	1	507	- A - - C T C T T - - - - A - A - G - - - - - T - - - G C G - - -
12 h	2	216	- A - - C T C - - - - - - - - - - G - - - - T - A - - G C G - - -
12 h	3	173	- A - A C T C T - - - - A A - A - G - - - - - T - - - G C G - - T
12 h	4	82	- A - - C T C T - - T - - A - A - G - - - - - T - - - G C G - - -
12 h	5	81	C A - - C T C T - - - - A C - A - G - - - - - T - - - G C G - - T
12 h	6	53	C A - A C T C T - - - - A A - A - G - - - - - T - - - G C G - - -
12 h	7	53	- A - - C T C T - - - G A A - A - G - - - - - T - - - G C G - - -
12 h	8	43	- A - - C T C T - - - - A A - A - G - - - - - - - - - G C G - - T
12 h	9	42	- A - - C T C T - - - - A A - - - G - - - - - T - - - G C G - - T
12 h	10	41	- A - - C T C T - - - - A A - A - G - A - - - T - - - G C G - - -
12 h	◎534	1	- - - - C - T - G C - T - -
Re-elevation (Post 2W)	◎1	2041	- T T - - - -
Re-elevation (Post 2W)	2	748	- - - - - - - - - - - - - - - - A - - - - - - - - - - T T - - - -
Re-elevation (Post 2W)	3	192	- T - - - - T T - - - -
Re-elevation (Post 2W)	4	140	- - - - - - - - - - - - - - - - - - A - - - - - - - - T T - - - -
Re-elevation (Post 2W)	5	112	- T T - - T -
Re-elevation (Post 2W)	6	80	- T T - - - T
Re-elevation (Post 2W)	7	43	- - - - - - - - - - - - - - - - A - - - - - - - - - - T T - - - T
Re-elevation (Post 2W)	8	43	- - - - - - - - - - - - - - - - - - A - - - - - - - C T T - - - -
Re-elevation (Post 2W)	9	36	- - - - C - T T - - - -
Re-elevation (Post 2W)	10	30	- - - - C - - - - - - - - - - - A - - - - - - - - - - T T - - - -
Last observation (Post 45W)	◎1	3639	- G C - - - -
Last observation (Post 45W)	2	1369	C - G C - - - -
Last observation (Post 45W)	3	398	- G C - - - T
Last observation (Post 45W)	4	167	- - - - C - G C - - - -
Last observation (Post 45W)	5	159	C - G C - - - T
Last observation (Post 45W)	6	93	C - - - C - G C - - - -
Last observation (Post 45W)	7	57	C - - - - - - - - - - - - C - - - - - - - - - - - - - G C - - - -
Last observation (Post 45W)	8	51	- G - - - - - - G C - - - -
Last observation (Post 45W)	9	47	- - - - - - - - - G - - - - - - - - - - - - - - - - - G C - - - -
Last observation (Post 45W)	10	35	- C - - - - - G C - - - -

A156 A

466 467 468
G C T

A156 A

A156 F

466 467 468
T T T

A156 A

E Patient 5 Relapse

Nucleotide positions: 84 93 115 117 123 135 141 156 162 190 192 203 216 228 240 252 267 276 280 282 288 297 303 312 315 316 327 330 333 337 342 357 358 365 384 388 402 417 423 426 427 429 438 444 468 471 475 487 492 500 507 508 516 531 534

Sample	Rank	Read count	Sequence
Reference			G A G G A G T C T T A A C T A T A C T A A C C C C T A T T G T G G C C A C G T G C G T G C T T G G T T G A C C
HCV-J			- G - A - - C T C C G - - C G C C - A G G - - T - - G - - - - - - G - - - - A A - - - - T - - - - - C A T T -
Baseline	1	461	- - - A G - - T - - - - - - - - - - C G G - - - - C G - - - - - - - - - - - - A - A C - - - - - - - - - - T - -
Baseline	◎2	262	- C - - - - - - - - - - - -
Baseline	3	178	- - - A G - - T - - - - - - - - - - C G - T - - - C G - - - - - - - - - - - - A - A C - - - - - - - - - - T - -
Baseline	4	154	- - - A G - - T - - - - - - - - - - C G - - - - - C G - - - - - - G - - - - - A - A C - - - - - - - - - - T - -
Baseline	5	141	- - - A G - - T - - - - - - - - - - C G G - - - T - G - - - - - - - - - - - - A A A C - - - - - - - - - - T - -
Baseline	6	141	- - - - - - - - - C - C - T - - - - - - - - - -
Baseline	7	115	- - - A G - - T - - - - - - - - - - C G G - - - - C G - - - - - - - - - - - - A - A C - - C - - - - - - T - -
Baseline	8	87	- - - - - - - - - C - C - T - - A - - - - - - -
Baseline	9	81	- - - A G - - T - - - - - - - - - - C G G - - - - C G - - - - - - - - - - - A A - A C - - - - - - - - - - T - -
Baseline	10	79	- - - A G - - T - - - - - - - - - - C G - - - - - C G - - - - - - G T - - - - A - A C - - - - - - - - - - T - -
12 h	1	406	- - - A G - - T - - - - - - - - - - C G G - - - - C G - - - - - - - - - - - - A - A C - - - - - - - - - - T - -
12 h	◎2	313	- C - - - - - - - - - - - -
12 h	3	288	- - - A G - - T C C - - - - G - C T C G - - - T - - G - C A - - - G - - - A - - - - C - - - - - - - - C A T - -
12 h	4	165	- - - A G - - T C C G - - C G - C T C G - - - T - - G - C - - - - G - - T A - - - - C - - - - - - - - C A T T -
12 h	5	155	- - - A G - - T - - - - - - - - - T C G G - - - - C G - - - - - - - - - - - - A - A C - - - - - - - - - - T - -
12 h	6	141	- - - A G - - T - - - - - - - - - - C G G - - - - C G - - - - - - - - - - - - A - A C - - - - - - - - - - T - T
12 h	7	126	- C - - - - - - C - - - - -
12 h	8	87	- - - A G - - T - - - - - - - - - - C G G - - - - C G - - - C - - - - - - - - A - A C - - - - - - - - - - T - -
12 h	9	86	- - - A G - - T C C - - - - G - C T C G - - - T - - G - C A - - - G - - - A - - - - C - - - - - - - - C A T - T
12 h	10	84	- C - - - - - - - - - - - T
Re-elevation (Post 7W)	◎1	4431	- -
Re-elevation (Post 7W)	2	121	- A -
Re-elevation (Post 7W)	3	79	- - - - - - - - - - - - T - - - - - - - - - - - - - - - - - A -
Re-elevation (Post 7W)	4	69	- A -
Re-elevation (Post 7W)	5	49	- C - - - - - - - -
Re-elevation (Post 7W)	6	42	- T
Re-elevation (Post 7W)	7	31	- G -
Re-elevation (Post 7W)	8	24	- - - - - - - - - C -
Re-elevation (Post 7W)	9	24	- G - - - - - - - - - - - - - - - - - - -
Re-elevation (Post 7W)	10	23	- - - - - - C -
Last observation (Post 28W)	◎1	3908	- -
Last observation (Post 28W)	2	868	- T
Last observation (Post 28W)	3	75	- C - - - - - - - - - - -
Last observation (Post 28W)	4	66	- T - A - - - - - -
Last observation (Post 28W)	5	57	- - - - - A -
Last observation (Post 28W)	6	57	- G -
Last observation (Post 28W)	7	55	A -
Last observation (Post 28W)	8	51	- - A -
Last observation (Post 28W)	9	51	- - - - - - - - - - - - - - - C - - - - - - - - - - - C -
Last observation (Post 28W)	10	45	- - - - - - - - - - - G -

FIG 4 continued

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F Patient 6 Relapse

Nucleotide position: 81 88 89 90 114 141 147 150 156 165 183 190 201 207 216 239 265 276 279 297 302 306 315 316 345 375 381 394 408 426 433 438 440 450 457 465 466 471 488 492 494 510 513 518 534

Reference: T G A G C T T T T C A C A T C A T T C C G G C T G C T G G G T C C T A G T T T A A A C A T

HCV-J: C - - T - C - C - - G - G - - - C C - - - - - - - - - A - A - T - - - - G - - G - - - - C

	Rank	Read count	Sequence
Baseline	1	434	C - T - - - - - - - - - - - - G - - - - - - - -
	2	339	C - - - - - C - C - - - - - - - - - - - - - - T - - - A - - - - - - - G - - - - - - - -
	3	288	C - - - - - C - - - - - C - T - C - T - - - T - - T - A - A - - - - - - G - - T - - - - -
	4	84	C - - - - - C - C - - - - - - - - - - - - - C - T - - - A - - - - - - G - - - - - - - -
	5	74	C - T - - - - - - - - - - - G C - - - - - - -
	6	72	C - - - - - C - - - G T C - T - C - T - - - T - - T - A - A - - - - - - G - - T - - - - -
	7	65	C - T - - - A - - - - - - - G - - - - - - - -
	8	62	C - - - - - - - - - - - - T - - - - - - - - - T - - - A - - - - - - - G - - - - - - A - -
	9	57	C - - - - - C - - T - - - G - - - - - - - - - T - - A A - - - - - - - G - - - - - - - -
	10	57	C - A T - - - - - - - - - - - G - - - - - - - -
	◎22	37	C - G - - - - - - - -
12 h	◎1	637	C - T - - - - - - - - - - - G - - - - - - - -
	2	234	C - - - - - - C - - - - - - - - - - - - - - - T - - - A - - - - - - - G - - - - - - - -
	3	211	C - - - - - C - C - - - - - - - - - - - - - - T - - - A - - - - - - - G - - - - - - - -
	4	208	C - - - T C C - - - - - - G - - - - T - - - - A T - - - A - - - - - - G - - - - G - - -
	5	172	C - T G - - - - - - - - - - G - - - - - - - -
	6	169	C - - - - - C - C - - - - - - - - - - - - - - T - - - A - - - C - - - G - - - - - - - -
	7	167	C - - - - - C C C - - - - - - - - - - - - - - T - - - A - - - - - - - G - - - - - - - -
	8	167	C - - - - - - - - - - - - - - - C - - A - - - T - - - - - - T - - - - G - - - - - - - -
	9	144	C - - - - - C - - - - - - - - - - - - - - - - T - - - A - - - - - - - G - - - - - - - -
	10	120	C - - - T - C - - - - - - - - - - T - - - - - T - - - A - - - - - - - G - - - - G - - -
Re-elevation (Post 17W)	◎1	1131	- T - - - - - - - -
	2	682	- G - T - - - - - - - -
	3	21	- T - - G - - - - -
	4	17	- T - C - - - - - -
	5	14	- C - - - - - - T - - - - - - - -
	6	14	- G - T - - - - - - C -
	7	14	- G - T - - G - - - - -
	8	13	- T - - - - - - - -
	9	10	- G - T - - - G - - -
	10	10	- T - - - T - - - - - - - -
Last observation (Post 47W)	1	3278	- G - - - - - - - -
	2	493	- A C - G - - - - - - - -
	3	176	- T G - - - - - - - -
	4	71	- - - C - G - - - - - - - -
	5	59	- G - - G - - - - -
	6	50	- G - - - - - - - C
	◎7	37	- T - - - - - - - -
	8	33	- A C - T G - - - - - - - -
	9	32	- - - - - - - - - - - - - - - - - - - A - - - - - - - - - - - - - - - G - - - - - - - -
	10	29	- - - - - - - - - - T - G - - - - - - - -

A156 A (466 467 468: G C T) → A156 A → A156 S (466 467 468: T C T) → A156 A

G Patient 7 No response

Nucleotide position / Reference / HCV-J / Rank / Read count

Baseline; T54A; 12 h; Re-elevation (Post2W); Last observation (Post 34W); T54A

T54 T (160 161 162: A C C)

T54 A (160 161 162: G C C) (0.28%)

↓ T54 T

↓ T54 A (99.84%)

↓ T54 T

T54 A (0.04%)

FIG 4 continued

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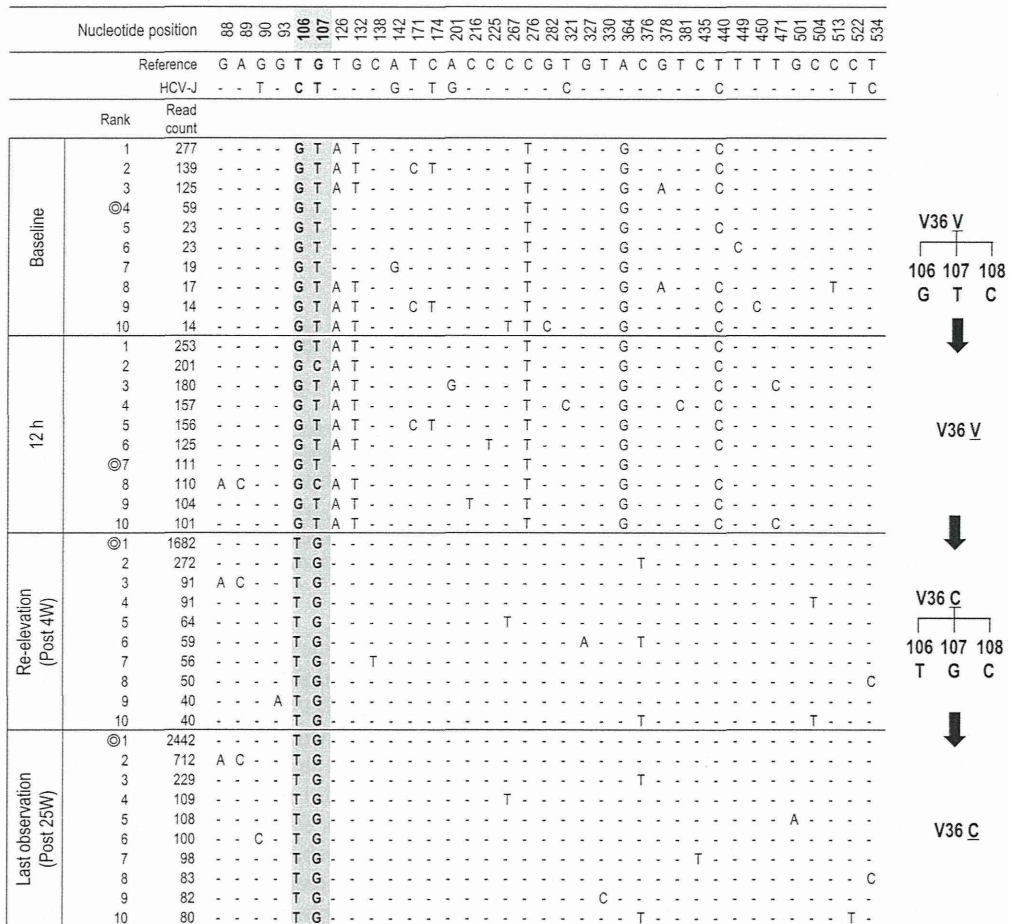

FIG 4 continued

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and at 12 h after introduction of treatment except in patient 3. In addition, these compositional changes were maintained even at the last observation. For statistical analysis of the results, 10 dominant isolates determined at each time point were used for the calculation and comparison of the genetic distance between the isolates at any 2 time points (baseline, reelevation, and last observation). As a result, the genetic distance between the isolates at baseline and reelevation or between the isolates at baseline and the last observation was significantly larger than that between the isolates at baseline and at the viral titer reelevation (Fig. 5) ($P = 0.01$), demonstrating that the changes in population composition induced by triple therapy were unlikely to return to the pretreatment composition.

Origin of HCV isolates showing clinical TVR resistance. To investigate which population at baseline developed a TVR-resistant mutation, deep sequencing results at 4 time points (baseline, 12 h after therapy, at reelevation of the viral titer, and at the last observation) were reanalyzed in 5 patients in whom clinically resistant mutations appeared (patients 3, 4, 6, 7, and 8) (Table 3). Among them, 3 patients (patients 4, 6, and 8) did not have any resistant mutant at baseline, while 2 patients (patients 3 and 7) had T54A mutants (0.36% and 0.28%, respectively) at baseline (Table 3).

In patient 3, the isolates with T54A accounted for 0.36% at baseline but had increased to 99.79% at the time of reelevation of the viral titer (Fig. 4C). On the other hand, from comparison of their sequences it was evident that those T54A isolates at baseline were different from the major T54A isolate at reelevation. The isolate at baseline closest to the major T54A isolate at reelevation was the wild-type isolate of rank 1 that existed as a major population before treatment, and we speculated that the T54A resistance mutation was developed by acquisition of a mutation to the T54T wild type in the 1-ranked isolate. In patient 7, who had a mixture of T54A variants as a minor population at baseline, similar to patient 3, the analysis showed that the T54A isolate that had become the dominant population after treatment was considered to have developed by acquisition of a mutation to the T54T wild type (Fig. 4G).

DISCUSSION

In this study, with the focus on the HCV NS3 protease region, viral quasispecies and changes associated with TVR/PEG-IFN/RBV triple therapy were investigated extensively by deep sequencing along with phylogenetic analysis, as well as their clinical significance. As a result, the associations between treatment response and changes in the complexity of the viral quasispecies at the early

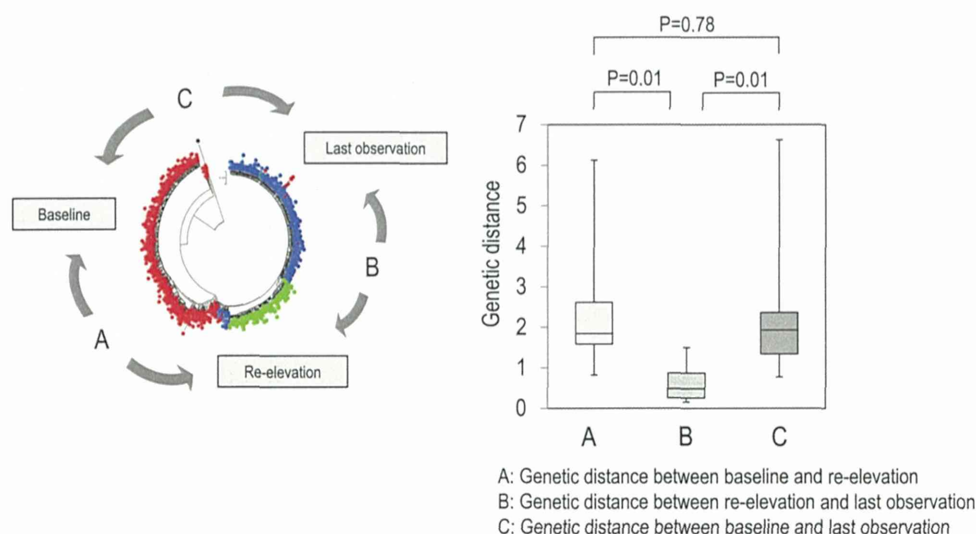

FIG 5 Genetic distances in the NS3 region between baseline and reelevation, between reelevation and the last observation, and between the last observation and baseline were compared in 8 non-SVR patients. The top 10 HCV isolates for each time point were selected to calculate the genetic distance for each patient. After obtaining these genetic distances for all 8 patients, statistical analysis was performed with the Wilcoxon rank sum test.

stage of treatment, changes in quasispecies composition associated with treatment in non-SVR patients, and the origin of TVR-resistant HCV variants were demonstrated.

First, focusing on changes in the complexity of the viral population (quasispecies) at the very early stage, that is, 12 h after the introduction of triple therapy, we observed that these early changes in viral complexity were correlated with the final treatment outcome or *IFNL3* status of the host. Previously, through the analysis of all HCV genomic fragments or E2-HVR-1 (hypervariable region 1), it was also indicated that an early decrease in the complexity of the quasispecies was associated with an early rapid viral response as well as an SVR after introduction to PEG-IFN/RBV dual therapy or IFN monotherapy (15, 33–35). However, it remained to be elucidated how the quasispecies was related to the final treatment outcome or an *IFNL3* SNP after TVR/PEG-IFN/RBV triple therapy. Employing Sn and Mf, we elucidated that the complexity of the quasispecies significantly decreased in Sn and marginally decreased in Mf as early as 12 h after the introduction of treatment in SVR patients but not in non-SVR patients and that changes in the complexity of the quasispecies were dependent on the host factor of the *IFNL3* SNP. Since the *IFNL3* SNP is a host factor regulating the IFN response (27, 36, 37), we speculated that the IFN response was greatly involved as a factor stipulating the change in the complexity of quasispecies at the early stage.

Next, dynamic changes in the composition of the viral population were examined in the non-SVR patients. First, a phylogenetic tree was constructed for all 8 patients with non-SVR using all determined viral isolates (Fig. 3). It was found that the composition of the population differed greatly between the time of treatment introduction and reelevation of the viral titer. Interestingly, the altered composition induced by the triple therapy did not return to the pretreatment status even after the end of therapy at the time of the last observation, except in patient 3 (Fig. 3). Successively, dominant populations were extracted and compared at baseline, at 12 h after treatment, at the reelevation of the viral titer, and at the last observation in all non-SVR patients (Fig. 4). Though the major population showed no marked change from before treatment to 12 h after the introduction of treatment, they greatly changed at the time of reelevation of the viral titer (time of appearance of TVR resistance), and a TVR-resistant mutation returned to the wild type after the end of treatment, whereas the compositional change in the population was maintained and the composition did not return to the pretreatment status (Fig. 4). The result was further confirmed statistically since genetic distances between baseline and reelevation were significantly longer than between reelevation and the last observation (Fig. 5). The influence of this compositional change in the viral population (quasispecies) on the future clinical course is unknown. However, it is possible that resistance may develop more easily during new therapies, since these compositions induced by TVR/PEG-IFN/RBV triple therapy were considered advantageous for viral proliferation in the environment of the triple therapy, although the TVR-resistant hot spot mutation itself returned to the wild type. Therefore, although clinical verification is necessary, we should be careful when introducing new therapies for these non-SVR patients.

Finally, analyses were performed to determine what pretreatment population gave birth to TVR-resistant HCV variants in the non-SVR patients. A clinically resistant mutation appeared in 5 of the 8 non-SVR patients (62.5%), and TVR resistance was considered to be acquired by a new mutation and not by selection of a preexisting variant during treatment in all 5 patients. For 3 of those 5 patients, deep sequencing showed no mixture of TVR-resistant populations at baseline, but 2 patients (patients 3 and 7) exhibited the T54A mutation (0.28% and 0.36%, respectively), which was thought to be the causal mutation for clinical resistance. However, these populations did not directly change into major populations exhibiting clinical resistance and disappeared at the reelevation of the viral titer, whereas it was likely that the population with wild-type T54T at baseline acquired clinical resistance by developing the T54A mutation. Previous reports demonstrated the possibility that a trace mixture of resistant mutations at baseline does not lead to clinical resistance (10, 11). The phenomenon was further clarified by the present study with the

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use of phylogenetic analysis, and it was considered difficult to predict TVR resistance at baseline. On the other hand, since the true depth needed to clarify the complete composition of quasispecies is unknown, it is still possible that deeper sequencing might identify a preexisting mutant variant that evolved into major clinically resistant populations, and this is one of the limitations of deep sequencing analysis. However, our study at least showed from phylogenetic analysis that preexisting mutant variants do not always evolve into clinically resistant populations.

It is unknown why a TVR-resistant hot spot mutation was undetected in patient 3 with relapse, even with deep sequencing. Although one possibility is that the TVR-resistant variants responsible for the development of TVR resistance had already disappeared at the time of analysis of relapse, it is also possible that some unknown mechanisms might exist that enable HCV to develop drug resistance even without drug-specific mutations, since the recent large-scale analysis of TVR resistance also detected mutants in only 77% of patients with non-SVR (38). In a recent *in vitro* study, it was reported that increased replication fitness through adaptation of the host's environment long after infection might lead to decreased drug sensitivity in HCV, even without specific mutations (39). Since various DAAs and various DAA combination regimens are still expected to appear, the mechanism of drug resistance lacking specific mutations should be clarified further.

What is the underlying mechanism for the association between the early change in quasispecies and the appearance of clinical TVR resistance? As shown in Fig. 2, genetic complexity 12 h after the introduction of therapy was decreased in the SVR group but not in the non-SVR group, demonstrating that viral populations disappeared vastly in the SVR patients but not in the SVR patients. Considering this result, it is possible that the non-SVR patients had a greater chance of developing TVR resistance since the larger number of various populations could function as a reservoir of drug-resistant HCV, though further study is needed.

In conclusion, focusing on the dynamic changes in quasispecies, this study demonstrated the association between treatment response and change in quasispecies of the virus at the early stage after the introduction of treatment, the origin of TVR-resistant HCV variants, and dynamic changes in virus populations after treatment by TVR/PEG-IFN/RBV triple therapy. These findings have important implications for responses to triple therapy and outcomes.

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Naturally occurring, resistance-associated hepatitis C virus NS5A variants are linked to IL28B genotype and are sensitive to interferon-based therapy.

Running Head: Naturally occurring RAVs and IFN therapy

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Abstract

BACKGROUND & AIMS: The presence of resistance-associated variants (RAVs) may attenuate the efficacy of direct acting antivirals (DAAs) in combination therapy for hepatitis C. The aim of this study was to characterize the NS3 and NS5A regions of hepatitis C virus (HCV) in naturally occurring RAVs.

METHODS: The NS3 and NS5A regions of HCV were amplified by nested PCR and their nucleotide sequences were determined by direct sequencing in 493 genotype 1b patients naive to DAA-based therapies. The effect of baseline RAVs on response to pegylated interferon and ribavirin therapy was analyzed in 65 patients after stratification by IL28B genotype.

RESULTS: The incidence of RAVs was 7.9% in NS3 (V36I/L: 1.2%, T54S: 2.8%, Q80K/R: 3.0%, A156S: 0.2%, and D168E/T: 2.4%), and 20.2% in NS5A (L31I/M: 2.2% and Y93H: 19.0%). The incidence in interferon experienced and naive patients was similar. The incidence of Y93H in NS5A was significantly higher in the IL28B TT genotype (rs8099917) than non-TT (27.1% vs. 9.5%, $p<0.001$). The virological response to peg-interferon plus ribavirin therapy was not affected by the presence of RAVs in IL28B TT genotype.

CONCLUSION: RAVs, especially Y93H in the NS5A region, were highly prevalent in DAA-naïve patients with genotype 1b HCV in Japan and were linked to IL28B TT genotype.

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Interferon-based therapy could be an alternative for patients with RAVs because these variants did not attenuate the response to that therapy. The analysis of RAVs may impact the selection of the optimal treatment strategy.

Key words: direct acting antivirals, HCV, IL28B genotype, interferon-based therapy, resistance-associated variants

Abbreviations

HCV, hepatitis C virus; IFN, interferon; ISDR, interferon sensitivity determining region; NVR, non-virological response; Peg-IFN, pegylated interferon; PCR, polymerase chain reaction; PR therapy, Peg-IFN plus RBV combination therapy; RAV, resistance-associated variant; RBV, ribavirin; RNA, ribonucleic acids; SVR, sustained virological response;

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Introduction

Interferon (IFN) has formed the basis of standard treatment for chronic hepatitis C since the 1990s. Combination therapy with pegylated IFN (Peg-IFN) and ribavirin (RBV) achieves a sustained virological response rate of 40-50% in genotype 1 and over 80% in genotype 2/3. The recent development of direct acting antivirals (DAAs), which specifically inhibit the activity of viral proteins essential for replication, has improved significantly the efficacy of therapy.

DAAs are classified according to the target HCV protein, NS3/4A, NS5A and NS5B (1-3). DAAs are highly potent but their efficacy is attenuated in the presence of HCV variants with resistance to their activity. Many such resistance-associated variants (RAVs) have been characterized and several hot spots for variation have been reported (4-9). Naturally occurring RAVs are present in a proportion of patients (10) but their prevalence has not been determined completely. The relationship between RAVs and response to interferon-based therapy is not known and their association with previously established factors that affect the efficacy of interferon-based therapy, such as mutations in the ISDR region of NS5A (11) and core protein (12) and SNPs in the human IL28B gene (13-15), also is not known.

Theoretically, the presence of RAVs could attenuate the efficacy of interferon-free combination therapy with DAAs. In fact, baseline RAVs involving amino acid position 168

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of NS3 and amino acid positions 31 and 93 of NS5A significantly attenuated the sustained virological response (SVR) rates of interferon-free Asunaprevir (NS3 protease inhibitor) and Daclatasvir (NS5A inhibitor) combination therapy; the SVR rate was 50% in patients with D168E in NS3, 48% in interferon-ineligible/intolerant patients with L31M/V and/or Y93H in NS5A, and 29% in non-responder patients with L31M/V and/or Y93H in NS5A(16). In Simeprevir plus Peg-IFN and RBV combination therapy, Q80K in NS3 attenuated the efficacy in genotype 1a patients (17). On the basis of this evidence, the treatment guidance for hepatitis C released by the American Association for the Study of Liver Disease and the Infectious Diseases Society of America (IDSA), and recommendations on treatment of hepatitis C released by the European Association for the Study of the Liver, recommend that Simeprevir combination therapy is not indicated in patients with Q80K in NS3 (18,19). As seen above, the analysis of RAVs at baseline may be crucial in the era of DAA-based therapy.

The aim of this study was to characterize naturally occurring RAVs in the NS3 and NS5A regions of hepatitis C virus.

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Patients and Method

Patients

Serum was obtained from a total of 493 HCV genotype-1b infected patients, who had not been exposed to DAAs. Of them, 308 had been treated previously by interferon-based therapy, 61 with standard IFN, 24 with standard IFN plus RBV, 23 with Peg-IFN and 190 with Peg-IFN plus RBV. The clinical backgrounds of patients are shown in Table 1. Fibrosis staging was categorized according to the METAVIR score: F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis with few septa; F3, numerous septa without cirrhosis; and F4, cirrhosis. Sequences of the ISDR and the core region of HCV were determined by direct sequencing after amplification by reverse-transcription and polymerase chain reaction, as reported previously. Genetic polymorphism in a SNP located near the IL28B gene (rs8099917) was determined by Taq-man PCR assay. Briefly, DNA was isolated from peripheral blood using the standard phenol-chloroform method. Genotyping was carried out using a predesigned TaqMan probe (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Written informed consent was obtained from each patient and the study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the institutional ethics review committee.

Accepted Article

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Amplification and sequencing of HCV genomes

We investigated the viral genome sequence by direct sequencing method. Viral RNA was extracted from serum using QIAamp Viral RNA Mini Kits (QIAGEN). The extracted RNA was reverse-transcribed and amplified by the PCR method using the SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen) with the pairs of primers as follows:, (sense, nucleotides 3302-3329) 5'-GGCAGACACCGCGGCGTGTGGGGACAT-3' and (antisense, 4286-4316) 5'-GCACTCATCACATATTATGATGTCATAGGC-3' for NS3 and (sense, 5872-5891) 5'-AAGAGGCTCCACCAGTGGAT-3' and (antisense, 6730-6749) 5'-CGCCGGAGCGTACCTGTGCA-3' for NS5A. The targeted HCV genome was amplified by nested PCR using PrimeSTAR Max DNA Polymerase ▪ (TaKaRa), with the pairs of primers as follows: (sense, 3305-3329) 5'-AGACACCGCGGCGTGTGGGGACAT-3' and (antisense, 4054-4074) 5'-AGACACCGCGGCGTGTGGGGACAT-3' for NS3 and (sense, 5893-5912) 5'-AATGAGGACTGCTCCACGCC-3' and (antisense, 6690-6709) 5'-GTGAAGAATTCGGGGGCCGG-3' for NS5A. The PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN) and sequenced using an automated DNA sequencer (3730xl DNA Analyzer, Applied Biosystems). Each sequence was confirmed for the sense and anti-sense strands. If minor sequences of RAV were detected in more than 10%

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of the strength of the major sequence, it was regarded as RAV positive.

For analysis, the predicted HCV amino acid sequences from the patients were compared with the sequence of the HCV-J strain (GenBank Accession No. AJ238799, http://www.ncbi.nlm.nih.gov/nuccore/AJ238799.1) as a reference. RAV positions in the HCV gene were determined according to previous reports (4-7, 20-27).

Response to Peg-IFN plus RBV in terms of RAVs

The impact of RAVs on the treatment response to Peg-IFN plus RBV combination therapy (PR therapy) was analyzed in 65 patients. The virological response (VR) was defined as a greater than 2.0 log reduction of HCV RNA at 12 weeks after therapy. The rate of VR was compared between patients with and without RAVs after stratification by IL28B genotype.

Statistical analysis

Categorical data were compared using the chi-square and Fisher's exact test. Continuous variables were analyzed using the Student's *t* test. A p value of <0.05 was considered statistically significant. Factors associated with Y93H were determined by multivariable logistic regression analysis. Statistical analyses were performed using the

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Statistical Package for the Social Sciences software version 18.0 (SPSS Inc, Chicago, IL, USA).

Results

Prevalence of RAVs

HCV from 69.8% of the patients had amino acid variations in the NS3 region (Table 2). Variations at positions Q80L (13.4%), S122G (18.9%) and V170I (48.9%) were detected at high frequency but these have not been reported to confer resistance to DAAs. Previously defined RAVs were detected in 7.9% of patients, including V36L (0.8%), T54S (2.8%), Q80K/R (3.0%), A156S (0.2%) and D168E/T (2.4%).

HCV from 83.9% of the patients had variations in the NS5A region (Table 3). Among them, F37L (50.2%) and Q54H (37.6%) were highly prevalent but these have not been reported to confer resistance to DAAs. Previously defined RAVs were detected in 20.2%, the frequency of the Y93H variant being the highest at 19.0%, followed by L31M (1.5%).

Multiple mutations within NS3 (T54S –D168E: 0.2%, T54S –A156S –D168E: 0.2%) or NS5A (L31I/M –Y93H: 0.6%) were infrequent. Dual mutations in NS3 and NS5A were also infrequent (T54S – Y93H: 0.2%, Q80K – Y93H: 0.2%, and D168E – Y93H: 0.9%).

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Factors associated with the presence of RAVs

The frequency of variations in terms of prior experience of interferon-based therapy was analyzed. As shown in Table 1, interferon experienced patients were older, more likely to have the IL28B (rs8099917) non-TT genotype and had a higher incidence of wild type ISDR sequences than interferon-naive patients. The frequency of RAVs in NS3 or NS5A did not differ between patients with and without prior IFN therapy (Tables 2 and 3).

Clinical features associated with the Y93H variant, the most prevalent RAV, also were analyzed (Table 4). Comparison of patients with the Y93 wild type virus and Y93H RAV showed that the Y93H group was older (66 vs. 62 years old, $p=0.02$), had lower platelet counts (141 vs. 162 $x10^9/L$, $p<0.01$), higher serum HCV RNA levels (6.9 vs. 6.5 Log IU/mL, $p<0.01$, Figure 1) and a higher prevalence of the IL28B (rs8099917) TT genotype (81% vs. 53%, $p<0.01$). By multivariate analysis, lower platelet counts, higher HCV RNA levels and IL28B TT genotype were independent factors contributing to the presence of the Y93H RAV (Table 4).

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