HCV RNA extraction, complementary DNA synthesis, amplification by two-step nested PCR from serum samples using primers specific for partial viral regions and direct sequencing were carried out as described previously [15, 16]. Generated sequence files were assembled using Vector NTI software (Invitrogen, Tokyo, Japan) and base-calling errors were corrected following inspection of the chromatogram.

This direct sequencing procedure was performed to determine the dominant viral sequences of the core [17], the interferon sensitivity-determining region (ISDR) [18] and the interferon-ribavirin resistance determining region (IRRDR) [19] from the serum of each patient.

IL28B SNP analysis

Recent reports have disclosed a significant correlation between polymorphisms in the interleukin (IL) 28B gene and patients' responses to pegylated-IFN plus ribavirin therapy for HCV [20-22]. Human genomic DNA was extracted from peripheral blood using a blood DNA extraction kit (QIAGEN, Tokyo, Japan) according to the manufacturer's protocol. The genotyping of each DNA sample was performed by real-time PCR with a model 7500 sequencer (ABI, Tokyo, Japan) using FAM- and VIC-labeled single nucleotide polymorphism (SNP) probes for the locus rs8099917 (ABI).

Deep sequencing

Deep sequencing of part of the viral NS5A region was performed for each of the 110 patients. Briefly, RNA was extracted from the stored sera and reverse transcribed to complementary DNA [23]. Then, two-step nested PCR was carried out

with primers specific for the NS5A region of the HCV genome. To avoid PCR selection bias, we searched for the most conserved DNA sequence regions around NS5A by examining sequence information published previously from 43 HCV-positive individuals from Japan [16] and designed novel primers for this study (Supplementary Table1). This PCR procedure amplified 436 viral nucleotides, including the 1st to 432nd nucleotide of the NS5A region. The primers for the second-round PCR had barcodes, 10 nucleotides (nt) in length, attached and these differed for each sample, so that the PCR products from each sample were identifiable. After the band densities of the PCR products were quantified using a Pico Green® dsDNA Assay Kit (Invitrogen™), the concentrations of 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 (Roche) was used for the analysis.

Statistical Analysis

Statistical differences in the parameters, including all available patients' demographic, biochemical, hematological, virological, and SNP data in the three groups (naïve, relapser and null responder), classified according to the response to previous PEG-IFN/RBV therapy, were determined using the Chi-square test for categorical variables and Kruskal-Wallis test for numerical variables. Statistical differences in the parameters in two groups (Y93H positive, Y93H negative) were determined by the Student t test or Mann-Whitney's U test for numerical variables and Fisher's exact

probability test or Chi-square test for categorical variables. Variables that achieved statistical significance ($p < 0.05$) in univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors. We also calculated the odds ratios and 95% confidence intervals. All p values of <0.05 by the two-tailed test were considered significant.

RESULTS

Average read numbers obtained by deep sequencing and the background error rate

To perform deep sequencing analysis of the NS5A region from many patients, simultaneous analysis was carried out using the barcode primers and approximately 3826 reads were obtained per sample from each group of patients (naïve, relapser and null responder) (Table 2). Because a previous clinical phase 2 study had yielded a significantly high SVR rate, especially in the patients with a null response to previous PEG-IFN/RBV combination therapy, we classified the patients according to their responses to previous PEG-IFN/RBV combination therapy with the assumption that differences in the response to PEG-IFN/RBV might influence the daclatasvir response.

The background error rate of pyrosequencing was calculated with a plasmid containing a cloned HCV sequence (pCV-J4L6S) [24] and the read number for the plasmid is also shown in Table 2. Though seven runs of the plasmid produced 2,277-7,000 reads, with an average of 5,448 reads, there was no background error at amino acid (aa) 31, 32 or 93 in NS5A. Because the background error rate was 0% at each position, the presence of mutations at 0.1% or higher was considered to be significant, based on the 95% confidence interval (0 - 0.1%) calculated for 0% in 2,227 reads. The background error rate coincided almost exactly with the background error rate obtained in our recent study [23].

Baseline characteristics

The baseline characteristics of the 110 patients are shown in Table 1. The data for viral factors (core aa 70, core aa 91, NS5A-ISDR and NS5A-IRRDR) in the table were obtained by direct sequencing as described in ~~the~~ Patients and Methods. As shown

in the table, there were significant differences among the three groups in AST, ALT, γGTP, alpha-fetoprotein, core aa 70, and IL28B SNP (rs8099917). Meanwhile, there was no significant difference in background factors of age and gender or liver fibrosis associated factors such as PLT and Alb.

Detection of NS5A resistance mutations by deep sequencing

Because previous reports showed that L31M/V/F, P32L, and Y93H are resistance mutations in NS5A of genotype 1b HCV, the presence of these mutations was analyzed by deep sequencing. Table 3 shows the rate of NS5A resistance mutations at aa 31, 32, and 93. At aa 32, no mutation was fond in any of the 110 patients. Regarding aa 31, resistance mutations (L31M/V/F) were observed in 13/110 patients (11.8%) and, despite no significant difference, tended to occur more frequently in the relapser group and naïve group than in the null group. Meanwhile, the aa 93 resistance mutation (Y93H) was observed in 34/110 (30.9%) and, despite no significant difference, also tended occur more frequently in the naïve group and relapser group than in the null group. Simultaneous aa 93 and 31 resistance mutations were observed in only 4/110 patients (3.6%) and these four patients all belonged to the naïve group. More detailed deep sequence results for the four patients with simultaneous mutation of L31M/V/F and Y93H are shown in Supplementary Table 2. Although the substitution rate of L31M/V/F in these patients was low, all isolates with L31M/V/F also featured the Y93H change.

Mutation rates of L31M/V/F and Y93H in each patient

Figure 1A and B show the mutation rates of L31M/V/F and Y93H in each

patient. One bar indicates the resistance mutation rate in one patient, obtained by deep sequencing. It was found that minor viral populations that were not detected by direct sequencing could be detected by deep sequencing.

In order to compare our deep sequencing data with previous direct sequencing data in terms of the frequency of NS5A mutations, the notion of "cut-offs" was introduced into our deep sequencing data, assuming that direct sequencing could detect minor populations existing above those cut-off levels. When the cut-off level of 50% was defined to detect minor populations by direct sequencing, L31M/V/F mutations and the Y93H mutations were detected in 1.8% (2/110 patients) and 7.3% (8/110) of our patients, respectively, while the values became 1.8% (2/110 patients) and 15.4% (15/110) when 20% was defined as the cut-off level. These results are comparable to the mutation rate determined previously by direct sequencing and that found in the database [25].

Univariate and multivariate analysis of factors related to the NS5A Y93H mutation

Focusing on the Y93H mutation that is found most frequently in daclatasvir-treatment naïve patients, clinical background factors that would determine efficacy of PEG-IFN/RBV combination therapy patients were investigated by univariate analysis of their association with the Y93H substitution (Table 4). Three factors, the IL28B SNP, core aa 70, and IRRDR, were found to be correlated with the Y93H substitution with statistical significance in the univariate analysis. In patients with the Y93H mutation, the major-type (TT) was frequently observed as the IL28B SNP, while arginine (R) was frequently observed at core aa 70 and the number of substitutions in the IRRDR was higher. There was no significant difference in the number of mutations

in the ISDR but that number tended to be higher in patients with the Y93H mutation, similar to the IRRDR.

The IL28B SNP, core aa 70, and IRRDR, which were correlated significantly with the aa 93 mutation by univariate analysis, were subjected to multivariate analysis (Table 4). The IL28B SNP major-type (TT) was extracted as an independent significant factor with the odds ratio of 3.67 ($p = 0.042$). The mutation rates of L31M/V/F and Y93H in each patient, classified by the IL28 SNP, are presented in Figure 2A and B. Y93H mutations were found significantly more frequently in IL28B TT patients than that in IL28B non-TT patients.

DISCUSSION

In this study, viral mutations conferring resistance to the NS5A replication complex inhibitor daclatasvir were investigated by deep sequencing in daclatasvir treatment-naïve genotype 1b HCV patients and the mutations, especially Y93H, were detected more frequently than predicted by direct sequencing. Interestingly and importantly, the presence of the Y93H mutation correlated with the IL28B SNP of the host, suggesting the possibility that IL28B major-type patients who might show a favorable response to IFN have a greater risk of being infected by daclatasvir-resistant HCV.

Regarding the daclatasvir-resistance mutations L31M/V/F, P32L, and Y93H in genotype-1b HCV, it has been reported that a single mutation produces 5- to 28-fold increased resistance and simultaneous mutations of L31M/V/F and Y93H yield 10,989 to 21,674-fold increased resistance in genotype 1b HCV infection [6]. Previously, the frequencies of L31 M/V/F and Y93H were reported to be 2.7% and 8.2%, respectively, with direct sequencing in genotype 1b daclatasvir-treatment naïve Japanese patients (n=294) and this was comparable with the frequency (3.8% and 8.3%, respectively) in genotype 1b patients, determined from the European HCV database (n=1796) [6, 25]. Among the regimens including daclatasvir for genotype 1b HCV infection, until now only the result of a phase II trial of daclatasvir/asunaprevir therapy for 43 patients has been reported [8, 9]. In that study, the pretreatment presence of HCVs carrying Y93H was significantly associated with non-SVR to that regimen and, moreover, that viruses carrying mutations in both regions of NS5A (L31M/V/F and Y93H) and of NS3 (D168A/V) emerged in most of non-SVR patients after virological failure.

In our study, the presence of L31 M/V/F and Y93H mutations in

daclatasvir-treatment naïve genotype 1b patients was comparable to a previous study which involved direct sequencing, when a cut-off value was introduced to our deep sequencing data, although the prevalence of NS5A mutants changed depending on the cut-off value. However, deep sequencing analysis revealed that NS5A L31M/V/F and Y93H mutations were detectable in 13/110 (11.8%) and in 34/110 (30.9%) patients, respectively, above the background error rate of 0.1% and significantly more frequently than detected by direct sequencing. These results demonstrate that deep sequencing is useful for the detection of viral mutants present as minor variants.

Do HCV populations with Y93H present as minor variants have any association with clinical characteristics? Interestingly, univariate analysis based on the relationship between the presence of theY93H variant and clinical factors or factors determining treatment efficacy to PEG-IFN/RBV combination therapy extracted three significant factors: the IL28B SNP, core aa 70 and the IRRDR (Table 4). All these factors were associated with a favorable response to PEG-IFN/RBV combination therapy in the group with the Y93H resistance mutation [26]. Despite that the difference did not reach statistical significance, the number of substitutions in the ISDR also tended to be higher in the group with the Y93H mutation, similar to the IRRDR. It was quite intriguing that multivariate analysis of the presence of Y93H extracted the IL28B major-type, the SNP was significantly associated with favorable IFN responses, as an independent factor (Table 4). On the other hand, because it is known that the IL28B SNP is closely linked with core aa 70, it is assumed that core 70R should be observed more frequently in the group with Y93H [16].

Then, do NS5A resistant variants with Y93H that are present prior to treatment affect the response to daclatasvir treatment? At present, in genotype-1b infection,

daclatasvir is scheduled to be used in combination with other DAAs but not with IFN. Considering the correlation between IL28B SNPs and Y93H, and the fact that Y93H variants might be sensitive to IFN but resistant to daclatasvir [27], patients with the IL28B major-type might be recommended to receive IFN-based therapy rather than DAA regimens including daclatasvir, because those patients have a greater chance of being infected with daclatasvir-resistant Y93H variants leading to treatment failure. In contrast, the IL28B minor-type patients who have poor responses to IFN might be more promising candidates.

The true clinical influence of Y93H on treatment responses remain unknown and further elucidation is mandatory after the approval of daclatasvir for clinical use. In particular, it is important to clarify the cut off values as to the mixture ratio of Y93H to Y93 wild type in establishing clinical resistance, if the presence of viruses with Y93H before treatment really does affect the response. If so, it is also important to clarify whether the proportion of Y93H variants changes during the clinical course (the natural course or during therapy including IFN) in order to determine the most appropriate timing for introducing daclatasvir. However, it is possible for Y93H variants to disappear after IFN treatment considering that Y93H variants might be sensitive to IFN. The mechanism of the relationship between the IL28B SNP and Y93H also is not clear at present. Considering that wild-type NS5A is known to be associated in its ISDR-region with IFN-resistance and with the IL28B minor SNPs (TG/GG) [28], it is possible that wild-type NS5A Y93 also is associated with IFN-resistance and with IL28B minor SNPs, although further elucidation is necessary.

We acknowledge that the PCR technique has a risk of producing biased amplicons according to the PCR primer sequences, and therefore, we designed novel

primers in this study by searching for the most conserved sequence regions around NS5A. We speculate that the sequence bias might have been avoided at least to some extent considering the fact that the NS5A mutation rate in this study was quite compatible with that of a previous study and that obtained from the public database.

In conclusion, we detected by deep sequencing the substantial presence of resistance mutations to daclatasvir, Y93H in particular, in daclatasvir treatment naïve patients and these were not detectable by direct sequencing. We also showed that IL28B major-type patients who have favorable responses to IFN may have a higher risk of being infected with Y93H HCV than IL28B minor-type patients, suggesting that those patients might have a higher risk of developing daclatasvir resistance, although further studies are needed.

FIGURE LEGENDS

Figure 1. The percentage of mutations in the NS5A region associated with resistance to daclatasvir is presented, classified by the response to previous pegylated-interferon/ribavirin (PEG-IFN/RBV) therapy (Naïve, Null Responder, and Relapser). (A) NS5A aa 31, (B) NS5A aa 93. Each bar indicates the mutation rate in one patient and a dot above a bar shows a patient with a mutation detected by deep sequencing.

Figure 2. The percentage of mutations at the NS5A region for resistance to daclatasvir is presented, classified by the IL28B SNP (TT or non-TT). (A) NS5A aa 31, (B) NS5A aa 93. Each bar indicates the mutation rate in one patient and a dot above a bar shows a patient with a mutation detected by deep sequencing.

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Table 1. Patient characteristics classified by their responses to previous PEG-IFN/RBV combination therapy

	Naïve N = 59	Relapser N = 30	Null responder N = 21	*p*
Age (years)	62.3 ± 11.5	62.7 ± 9.1	61.2 ± 7.7	*0.719*
Sex F/M	35 / 24	16 / 14	9 / 12	*0.427*
AST (IU/l)	35.4 ± 12.6	43.9 ± 53.4	45.3 ± 14.6	*0.008*
ALT (IU/L)	34.6 ± 18.5	45.3 ± 73.2	51.8 ± 23.5	*<0.001*
PLT ($x10^{-4}/\mu l$)	15.1 ± 5.6	14.3 ± 3.8	13.8 ± 4.8	*0.582*
Alb (g/dl)	4.2 ± 0.4	4.3 ± 0.3	4.2 ± 0.5	*0.334*
γGTP (IU/L)	35.2 ± 37.7	37.6 ± 45.1	67.1 ± 55.2	*<0.001*
AFP (ng/ml)	5.7 ± 6.3	4.5 ± 3.6	14.7 ± 29.0	*<0.001*
Core aa 70 R	35 (59.3%)	23 (76.7%)	6 (28.6%)	*0.003*
Core aa 91 L	41 (69.5%)	18 (60.0%)	14 (66.7%)	*0.672*
ISDR 2-	14 (23.7%)	5 (16.7%)	2 (9.5%)	*0.340*
IRRDR 5-	29 (49.2%)	13 (43.3%)	8 (38.1%)	*0.181*
IL28B SNP TT	38 (64.4%)	27 (90.0%)	6 (25.6%)	*<0.001*

PEG-IFN/RBV, pegylated-interferon/ribavirin; ISDR, interferon sensitivity-determining region; IRRDR, interferon-ribavirin resistance determining region.

Table 2. Amplicon read numbers obtained by deep sequencing

	N	Average reads ± SD*(range) / sample
Naïve	59	3603.9 ± 1758.4 (655-10293)
Relapser	30	3980.4 ± 3295.9 (445-14330)
Null responder	21	4601.6 ± 2385.5 (1187-9579)
Plasmid	7	5448.3 ± 1299.1 (2277-7000)

*SD; standard deviation.

Table 3. Presence of daclatasvir-resistance amino acid substitutions in daclatasvir-treatment naïve patients, determined by deep sequencing

	Naïve	Relapser	Null responder	Naïve vs. Relapser	Naïve vs. Null	Relapser vs. Null
	N = 59	N = 30	N = 21	*p*	*p*	*p*
L31M/V/F %, median (range) *	2.0 (0.0-99.8)	4.1 (0.0-100.0))	0.2 (0.0-3.4)	*0.895*	*0.295*	*0.317*
Pts with L31M/V/F (%)**	8 (13.6%)	4 (13.3%)	1 (4.8%)	*1.000*	*0.510*	*0.612*
P32L %, median (range) *	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	*1.000*	*1.000*	*1.000*
Pts with P32L (%)**	0 (0%)	0 (0%)	0 (0%)	*1.000*	*1.000*	*1.000*
Y93H %, median (range) *	11.7 (0.0-99.1)	7.9 (0.0-100.0)	4.1 (0.0-45.3)	*0.824*	*0.190*	*0.301*
Pts with Y93H (%)**	21 (35.6%)	10 (33.3%)	3 (14.3%)	*1.000*	*0.112*	*0.224*

* Median proportion per patient

** Number of patients with the mutant

Table 4. Univariate and multivariate analysis of factors associated with NS5A-Y93H

Variables	No. of patients	NS5A-Y93H substitution		Univariate Analysis (N = 110)		Multivariate Analysis (N = 110)	
		Positive (N = 34)	Negative (N = 76)	Odds Ratio (95% CI)	p Value	Odds Ratio (95% CI)	p Value
Age (years) ≥65	48	16 (47.1%)	32 (42.1%)	1.22 (0.54-2.76)	0.629		
Sex Male	50	16 (47.1%)	34 (44.7%)	1.10 (0.49-2.47)	0.821		
AST (IU/L) ≥41	38	11 (32.4%)	27 (35.5%)	0.87 (0.37-2.05)	0.746		
ALT (IU/L) ≥41	33	9 (26.5%)	24 (31.6%)	0.78 (0.32-1.92)	0.590		
Platelets ($x10^4/mm^3$) ≤12	35	12 (35.3%)	23 (30.3%)	1.43 (0.61-3.33)	0.601		
Albumin (g/dL) ≤4	25	9 (26.5%)	16 (21.1%)	0.69 (0.28-1.70)	0.422		
γGTP (IU/L) ≥41	30	10 (29.4%)	20 (26.3%)	1.25 (0.51-3.08)	0.628		
AFP ≥10	16	5 (14.7%)	11 (14.5%)	1.02 (0.32-3.20)	0.974		
IL28B TT	71	29 (85.3%)	42 (55.3%)	4.70 (1.64-13.43)	0.004	3.67 (1.05-12.88)	0.042
Core aa 70 R	64	25 (73.5%)	39 (51.3%)	2.64 (1.09-6.38)	0.032	1.19 (0.40-3.55)	0.759
Core aa 91 L	73	24 (70.6%)	49 (64.5%)	1.32 (0.55-3.17)	0.531		
ISDR* ≥2	21	8 (23.5%)	13 (17.1%)	1.49 (0.55-4.02)	0.430		
IRRDR** ≥5	54	23 (67.5%)	32 (42.1%)	2.88 (1.23-6.73)	0.015	2.37 (0.98-5.74)	0.056
NS5A L31 M/V/F positive	11	2 (5.9%)	9 (11.8%)	0.46 (0.10-2.28)	0.345		
History of IFN therapy	59	21 (61.8%)	38 (50.0%)	1.62 (0.71-3.69)	0.255		

* ISDR mutation number

** IRRDR mutation number

Figure 1A