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%

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

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%).

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).

Variations and early response of Peg-IFN / RBV combination therapy

The impact of RAVs on treatment response to Peg-IFN plus RBV combination therapy was analyzed in 65 patients. The virological response (VR), defined as a greater than 2.0 log reduction of HCV RNA at 12 weeks after therapy, did not differ between patients with or without Y93H RAV among IL28B TT genotype. For comparison, the impacts of the Q80L and S122 variants also were analyzed and these variants also did not affect the VR (Figure 2). The number of patients with IL28B non-TT genotype was too small to analyze the effect of Y93 RAVs on VR.

Discussion

Treatment of HCV has entered the era of DAA-based therapy. Early clinical trials and in vitro studies have shown that treatment with a single DAA can suppress the replication of HCV but the emergence of RAVs is rapid and this may negate the inhibitory effect of the drug. Several previous studies have reported the incidence of RAVs in patients naive to treatment with DAAs. Here, we studied a large number of patients and revealed that naturally occurring RAVs in NS3 and NS5A are not rare in genotype 1b HCV. The frequency of RAVs was 7.9% in NS3 and 20.2% in NS5A. Caution should be used because the presence of these RAVs could attenuate the efficacy of DAA-based therapy.

RAVs for the NS3 protease inhibitors vary for different DAAs and HCV genotypes

(4-7, 20-24). RAVs common to first generation protease inhibitors include amino acid

positions 36, 54, 155 and 156, while those for second generation protease inhibitors include

positions 80,156, and 168 (6,8). The frequency of RAVs in NS3 was higher in the present

study than about 1% in a previous report (8,9). The impact of these RAVs on treatment

outcome should be evaluated separately for interferon-based therapy and interferon-free

combination therapy using DAAs. Bartels et al. reported that the SVR rates of Peg-IFN, RBV

and TVR triple therapy were similar in patients with or without TVR-associated RAVs at

baseline (9). This observation suggested that RAVs are susceptible to interferon. The result of

our study supports this conclusion because the VR to PR therapy did not differ between

patients with or without RAVs. On the other hand, several reports have revealed that baseline

RAVs significantly attenuated the SVR rate of interferon-free Asunaprevir (NS3 protease

inhibitor) and Daclatasvir (NS5A inhibitor) combination therapy. Thus, the presence of

RAVs in NS3 could impact the selection of optimal treatment. In cases with NS3 RAVs,

interferon-based therapy or interferon-free combination therapy with DAAs other than

against NS3 should be preferred for patients with RAVs.

In Japan, an NS5A inhibitor is now becoming a key drug for the interferon-free

combination therapy using DAAs. The frequency reported for RAVs in NS5A is higher in

Japan than in Western countries (9,28,29). A strikingly high incidence of the Y93H mutation should influence the outcome of Asunaprevir and Daclatasvir combination therapy, the first interferon-free combination therapy using DAAs approved in Japan, if treatment is given without assessment of baseline RAVs. Previous reports clearly indicated that the rate of SVR decreased to below 50% in patients with baseline RAVs. Because susceptibility to interferon was not attenuated in patients with Y93H RAV, interferon-based therapy, such as with the NS3 inhibitor Simeprevir plus peg-interferon and RBV, may be preferable for these patients. It was shown recently that Daclatasvir plus peg-interferon and RBV combination therapy achieved a high rate of SVR in treatment naïve Japanese patients (30). Taking into account the high prevalence of NS5A RAVs at baseline, as shown here, the result of this report suggested that RAVs in NS5A may not impact the outcomes of interferon-based therapies. Furthermore, newer combination therapies with DAAs are expected to be effective against these naturally occurring RAVs (31-33).

The association between the IL28B genotype and Y93H mutation was an unexpected finding but is in accordance with a recent report of an independent cohort of Japanese patients (29). No other variations in NS3 or NS5A showed an association with IL28B. Prior exposure to interferon therapy was not associated with the presence of Y93H and the precise reason for the association is unclear. Another finding was an association between Y93H and a

high HCV RNA titer. This finding and the high prevalence of Y93H suggest that the Y93H

RAV may be replication competent. This is in contrast to RAVs in NS3, where the

replication fitness of the variants is reduced compared to the wild type virus, a finding

supported clinically by the observation that treatment induced RAVs in NS3 become

undetectable after long term follow-up (34-36). However, this result should be considered

carefully, since there is no *in vitro* data to support the enhancement of replication by Y93H

mutations. Adaptive mutations other than Y93H may be linked to high serum HCV RNA titer.

Further studies are necessary to confirm this.

Another factor associated with Y93H was a lower platelet counts. This finding may

suggest a more advanced stage of disease in Y93H-infected patients. A prospective

observational study is needed to confirm this relationship.

In conclusion, RAVs, especially Y93H in the NS5A region, were highly prevalent in

DAA-naive patients with genotype 1b hepatitis C in Japan. The presence of RAV Y93H has

been reported to attenuate the efficacy of interferon-free combination therapy with DAAs,

while the present study revealed that this RAV may be linked to the IL28B TT genotype in

Japanese and was susceptible to interferon-based therapy. Thus, the analysis of RAVs in NS3

and NS5A may impact the selection of optimal treatment strategies, where interferon-based

therapy with or without combination of DAAs could be an alternative for patients with

RAVs.

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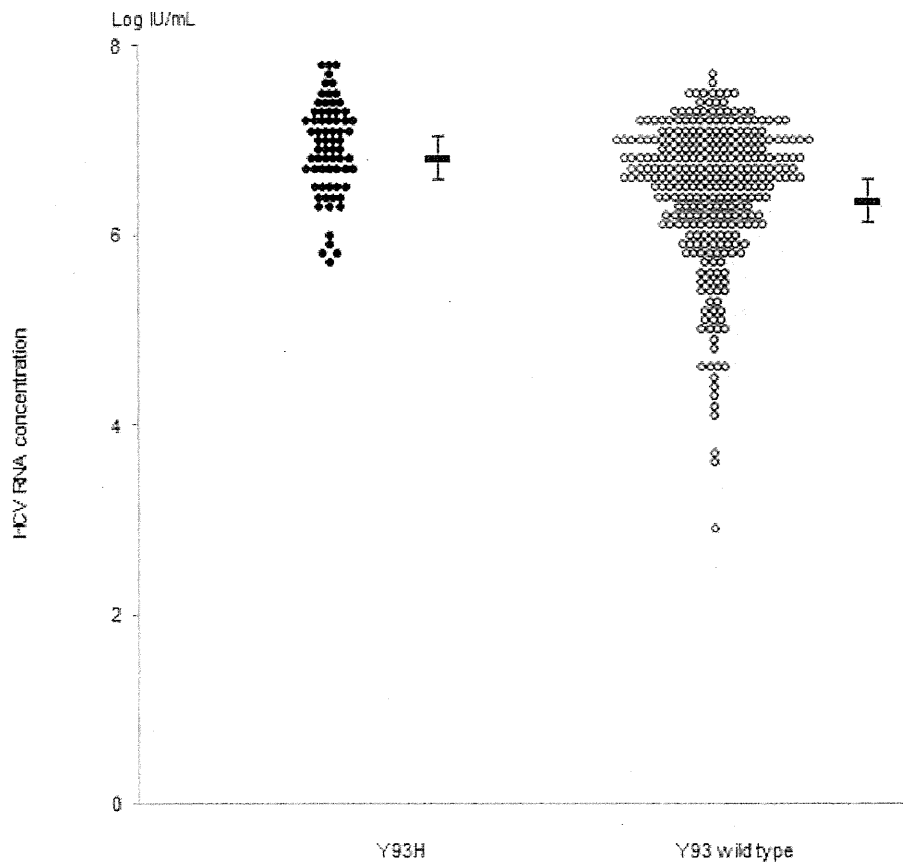
Figure 1. HCV RNA levels according to the presence of Y93H in NS5A

The serum level of HCV RNA was compared between patients with and without Y93H RAV

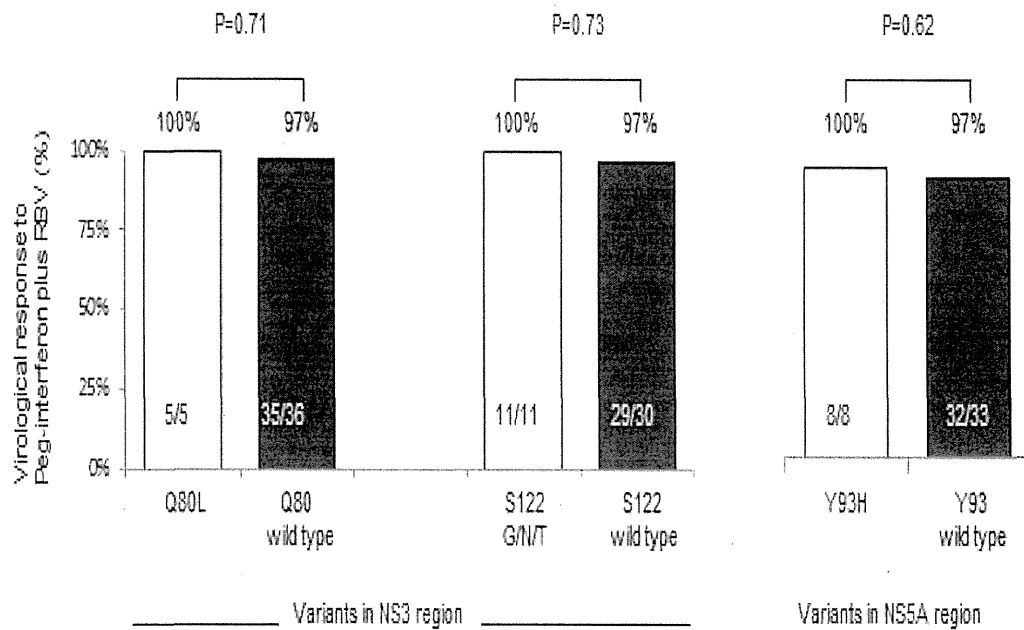mutations in NS5A. Patients with Y93H had significantly higher HCV RNA levels.

Figure 2. Virological responses to Peg-interferon plus RBV therapy in terms of variations in NS3 and NS5A

The rate of virological response to peg-interferon plus RBV therapy was analyzed in patients with or without frequently observed variations in the NS3 region (Q80L and S122G/N/T) and NS5A region (Y93H), after stratification by IL28B genotype TT.

Table 1. Baseline characteristics

| | Total | Prior interferon treatment | | |
| | | Naïve | Experienced | P-value |
	(n=493)	(n=185)	(n=308)	
Age	62.9±11.8	59.8±14.1	64.7±9.8	<0.01
Male / Female	175 / 318	64 / 121	111 / 197	0.75
ALT (U/L)	50.1±38.6	55±40.1	47±37.5	0.045
Platelet (x10^{-9}/L)	158±58.8	16.6±6.2	15.3±5.7	0.46
Albumin (g/dl)	4.1±0.5	4.1±0.5	4.0±0.5	0.99
AFP (ng/ml)	9.6±18.8	8.9±21.6	10.1±17.1	0.50
HCV RNA (log IU/ml)	6.5±0.8	6.5±0.7	6.5±0.8	0.71
Fibrosis stage (0-1/ 2/ 3-4)	138/ 101/ 125	56/ 34/ 33	82/ 67/ 92	0.16
IL28B (rs8099917) (TT/ TG or GG)	259/ 173	101/ 52	158/ 121	0.06
ISDR mutation (0/ 1-2/ ≥3)	214/ 124/ 30	67/ 46/ 17	147/ 78/ 13	0.03
Core amino acid 70 (Wild type/ Mutant)	189/ 102	89/ 38	100/ 64	0.11

ALT; alanine aminotransferase, AFP; alpha-fetoprotein, IFN; interferon, peg-IFN; pegylated interferon, RBV; ribavirin, ISDR; interferon sensitivity determinant region

Table 2. Incidence of variants in the NS3 region

Position	Total (n=493)	Prior interferon treatment		
		Naïve (n=185)	Experienced (n=308)	P-value
V36I	2 (0.4)	1 (0.5)	1 (0.3)	0.72
V36L	4 (0.8)	4 (2.1)	0 (0)	0.01
T54S*	14 (2.8)	8 (4.3)	6 (1.9)	0.12
V55I	1 (0.2)	0 (0)	1 (0.3)	0.44
N77K	1 (0.2)	0 (0)	1 (0.3)	0.44
N77S	4 (0.8)	2 (1.1)	1 (0.6)	0.61
Q80G	2 (0.4)	2 (1.1)	0 (0)	0.07
Q80K*	11 (2.2)	6 (3.2)	5 (1.6)	0.24
Q80L	77 (13.4)	27 (11.4)	50 (14.6)	0.32
Q80M	1 (0.2)	0 (0)	1 (0.3)	0.44
Q80R*	4 (0.8)	1 (0.2)	3 (0.6)	0.60
Q80stop	1 (0.2)	0 (0)	1 (0.3)	0.43
S122G	93 (18.9)	35 (18.9)	58 (18.8)	0.98
S122I	1 (0.2)	0 (0)	1 (0.3)	0.44
S122N	24 (4.9)	7 (3.8)	17 (5.5)	0.39
S122R	3 (0.6)	2 (1.1)	1 (0.3)	0.30
S122T	36 (7.3)	16 (8.7)	20 (6.5)	0.37
A156S*	1 (0.2)	1 (0.5)	0 (0)	0.20
D168E*	11 (2.2)	6 (3.2)	5 (1.6)	0.24
D168T*	1 (0.2)	0 (0)	1 (0.3)	0.44
V170I	241 (48.9)	100 (54.1)	141 (45.8)	0.14
V170M	5 (1.0)	0 (0)	5 (1.6)	0.08
V170T	1 (0.2)	0 (0)	1 (0.3)	0.44
Any variants	344 (69.8)	133 (71.9)	211 (68.5)	0.43
RAV*	39 (7.9)	19 (10.3)	20 (6.5)	0.13

Data are expressed as the numbers of patients with variations and percentages in parentheses.

RAV*: resistance-associated variants

Table 3. Incidence of variants in the NS5A region

	Total	Naïve	Experienced	P value
		Prior interferon treatment		
Position	(n=410)	(n=136)	(n=274)	
L23I	3 (0.7)	0 (0)	3 (1.1)	0.22
L28M	26 (6.3)	6 (4.4)	20 (7.3)	0.26
R30H	1 (0.2)	1 (0.7)	0 (0)	0.16
R30L	1 (0.2)	1 (0.7)	0 (0)	0.16
R30Q	44 (10.7)	14 (10.3)	30 (10.9)	0.84
R30S	1 (0.2)	0 (0)	1 (0.4)	0.48
L31I*	3 (0.7)	2 (1.5)	1 (0.4)	0.22
L31M*	6 (1.5)	3 (2.2)	3 (1.1)	0.42
F37I	8 (2.0)	2 (1.5)	6 (2.2)	0.62
F37L	206 (50.2)	61 (44.9)	145 (52.9)	0.12
F37V	3 (0.7)	1 (0.7)	2 (0.7)	1.00
Q54C	1 (0.2)	0 (0)	1 (0.4)	0.48
Q54E	1 (0.2)	0 (0)	1 (0.4)	0.48
Q54H	154 (37.6)	53 (39.0)	101 (36.9)	0.68
Q54L	9 (2.2)	3 (2.2)	6 (2.2)	0.99
Q54N	7 (1.7)	2 (1.5)	5 (1.8)	0.79
Q54V	2 (0.5)	1 (0.7)	1 (0.4)	0.61
Q54Y	12 (2.9)	4 (2.9)	8 (2.9)	0.99
P58A	4 (1.0)	2 (1.5)	2 (0.7)	0.47
P58L	6 (1.5)	1 (0.7)	5 (1.8)	0.39
P58Q	2 (0.5)	0 (0)	2 (0.7)	0.32
P58R	2 (0.5)	0 (0)	2 (0.7)	0.32
P58S	21 (5.1)	9 (6.6)	12 (4.3)	0.49
P58T	2 (0.5)	2 (1.5)	0 (0)	0.04
P58V	1 (0.2)	1 (0.7)	0 (0)	0.16
Y93H*	78 (19.0)	21 (15.4)	57 (20.8)	0.19
Y93N	1 (0.2)	0 (0)	1 (0.4)	0.48
Y93P	1 (0.2)	0 (0)	1 (0.4)	0.48

Y93S	2 (0.5)	0 (0)	2 (0.7)	0.32
Y93T	1 (0.2)	0 (0)	1 (0.4)	0.48
Any variants	344 (83.9)	109 (80.1)	235 (85.8)	0.15
RAV*	83 (20.2)	24 (17.6)	59 (21.5)	0.36

Data are expressed with number of patients with variations and percentage in parenthesis.

RAV,*: resistance-associated variants