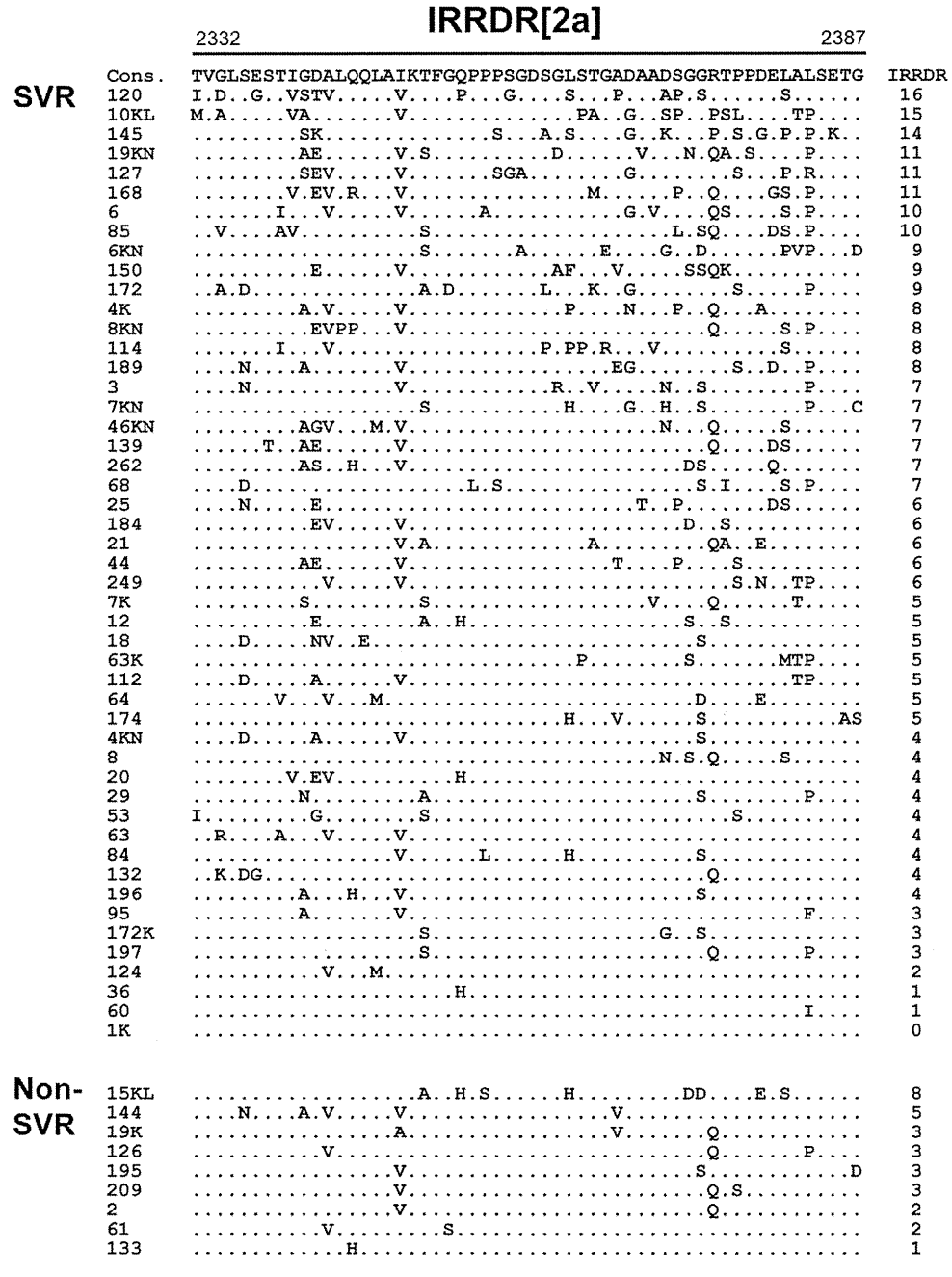
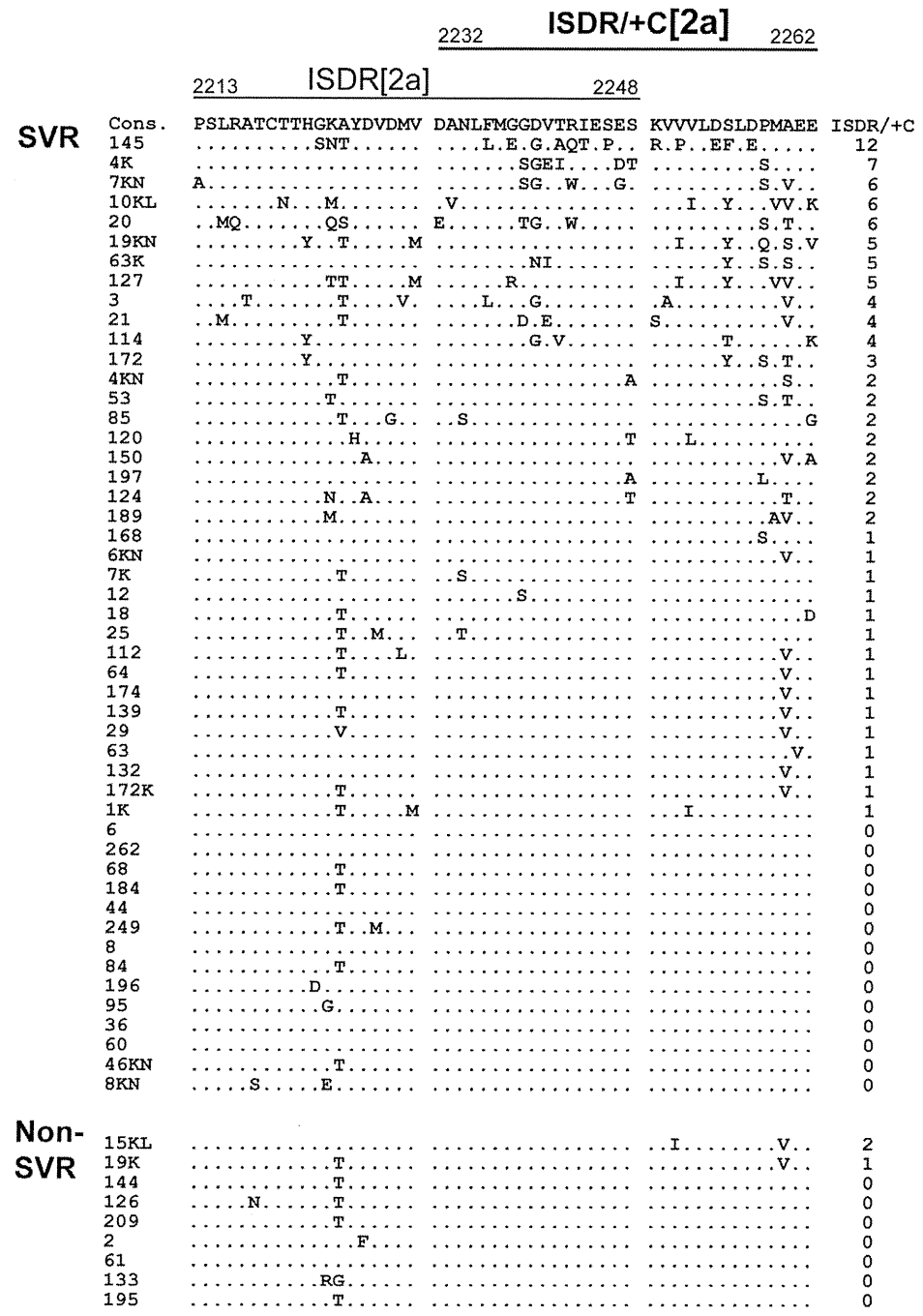


Figure 1. Sequence alignment of IRRDR[2a]. Sequences of IRRDR[2a] (interferon/ribavirin resistance-determining region of HCV-2a) obtained from SVR and non-SVR patients are aligned. The consensus sequence (Cons) is shown on the top. The numbers along the sequence indicate the aa positions. Dots indicate residues identical to those of the Cons sequence. The numbers of the mutations in IRRDR[2a] are shown on the right.
doi:10.1371/journal.pone.0030513.g001

As for HCV-2b infection, the receiver operating characteristic analysis identified "two" as the optimal threshold of the number of mutations in IRRDR/N[2b] by which to predict RVR (data not shown). Accordingly, we found that 65% (22/34) of RVR, and 18% (3/17) of non-RVR patients, were infected with HCV-2b isolates of IRRDR/N[2b]≥2 (Table 4). On the other hand, 35% (12/34) of RVR, and 82% (14/17) of the non-RVR patients, were infected with IRRDR/N[2b]≤1. These results suggested that IRRDR/N[2b]≥2 was significantly associated with RVR ($P=0.0025$). However, no correlation, or even no tendency toward significant correlation, was observed between IRRDR/N[2b]≥2 and SVR in HCV-2b infection.

Correlation between NS5A Sequence Heterogeneity and Viremia Titers in the Serum of patients infected with HCV-2a and HCV-2b before PEG-IFN/RBV Therapy

Next, we examined the impact of IRRDR sequence heterogeneity on HCV titers in the serum before the initiation of the treatment. As shown in Figure 4A, patients infected with IRRDR[2a]≥4 had significantly lower pretreatment serum



	Cons.	PSLRATCTTHGKAYDVDMV	DANLFMGGDVTRIESES	KVVVLDSLDPMAEE	ISDR/+C
SVR	145	.....SNT.....	....L.E.G.AQT.P..	R.P..EF.E.....	12
	4K	.....	.....SGEI....DT	.....S....	7
	7KN	A.....	.....SG..W...G.	.....S.V..	6
	10KL	.....N...M.....	.V.....	...I..Y...VV.K	6
	20	..MQ.....QS.....	E.....TG..W.....	.....S.T..	6
	19KN	.....Y..T.....M	.....	..I...Y..Q.S.V	5
	63K	.....	.....NI.....	.....Y..S.S..	5
	127	.....TT.....M	.....R.....	..I...Y...VV..	5
	3	....T.....T....V.	....L...G.....	.A.....V..	4
	21	..M.....T.....	.....D.E.....	S.....V..	4
	114	.....Y.....	.....G.V.....	.....T.....K	4
	172	.....Y.....	.....	.....Y..S.T..	3
	4KN	.....T.....	.....A	.....S..	2
	53	.....T.....	.....	.....S.T..	2
	85	.....T...G..	..S.....	.....G	2
	120	.....H.....	.....T	...L.....	2
	150	.....A.....	.....	.....V.A	2
	197	.....	.....A	.....L....	2
	124	.....N..A....	.....T	.....T..	2
	189	.....M.....	.....	.....AV..	2
	168	.....	.....	.....S....	1
	6KN	.....	.....	.....V..	1
	7K	.....T.....	..S.....	.....	1
	12	.....	.....S.....	.....	1
	18	.....T.....	.....	.....D	1
	25	.....T..M...	..T.....	.....	1
	112	.....T....L.	.....	.....V..	1
	64	.....T.....	.....	.....V..	1
	174	.....	.....	.....V..	1
	139	.....T.....	.....	.....V..	1
	29	.....V.....	.....	.....V..	1
	63	.....	.....	.....V.	1
	132	.....	.....	.....V..	1
	172K	.....T.....	.....	.....V..	1
	1K	.....T.....M	.....	...I.....	1
	6	.....	.....	.....	0
	262	.....	.....	.....	0
	68	.....T.....	.....	.....	0
	184	.....T.....	.....	.....	0
	44	.....	.....	.....	0
	249	.....T..M...	.....	.....	0
	8	.....	.....	.....	0
	84	.....T.....	.....	.....	0
	196	.....D.....	.....	.....	0
	95	.....G.....	.....	.....	0
	36	.....	.....	.....	0
	60	.....	.....	.....	0
	46KN	.....T.....	.....	.....	0
	8KN	.....S.....E.....	.....	.....	0
Non-SVR	15KL	.....	.....	..I.....V..	2
	19K	.....T.....	.....	.....V..	1
	144	.....T.....	.....	.....	0
	126	.....N.....T.....	.....	.....	0
	209	.....T.....	.....	.....	0
	2	.....F.....	.....	.....	0
	61	.....	.....	.....	0
	133	.....RG.....	.....	.....	0
	195	.....T.....	.....	.....	0

Figure 2. Sequence alignment of ISDR/+C[2a]. Sequences of ISDR/+C[2a] (part of interferon sensitivity determining-region plus its carboxy-flanking region of HCV-2a) obtained from SVR and non-SVR patients are aligned. The consensus sequence (Cons) is shown on the top. The numbers along the sequence indicate the aa positions. Dots indicate residues identical to those of the Cons sequence. The numbers of the mutations in ISDR/+C[2a] are shown on the right.
doi:10.1371/journal.pone.0030513.g002

HCV core antigen titers than those infected with IRRDR[2a]≤3. On the other hand, there was no significant difference in HCV viremia titers between ISDR/+C[2a]≥1 and ISDR/+C[2a] = 0 (Figure 4B). Also, in HCV-2b infection, there was no significant difference in pretreatment HCV viremia titers between IRRDR/N[2b]≥2 and IRRDR/N[2b]≤1 (Figure 4C).

Correlation between Core Protein Sequence Heterogeneity and RVR or SVR

A close correlation between core protein sequence patterns and treatment outcome has been proposed in HCV-1b infection [12,13]. To examine this hypothesis in HCV-2a and -2b infections, core regions of the virus genome were amplified from the pretreated sera, and the aa sequences deduced and aligned

Table 3. Average numbers of aa mutations within IRRDR[2a], ISDR/+C[2a] and IRRDR/N[2b] of HCV NS5A obtained form pre-treated sera of HCV-2a and -2b-infected patients with SVR, non-SVR, RVR and non-RVR.

NS5A region	No. of mutations			No. of mutations		
	SVR	Non-SVR	*P* value	RVR	Non-RVR	*P* value
IRRDR[2a] (aa 2332–2387)	6.4±3.4*	3.3±2.1	**0.01**	6.8±3.3	3.3±1.9	0.0003
ISDR/+C[2a] (aa 2232–2262)	2.0±2.4	0.3±0.7	**0.047**	2.1±2.5	0.6±0.7	0.025
IRRDR/N[2b] (aa 2332–2357)	1.8±1.5	1.4±1.3	0.45	2.0±1.4	1.0±1.2	0.01

*Mean ± S.D.

Abbreviations: SVR, sustained virological response; RVR, rapid virological response; IRRDR[2a], interferon/ribavirin resistance-determining region of HCV-2a; ISDR/+C[2a], part of interferon sensitivity determining-region plus its carboxy-flanking region of HCV-2a; IRRDR/N[2b], an N-terminal part of interferon/ribavirin resistance-determining region of HCV-2b.

doi:10.1371/journal.pone.0030513.t003

IRRDR[2b] 2387

2332 **IRRDR/N[2b]** 2357

	Cons.	AKVLTQDNVEGVLREMADKVLSPLQD	CNDSGHSTGVDTGGDSVQQPSDETAASEAG	IRRDR/N(2b)	Final response
RVR	73	.....S...I.K.....P..	S....R.....NI...P.....	4	SVR
	138	.....FK...G..F.....	H....R...A.....S.	4	SVR
	143	T.....D...R...S...	H.....AEA.....TA	4	SVR
	166	.R.....K.....SF..	Y.....A.....D..	4	Non-SVR
	183	.....T...I.K.....P..	S....R.....NI..L...A.V....	4	SVR
	231	.....FN.PR.	.....A.....S.D..	4	SVR
	2KL	.....KE.....P..	.....A.....T....	3	SVR
	7	.R.....I.....A.....	.....L...A.....D..	3	SVR
	21KN	.....I....VE.....	HT.....A.....	3	SVR
	116	.....GE..K.....	.....T.	3	SVR
	185	.....I.....I..E.	.....A.....D..	3	Non-SVR
	193	.....E...G.....F.....	.....A.....T.	3	SVR
	205	.....G....F.....F.....	Q.....A.....	3	Non-SVR
	233	.T.....R...K.....	.....T.	3	SVR
	4	V.....S.....	.Q.....T.	2	Non-SVR
	5KN	.R.....K.....	H.....A.....	2	SVR
	9KN	.....K.P..	N....L...AE.....D..	2	SVR
	94	.....F..P..	.....A.....T.	2	SVR
	106K	.....V.....G	.D...R.....	2	SVR
	212	.....E.....A.....	.....D..	2	SVR
	147	.R.....K.....	H.....A.....	2	Unknown
	229	.R.....I....	H.....E.....	2	Non-SVR
	11KL	.....I.....	.H.....M.....V..T.	1	SVR
	55KN	.R.....	H.....A.....A.....	1	SVR
	87	.....K.....	.....T....	1	SVR
	103	..I.....	.....AD.....	1	SVR
	165	.....F.....	H.....A.....S.	1	SVR
	1K	.....	.....A..A....T...T.	0	SVR
	10K	.....	.....I.L.....	0	SVR
	46	.....	.....	0	SVR
	99	.....	.....S..T.	0	SVR
	113	.....	.....T...T.	0	SVR
	179	.....	H.....A....N.A.....T...S.	0	SVR
	187	.....	Y.....T..T.	0	SVR
Non-RVR	18KN	.....G.....F.LP.E	.D.....T.	5	SVR
	13K	.....E...K.....	.D.....E....K.....	2	Non-SVR
	110	.....H...E.....	.H.....N.I..A.....T.	2	Unknown
	11K	.....N.....	.....C...A.....C.....G..T.	1	SVR
	23KN	.....E.....	.D.....M.....A.....S.	1	Non-SVR
	40KN	.....P..	HS...R.....D.E	1	SVR
	89	.....F.....	.....TE	1	Non-SVR
	157	.....N.....	YS.....P.....E	1	SVR
	164	.R.....	S.....I.....D.....T.	1	SVR
	170	.....E.....	SD.....N....I.....T.	1	Unknown
	265KL	.....I.....	.....E.	1	Non-SVR
	1	.....	YH.....A.....I...P.....	0	Non-SVR
	32KN	.....	.....G....	0	Non-SVR
	52	.....	H.....A.....T..T.	0	SVR
	105	.....	Y.....	0	Non-SVR
	107	.....	.....R.....	0	Non-SVR
	134	.....	.....G..T.	0	Unknown

Figure 3. Sequence alignment of NS5A of HCV-2b isolates. Sequences of IRRDR/N[2b] (an N-terminal part of interferon/ribavirin resistance-determining region of HCV-2b) obtained from RVR and non-RVR patients are aligned. The consensus sequence (Cons) is shown on the top. The numbers along the sequence indicate the aa positions. Dots indicate residues identical to those of the Cons sequence. The numbers of the mutations in IRRDR/N[2b] and the final treatment outcome of each patient are shown on the right.

doi:10.1371/journal.pone.0030513.g003

Table 4. Correlation between NS5A sequence heterogeneity and SVR or RVR in HCV-2a and HCV-2b infections.

Factor	SVR	Non-SVR	*P* value	RVR	Non-RVR	*P* value
IRRDR[2a]≥4	42/49* (86%)	2/9 (22%)	**0.0003**	42/46 (93%)	5/15 (33%)	<0.0001
IRRDR[2a]≤3	7/49 (14%)	7/9 (78%)		4/46 (7%)	10/15 (67%)	
ISDR/+C[2a]≥1	35/49 (71%)	2/9 (22%)	**0.008**	32/46 (70%)	7/15 (47%)	0.1
ISDR/+C[2a] = 0	14/49 (29%)	7/9 (78%)		14/46 (30%)	8/15 (53%)	
IRRDR/N[2b]≥2	17/34 (50%)	6/13 (46%)	1.0	22/34 (65%)	3/17 (18%)	0.0025
IRRDR/N[2b]≤1	17/34 (50%)	7/13 (54%)		12/34 (35%)	14/17 (82%)	

*No. of isolates with a given factor/total no. of SVR or RVR.
Abbreviations: SVR, sustained virological response; RVR, rapid virological response; IRRDR[2a], interferon/ribavirin resistance-determining region of HCV-2a; ISDR/+C[2a], part of interferon sensitivity determining-region plus its carboxy-flanking region of HCV-2a; IRRDR/N[2b], an N-terminal part of interferon/ribavirin resistance-determining region of HCV-2b.
doi:10.1371/journal.pone.0030513.t004

with the prototype sequences (HCV-J6 [18] and HCV-J8 [19]). The residues at positions 70 and 91, which were reported to be associated with the treatment outcome in HCV-1b infection [13], were both well conserved among HCV-2a and -2b isolates and, therefore, no correlation with treatment outcome was expected for these residues (Figures S1 and S2). In this connection, the residues at positions 48 and 110 of HCV-2a isolates showed certain degrees of variation. However, there was no significant correlation between the sequence patterns and the treatment outcome.

Identification of Independent Predictive Factors for SVR and RVR in HCV-2a and HCV-2b infections

In order to identify significant independent predictors of SVR in HCV-2a and HCV-2b infections, univariate and multivariate logistic regression analyses were carried out using all available data of baseline patients' parameters and viral genetic polymorphic factors. Univariate analysis identified 3 factors that were significantly associated with SVR in HCV-2a infection; the heterogeneity of IRRDR[2a] (≥4 vs. ≤3), ISDR/+C[2a] (≥1 vs. =0) and patients' age (<55 years) (Table 5). Subsequently, these factors were entered in multivariate regression analysis. The result obtained revealed that the IRRDR[2a] heterogeneity was the only independent predictive factor for SVR in HCV-2a infection ($P = 0.001$). The IRRDR[2a] heterogeneity was also the independent predictive factor for RVR (Table S1).

As for HCV-2b infection, univariate analysis identified two host factors that were significantly, or almost significantly, associated with SVR; γ-GTP levels (<30 IU/L) and body weight (<65 kg) (Table 5). No viral factor was identified in this analysis. In subsequent multivariate analysis, γ-GTP levels was identified as an independent predictive factor for SVR in HCV-2b infection. In this connection, the heterogeneity of IRRDR/N[2b], a viral factor, was identified to be significantly associated with RVR in HCV-2b infection (Table S1).

Discussion

The clinical outcome of PEG-IFN/RBV therapy for HCV infection is influenced by a number of host and viral factors [20]. It has recently been reported that host genetic polymorphisms near or within the IL28B gene on the chromosome 19 show a critical impact on the treatment outcome of patients infected with HCV-1a and -1b [21–23]. Also, HCV genetic polymorphisms have been known to contribute to differences in the treatment outcome, as demonstrated by the observations that SVR rates for patients infected with HCV genotypes 2 and 3 are higher than those for patients infected with HCV genotype 1 [2,6]. Moreover,

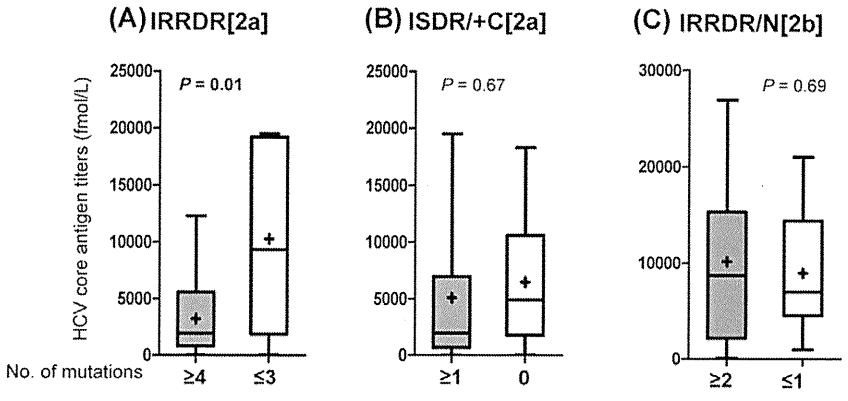


Figure 4. Correlation between NS5A sequence heterogeneity and pretreatment serum HCV core antigen titers in HCV-2a and HCV-2b infections. Pretreatment serum HCV core antigen titers of patients classified on the basis of the number of mutations in IRRDR[2a] (interferon/ribavirin resistance-determining region of HCV-2a) (≥4 vs. ≤3) (A), ISDR/+C[2a] (part of interferon sensitivity determining-region plus its carboxy-flanking region of HCV-2a) (≥1 vs. =0) (B) and IRRDR/N[2b] (≥2 vs. ≤1) (an N-terminal part of interferon/ribavirin resistance-determining region of HCV-2b) (C) are depicted. Maximum and minimum values are indicated by the upper and lower bars, respectively. Distribution ranges are displayed as boxes. Mean and median values are also indicated inside the boxes as+and horizontal bars, respectively.
doi:10.1371/journal.pone.0030513.g004

Table 5. Univariate and multivariate analyses for identification of independent predictive factors for SVR in HCV-2a- and -2b-infected patients treated with PEG-IFN/RBV therapy.

Genotype	Variable	Univariate		Multivariate	
		Odds ratio (95% CI)	*P* value	Odds ratio (95% CI)	*P* value
HCV-2a	IRRDR[2a] mutations	21.0 (3.6–122.5)	0.0003	21.0 (3.6–122.5)	**0.001**
	ISDR/+C[2a] mutations	8.8 (1.6–47.4)	0.008		
	Age (<55 years)	9.8 (1.1–84.7)	0.026		
HCV-2b	γ-GTP (<30 IU/L)	26.0 (1.3–504.7)	0.004	6.2 (1.1–36.2)	0.04
	Body weight (<65 kg)	3.8 (1.0–13.9)	0.06		

Abbreviations: SVR, sustained virological response; IRRDR[2a], interferon/ribavirin resistance-determining region of HCV-2a; ISDR/+C[2a], part of interferon sensitivity determining-region plus its carboxy-flanking region of HCV-2a; γ-GTP, gamma glutamyl transpeptidase.
doi:10.1371/journal.pone.0030513.t005

polymorphisms of NS5A and core regions of a given HCV genotype, in particular HCV-1b, have been linked to the difference in SVR rates [7,8,11–13,17]. It should be noted that the significant link between polymorphisms of NS5A and core regions of HCV-1b and treatment outcome was inferred mostly from studies carried out on patients in Asian countries, in particular Japan, and that somewhat controversial results were obtained from studies carried out on patients infected with HCV-1a or -1b in non-Asian countries [24–31]. However, we would like to point out that most of these publications focused mainly on ISDR and core mutations, but not on IRRDR. In addition, the impact of viral genetic variation on treatment outcome in non-HCV-1 infection, either in Asian or non-Asian countries, is still unclear.

In our previous study, we identified IRRDR in NS5A of HCV-1b as a significant determinant for PEG-IFN/RBV treatment outcome; EVR and, more importantly, SVR [11,12]. Consistent with the previous observation, we have demonstrated in the present study that sequence heterogeneity within IRRDR is closely correlated with the treatment responses in HCV-2a and -2b infections. In HCV-2a infection, IRRDR[2a]≥4 was closely associated with RVR (Table S1) and SVR (Table 5). In HCV-2b infection, the sequence heterogeneity within an N-terminal part of IRRDR (IRRDR/N[2b]) was significantly associated with RVR (Table S1). Furthermore, both IRRDR[2a]≥4 and ISDR/+C[2a]≥1 showed remarkable positive predictive values (95%) for SVR prediction (Table S2), suggesting the clinical usefulness of these markers to encourage those patients to receive PEG-IFN/RBV treatment. On the other hand, their negative predictive values for non-SVR were rather low (50% and 33%). This suggests the possible involvement of another factor(s) that determines non-SVR and may limit the clinical usefulness of these markers to accurately predict non-SVR.

The present results were dependent upon the small number of non-SVR patients due to the high response rates of HCV-2a and -2b. In spite of this, the parallels between the RVR/non-RVR and the SVR/non-SVR analyses, especially in HCV-2a infection, support the possibility that the sequences presented in this study are truly representative of the viruses in general circulation.

The clinical correlation between IRRDR sequence heterogeneity and virological responses of IFN-based therapy in HCV infection can be linked to a recent experimental observation by Tsai et al. [32] that an HCV subgenomic RNA replicon containing NS5A of HCV-1b exerted more profound inhibitory effects on IFN activities than the original HCV-2a replicon, and that domain swapping between NS5A sequences of HCV-1b and -2a in the V3 and/or a C-terminal region including IRRDR resulted in a transfer of their anti-IFN activities. Also, it is worthy to note that IRRDR is among the most variable sequences across the different genotypes and subtypes of HCV [33] whereas its upstream and downstream sequences show a higher degree of sequence conservation (Figure 5). This may suggest that whereas the upstream and downstream sequences have a conserved function(s) across all the HCV genotypes, IRRDR sequences have a genotype-dependent or even a strain-dependent function(s). Indeed, the upstream sequences, especially a Pro-rich motif, play key roles in multiple stages of viral replication [34] while the downstream sequence in viral particle assembly and production [35]. Therefore, the sequence heterogeneity of IRRDR and its significant correlation with IFN-responsiveness imply the possibility that IRRDR is involved, at least partly, in the viral strategy to evade IFN-mediated antiviral host defense mechanisms. Its possible molecular mechanism, however, is yet to be elucidated. The IRRDR sequence heterogeneity also suggests genetic flexibility of this region and, indeed, the C-terminal portion of NS5A was shown to tolerate sequence insertions and deletions [36]. This flexibility might play an important role in modulating the interaction with various host systems, including IFN-induced antiviral machineries. It is also possible that the genetic flexibility of IRRDR is accompanied by compensatory changes elsewhere in the viral genome and that these compensatory changes affect overall viral fitness and responses to IFN-based therapy [37].

The relapse rate was higher in HCV-2b infection than in HCV-2a (Table 1). It should be noted that while the sequence heterogeneity within IRRDR[2a] was significantly correlated with both RVR and SVR in HCV-2a infection, IRRDR/N[2b] was correlated only with RVR in HCV-2b infection. These observations might be linked to an intrinsic difference in IFN- and/or RBV-sensitivity between HCV-2a and -2b isolates [8,38]. We assume that HCV-2b is considered between HCV-1b and HCV-2a in terms of resistance to PEG-IFN/RBV treatment and that an extended treatment for a total of 36~48 weeks would be needed to prevent relapse in HCV-2b infection, especially for patients who have risk factors that do not fit the SVR or RVR prediction criteria (Table 5 and Table S1).

A mutation at position 70 of the core protein of HCV-1b has been reported to be correlated with PEG-IFN/RBV treatment outcome [12,13]. In the present study, however, we found no significant correlation between core protein polymorphism and treatment outcome in HCV-2a or -2b infections. The residue at position 70 of the core protein of HCV-2a and -2b isolates was Arg, which is known to be associated with SVR in HCV-1b infection [12,13], and was well conserved in all the isolates tested in the present study (Figures S1 and S2). The observed sequence

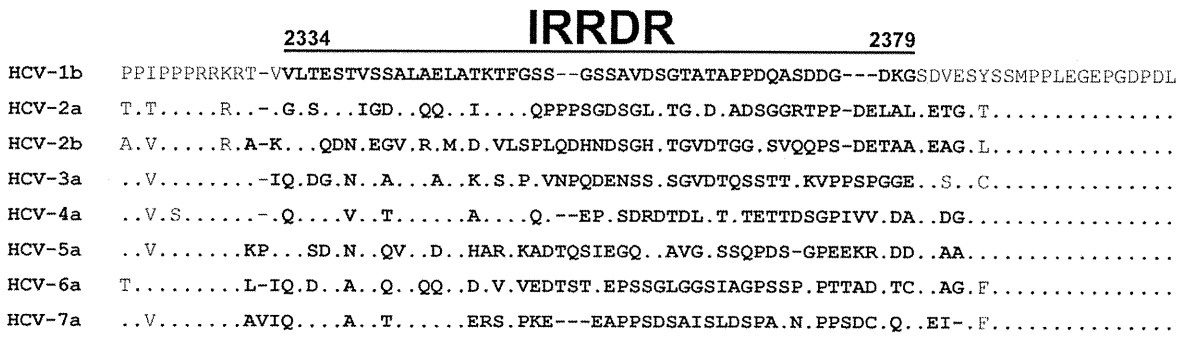


Figure 5. Sequence alignment of IRRDR (interferon/ribavirin resistance-determining region) and its upstream and downstream sequences of different HCV genotypes. The residues in the region that corresponds to IRRDR of HCV-1b [11] are written in boldface letters. Dots indicate residues identical to the HCV-1b sequence. References of aligned sequences are: HCV-1b, El-Shamy et al. [11]; HCV-2a and -2b, Murakami et al. [8]; HCV-3a, X76918; HCV-4a, Y11604; HCV-5a, AF064490; HCV-6a, D84262; HCV-7a, EF108306.
doi:10.1371/journal.pone.0030513.g005

conservation at position 70 might be the reason for the lack of significant correlation between core protein polymorphism and treatment outcome in HCV-2a or -2b infections. On the other hand, Thr at position 110 of the core protein of HCV-2a has recently been reported to be significantly associated with SVR [10]. In the present study, Thr at position 110 was found in 35% (14/40) and 14% (1/6) of SVR and non-SVR cases, respectively (Figure S1). Similarly, Thr at position 48 was found in 35% (14/40) of SVR cases, but not in non-SVR cases (0/6). The observed differences between SVR and non-SVR, however, were not statistically significant due possibly to the small number of samples tested. A larger-scale study would be needed to determine the possible importance of those residues.

We preliminarily analyzed a host genetic factor, the single nucleotide polymorphism (SNP) at rs8099917 near the IL28B gene [21–23], of a portion of the patients examined in the present study. The result showed that the minor genotypes (T/G and G/G) were found in 5.1% (2/39) and 15.4% (2/13) of RVR and non-RVR patients, respectively, and 2.8% (1/36) and 20.0% (2/10) of SVR and non-SVR patients, respectively (Kim et al., unpublished observation). Although the differences were not statistically significant due probably to the small number of the patients tested, the minor genotypes showed a trend toward being associated with non-SVR, and with non-RVR to a lesser extent, in HCV-2a and -2b infections, as has been reported for HCV-1a and -1b infections [21–23]. The impact of the IL28B SNP, however, appeared to be weaker in HCV-2a and -2b infections than that seen in HCV-1a and -1b infections, and also weaker than that of the most powerful viral factor, IRRDR[2a]≥4, in HCV-2a infection. In this context, we found that, of the four patients with the minor IL28B genotypes, two patients (nos. 2 and 105), who underwent unfavorable treatment response (non-RVR and non-SVR), were infected with HCV isolates of IRRDR[2a]≤3 or IRRDR/N[2b]≤1 while the other two patients (no. 63 and 106), who achieved favorable treatment response (SVR and/or RVR), were infected with HCV isolates of IRRDR[2a]≥4. This might imply the possibility that, in HCV-2 infection, the combination of the minor IL28B genotypes and a low degree of IRRDR sequence heterogeneity has a strong power to predict unfavorable treatment responses whereas a high degree of IRRDR sequence heterogeneity has a dominant predictive power for favorable treatment responses regardless the IL28B genotype. Analysis in a large-scale multicenter study is needed to clarify this issue.

In conclusion, our data suggest that the sequence heterogeneity of NS5A, i.e., IRRDR[2a]≥4, and ISDR/+C[2a]≥1 to a lesser extent, would be a useful predictive marker for SVR in HCV-2a infection. Also, IRRDR/N[2b]≥2 is significantly associated with RVR in HCV-2b infection. These results further emphasize the importance of NS5A, a viral factor, in determining the responsiveness to PEG-IFN/RBV therapy.

Supporting Information

Figure S1 Sequence alignment of the core protein of HCV-2a isolates. Core protein sequences (aa 1 to 120) of HCV-2a obtained from SVR and non-SVR patients are aligned. Prototype sequence of HCV-J6 [18] is shown on the top. The numbers along the sequence indicate the aa positions. Dots indicate residues identical to those of the prototype sequence.
(TIF)

Figure S2 Sequence alignment of the core protein of HCV-2b isolates. Core protein sequences (aa 1 to 120) of HCV-2b obtained from SVR and non-SVR patients are aligned. Prototype sequence of HCV-J8 [19] is shown on the top. The numbers along the sequence indicate the aa positions. Dots indicate residues identical to those of the prototype sequence.
(TIF)

Table S1 Univariate and multivariate analyses for identification of independent predictive factors for RVR in HCV-2a- and -2b-infected patients treated with PEG-IFN/RBV therapy.
(DOC)

Table S2 Positive and negative predictive values (PPV and NPV) of NS5A polymorphic factors for SVR prediction.
(DOC)

Acknowledgments

The authors are grateful to Dr. N. Enomoto (Yamanashi University, Yamanashi, Japan) and Dr. Y. Tanaka (Nagoya City University, Nagoya, Japan) for their critical comments and suggestions. Thanks are also due to Ms. M. Kohmoto and Ms. A. Asahi for their technical assistance.

Author Contributions

Conceived and designed the experiments: AE SRK HH. Performed the experiments: AE IS YI LD. Analyzed the data: AE IS YI LD SI SY TF ST YY YS TA HH. Contributed reagents/materials/analysis tools: SRK SI SY TF ST YY YS TA. Wrote the paper: AE SRK HH. Obtained permissions from the Ethics Committees: AE SRK HH.

References

1. Micallef JM, Kaldor JM, Dore GJ (2006) Spontaneous viral clearance following acute hepatitis C infection: a systematic review of longitudinal studies. J Viral Hepat 13: 34–41.
2. Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, et al. (2002) Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 347: 975–982.
3. Sherman KE, Flamm SL, Afdhal NH, Nelson DR, Sulkowski MS, et al. (2011) Response-guided telaprevir combination treatment for hepatitis C virus infection. N Engl J Med 365: 1014–1024.
4. Limaye AR, Draganov PV, Cabrera R (2011) Boceprevir for chronic HCV genotype 1 infection. N Engl J Med 365: 176; author reply 177–178.
5. Enomoto N, Takada A, Nakao T, Date T (1990) There are two major types of hepatitis C virus in Japan. Biochem Biophys Res Commun 170: 1021–1025.
6. Sarasin-Filipowicz M (2009) Interferon therapy of hepatitis C: molecular insights into success and failure. Swiss Med Wkly 140: 3–11.
7. Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, et al. (1996) Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. N Engl J Med 334: 77–81.
8. Murakami T, Enomoto N, Kurosaki M, Izumi N, Marumo F, et al. (1999) Mutations in nonstructural protein 5A gene and response to interferon in hepatitis C virus genotype 2 infection. Hepatology 30: 1045–1053.
9. Akuta N, Suzuki F, Tsubota A, Suzuki Y, Hosaka T, et al. (2003) Association of amino acid substitution pattern in nonstructural protein 5A of hepatitis C virus genotype2a low viral load and response to interferon monotherapy. J Med Virol 69: 376–383.
10. Kadokura M, Maekawa S, Sueki R, Miura M, Komase K, et al. (2011) Analysis of the complete open reading frame of hepatitis C virus in genotype 2a infection reveals critical sites influencing the response to peginterferon and ribavirin therapy. Hepatol Int 5: 789–799.
11. El-Shamy A, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, et al. (2008) Sequence variation in hepatitis C virus nonstructural protein 5A predicts clinical outcome of pegylated interferon/ribavirin combination therapy. Hepatology 48: 38–47.
12. El-Shamy A, Kim SR, Ide YH, Sasase N, Imoto S, et al. (2012) Polymorphisms of hepatitis C virus non-structural protein 5A and core proteins and clinical outcome of pegylated-interferon/ribavirin combination therapy. Intervirology 55: 1–11.
13. Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, et al. (2007) Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b: Amino acid substitutions in the core region and low-density lipoprotein cholesterol levels. J Hepatol 46: 403–410.
14. Okamoto H, Sugiyama Y, Okada S, Kurai K, Akahane Y, et al. (1992) Typing hepatitis C virus by polymerase chain reaction with type-specific primers: application to clinical surveys and tracing infectious sources. J Gen Virol 73(Pt 3): 673–679.
15. El-Shamy A, Sasayama M, Nagano-Fujii M, Sasase N, Imoto S, et al. (2007) Prediction of efficient virological response to pegylated interferon/ribavirin combination therapy by NS5A sequences of hepatitis C virus and anti-NS5A antibodies in pre-treatment sera. Microbiol Immunol 51: 471–482.
16. Lusida MI, Nagano-Fujii M, Nidom CA, Soetjipto, Handajani R, et al. (2001) Correlation between mutations in the interferon sensitivity-determining region of NS5A protein and viral load of hepatitis C virus subtypes 1b, 1c, and 2a. J Clin Microbiol 39: 3858–3864.
17. Akuta N, Suzuki F, Hirakawa M, Kawamura Y, Yatsuji H, et al. (2009) Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 2a high viral load and virological response to interferon-ribavirin combination therapy. Intervirology 52: 301–309.
18. Okamoto H, Okada S, Sugiyama Y, Kurai K, Iizuka H, et al. (1991) Nucleotide sequence of the genomic RNA of hepatitis C virus isolated from a human carrier: comparison with reported isolates for conserved and divergent regions. J Gen Virol 72(Pt 11): 2697–2704.
19. Okamoto H, Kurai K, Okada S, Yamamoto K, Lizuka H, et al. (1992) Full-length sequence of a hepatitis C virus genome having poor homology to reported isolates: comparative study of four distinct genotypes. Virology 188: 331–341.
20. Kau A, Vermehren J, Sarrazin C (2008) Treatment predictors of a sustained virologic response in hepatitis B and C. J Hepatol 49: 634–651.
21. Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, et al. (2009) Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 461: 399–401.
22. Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, et al. (2009) Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet 41: 1105–1109.
23. Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, et al. (2009) IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet 41: 1100–1104.
24. Duverlie G, Khorsi H, Castelain S, Jaillon O, Izopet J, et al. (1998) Sequence analysis of the NS5A protein of European hepatitis C virus 1b isolates and relation to interferon sensitivity. J Gen Virol 79: 1373–1381.
25. Squadrito G, Orlando ME, Cacciola I, Rumi MG, Artini M, et al. (1999) Long-term response to interferon alpha is unrelated to "interferon sensitivity determining region" variability in patients with chronic hepatitis C virus-1b infection. J Hepatol 30: 1023–1027.
26. Sarrazin C, Berg T, Lee JH, Ruster B, Kronenberger B, et al. (2000) Mutations in the protein kinase-binding domain of the NS5A protein in patients infected with hepatitis C virus type 1a are associated with treatment response. J Infect Dis 181: 432–441.
27. Chung RT, Monto A, Dienstag JL, Kaplan LM (1999) Mutations in the NS5A region do not predict interferon-responsiveness in American patients infected with genotype 1b hepatitis C virus. J Med Virol 58: 353–358.
28. Zeuzem S, Lee JH, Roth WK (1997) Mutations in the nonstructural 5A gene of European hepatitis C virus isolates and response to interferon alfa. Hepatology 25: 740–744.
29. Pascu M, Martus P, Hohne M, Wiedenmann B, Hopf U, et al. (2004) Sustained virological response in hepatitis C virus type 1b infected patients is predicted by the number of mutations within the NS5A-ISDR: a meta-analysis focused on geographical differences. Gut 53: 1345–1351.
30. Alestig E, Arnholm B, Eilard A, Lagging M, Nilsson S, et al. (2011) Core mutations, IL28B polymorphisms and response to peginterferon/ribavirin treatment in Swedish patients with hepatitis C virus genotype 1 infection. BMC Infect Dis 11: 124.
31. Donlin MJ, Cannon NA, Aurora R, Li J, Wahed AS, et al. (2010) Contribution of genome-wide HCV genetic differences to outcome of interferon-based therapy in Caucasian American and African American patients. PLoS One 5: e9032.
32. Tsai YH, Kuang WF, Lu TY, Kao JH, Lai MY, et al. (2008) The non-structural 5A protein of hepatitis C virus exhibits genotypic differences in interferon antagonism. J Hepatol 85: 2485–502.
33. Macdonald A, Harris M (2004) Hepatitis C virus NS5A: tales of a promiscuous protein. J Gen Virol 85: 2485–2502.
34. Hughes M, Gretton S, Shelton H, Brown DD, McCormick CJ, et al. (2009) A conserved proline between domains II and III of hepatitis C virus NS5A influences both RNA replication and virus assembly. J Virol 83: 10788–10796.
35. Tellinghuisen TL, Foss KL, Treadaway J (2008) Regulation of hepatitis C virion production via phosphorylation of the NS5A protein. PLoS Pathog 4: e1000032.
36. Moradpour D, Evans MJ, Gosert R, Yuan Z, Blum HE, et al. (2004) Insertion of green fluorescent protein into nonstructural protein 5A allows direct visualization of functional hepatitis C virus replication complexes. J Virol 78: 7400–7409.
37. Yuan HJ, Jain M, Snow KK, Gale Jr. M, Lee WM (2009) Evolution of hepatitis C virus NS5A region in breakthrough patients during pegylated interferon and ribavirin therapy. J Viral Hepat 17: 208–216.
38. Sakamoto N, Nakagawa M, Tanaka Y, Sekine-Osajima Y, Ueyama M, et al. (2011) Association of IL28B variants with response to pegylated-interferon alpha plus ribavirin combination therapy reveals intersubgenotypic differences between genotypes 2a and 2b. J Med Virol 83: 871–878.

J Gastroenterol
DOI 10.1007/s00535-012-0578-z

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Prediction of response to pegylated interferon/ribavirin combination therapy for chronic hepatitis C genotype 1b and high viral load

Soo Ryang Kim · Ahmed El-Shamy · Susumu Imoto · Ke Ih Kim · Yoshi-hiro Ide · Lin Deng · Ikuo Shoji · Yasuhito Tanaka · Yutaka Hasegawa · Mitsuhiro Ota · Hak Hotta

Received: 20 October 2011 / Accepted: 27 February 2012

Abstract
Background This study explores pretreatment predictive factors for ultimate virological responses to pegylated interferon-α (1.5 μg/kg/week) and ribavirin (600–1000 mg/day) (PEG-IFN/RBV) combination therapy for patients infected with hepatitis C virus (HCV)-1b and a high viral load.
Methods A total of 75 patients underwent PEG-IFN/RBV combination therapy for 48 weeks. HCV amino acid (aa) substitutions in non-structural protein 5a, including those in the IFN/RBV resistance-determining region (IRRDR) and the IFN sensitivity-determining region and the core regions, as well as the genetic variation (rs8099917) near the interleukin 28B (IL28B) gene (genotype TT) were analyzed.
Results Of the 75 patients, 49 % (37/75) achieved a sustained virological response (SVR), 27 % (20/75) showed relapse, and 24 % (18/75) showed null virological response (NVR). Multivariate logistic regression analysis identified IRRDR with 6 or more mutations (IRRDR ≥6) [odds ratio (OR) 11.906, $p < 0.0001$] and age <60 years (OR 0.228, $p = 0.015$) as significant determiners of SVR and IL28B minor (OR 14.618, $p = 0.0019$) and platelets $<15 \times 10^4/mm^3$ (OR 0.113, $p = 0.0096$) as significant determiners of NVR. A combination of IRRDR ≥6 and age <60 years improved SVR predictability (93.3 %), and that of IRRDR ≤5 and age ≥60 years improved non-SVR predictability (84.0 %). Similarly, a combination of IL28B minor and platelets $<15 \times 10^4/mm^3$ improved NVR predictability (85.7 %), and that of IL28B major and platelets $\geq 15 \times 10^4/mm^3$ improved non-NVR (response) (97.1 %) predictability.
Conclusion IRRDR ≥6 and age <60 years were significantly associated with SVR. IL28B minor and platelets $<15 \times 10^4/mm^3$ were significantly associated with NVR. Certain combinations of these factors improved SVR and NVR predictability and could, therefore, be used to design therapeutic strategies.

Keywords IRRDR · IL28B · SVR · Relapse · NVR

S. R. Kim (✉) · S. Imoto · K. I. Kim
Department of Gastroenterology, Kobe Asahi Hospital, 3-5-25 Bououji-cho, Nagata-ku, Kobe 653-0801, Japan
e-mail: asahi-hp@arion.ocn.ne.jp

A. El-Shamy · Y. Ide · L. Deng · I. Shoji · H. Hotta
Division of Microbiology, Center for Infectious Diseases, Kobe University Graduate School of Medicine, Kobe, Japan

A. El-Shamy
Department of Virology, Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt

Y. Tanaka
Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

Y. Hasegawa
Educational Center for Clinical Pharmacy, Kobe Pharmaceutical University, Kobe, Japan

M. Ota
Medical Biochemistry, Kobe Pharmaceutical University, Kobe, Japan

Introduction

Hepatitis C virus (HCV) is the major cause of chronic liver diseases worldwide [1]. As a consequence of the long-term persistence of chronic hepatitis C (CHC), the number of patients with hepatocellular carcinoma is expected to increase over the next 20 years [2]. To reduce the impact of this worldwide health problem, efficient treatment is required. Currently, combination therapy with pegylated

Published online: 24 March 2012

interferon-α and ribavirin (PEG-IFN/RBV) is the standard treatment for CHC. The therapy is sometimes not easily tolerated, however, and sustained virological response (SVR) is achieved in only ~50 % of patients, with SVR rarely being achieved in those infected with the most resistant genotypes—HCV-1a and HCV-1b involving high viral loads [3]. In Japan, the most common genotype is HCV-1b. Given the considerable side effects of the PEG-IFN/RBV therapy, the possibility of its discontinuation, and its high cost, being able to predict treatment outcome is desirable. A wide range of predictors would assist clinicians and patients in more accurately assessing the likelihood of SVR and thus in making more informed treatment decisions [4]. One of the most reliable methods of predicting response is to monitor the early drop in serum HCV RNA levels during treatment [5]; however, there is no established method of predicting such an outcome before treatment [6].

Although host factors including age, sex, ethnicity, platelets, liver fibrosis, obesity, and viral factors including genotype and viral load have been associated with the outcome of PEG-IFN/RBV therapy [6], little was known until recently about host genetic factors and viral genetic polymorphisms within a given genotype of HCV that might be associated with response to the therapy. Recent reports have revealed factors associated with response to PEG-IFN/RBV therapy: single nucleotide polymorphisms, as host genetic factors, located in interleukin (IL) 28B (rs8105790, rs11881222, rs8103142, rs28416813, rs4803219, rs8099917, rs7248668, and rs12979860) on chromosome 19 [7–10] and amino acid (aa) substitutions in non-structural protein 5a (NS5A), especially those in the IFN/RBV resistance-determining region (IRRDR) [11–13] and the IFN sensitivity-determining region (ISDR) [14], and the core region of HCV [15, 16], as viral genetic polymorphisms.

In this study, we compare the impact of host genetic factors such as IL28B and viral genetic polymorphisms including those in IRRDR, ISDR, and core mutations of HCV, as pretreatment predictive factors of PEG-IFN/RBV treatment outcome, and aim at establishing a rational strategy for the treatment of CHC patients infected with HCV-1b with high viral loads.

Methods

Patients

A total of 75 patients (43 men and 32 women; median age 60 years; range 30–74) who completed PEG-IFN/RBV combination therapy for 48 weeks were enrolled in the study. They were seen at Kobe Asahi Hospital in Kobe, Japan, and diagnosed with chronic HCV-1b infection on the basis of the presence of anti-HCV antibodies and HCV RNA. Informed consent in writing was obtained from each patient, and the study protocol, conforming to ethical guidelines, was approved by the Ethics Committee of Kobe Asahi Hospital. The HCV genotype was determined according to the method of Okamoto et al. [17]. The inclusion and exclusion criteria for the 75 patients in this study were as follows: patients were required to have hemoglobin levels of ≥11 g/dL (women) or ≥12 g/dL (men), platelet counts of $\geq 9 \times 10^4/mm^3$, HCV RNA ≥5.0 Log IU/mL, neutrophil count $\geq 1500/mm^3$, and thyroid-stimulating hormone levels within normal limits. Patients were excluded if they had human immunodeficiency virus (HIV) or hepatitis B coinfection, creatinine clearance <50 mL/min, cause of liver disease other than CHC, evidence of advanced liver disease, preexisting psychiatric conditions, or a history of severe psychiatric disorder. Patients were treated with PEG-IFN α-2b (1.5 μg/kg body weight, once a week subcutaneously) and RBV (600–1000 mg daily, per os) for 48 weeks, according to the standard treatment protocol for Japanese patients established by a hepatitis study group of the Ministry of Health, Labor and Welfare, Japan. Serum samples were collected from the patients at intervals of 4 weeks before, during, and after the treatment, and tested for HCV RNA based on the COBAS TaqMan HCV test (Roche Diagnostics, Basel, Switzerland).

Sequence analysis of HCV NS5A and HCV core regions

HCV RNA was extracted from 140 μL of serum with the use of a commercially available kit (QIAmp viral RNA kit; QIAGEN, Tokyo, Japan). Amplification of full-length NS5A and the core regions of the HCV genome was carried out as described [11, 12, 18]. The sequences of the amplified fragments of NS5A and the core regions were determined by direct sequencing without subcloning. The aa sequences were deduced and aligned with the use of GENETYX Win software version 7.0 (GENETYX., Tokyo, Japan).

Genetic variation near the IL28B gene

Genetic polymorphism rs8099917 around the IL28B gene was determined by real-time polymerase chain reaction (PCR) with the TaqMan assay [7]. We defined the IL28B major allele as homozygous for the major sequence (TT) and the IL28B minor allele as homozygous (GG) or heterozygous (TG) for the minor sequence.

Statistical analysis

Statistically significant differences in treatment responses according to patient baseline parameters of age, sex, body mass index (BMI), HCV RNA load, alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γ-GTP), hemoglobin, platelets, total cholesterol, and drug doses of PEG-IFN and RBV were determined by the Wilcoxon two-sample test for numerical variables and Fisher's exact probability test for categorical variables. Likewise, statistically significant differences in treatment responses according to NS5A and core mutations and genetic variation near the IL28B gene (genotype TT) were determined by Fisher's exact probability test. Variables with a p value of <0.1 in univariate analysis were included in stepwise multivariate logistic regression analysis. Variables with a p value of <0.05 in multivariate analysis were considered statistically significant. The odds ratio was also calculated. All statistical analyses were carried out with SAS software version 9.2 (SAS, Chicago, IL, USA).

Nucleotide sequence accession numbers

The sequence data reported in this paper have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases under accession numbers AB285035 through AB285081, AB354116 through AB354118, and AB518774 through AB518861.

Results

Patient responses to PEG-IFN/RBV combination therapy

Among the 75 patients enrolled in this study, rapid virological response (RVR) at week 4 was achieved by 13 % (10/75), complete early virological response (cEVR) at week 12 by 60 % (45/75), end-of-treatment response (ETR) by 72 % (54/75), and SVR by 49 % (37/75). SVR was seen in 90 % (9/10), 76 % (34/45), and 69 % (37/54) of the RVR, cEVR, and ETR patients, respectively (data not shown). Continuous viremia throughout the observation period (72 weeks), referred to as null virological response (NVR), was observed in 24 % (18/75), while transient disappearance of serum HCV RNA at a certain point in time followed by a rebound in viremia either before or after the end of the treatment course, referred to as a relapse, was observed in 27 % (20/75).

The numbers of patients who received ≥1.4 μg/kg/week of the dose of PEG-IFN were 23 of 37 in SVR, 15 of 20 in relapse, and 14 of 18 in NVR. Similarly, the numbers of patients who received ≥11.0 mg/kg/day of the dose of RBV were 16 of 37 in SVR, 7 of 20 in relapse, and 6 of 18 in NVR.

Correlation between patient demographic characteristics and treatment responses

The baseline characteristics and the clinical responses of the patients are shown in Table 1. By univariate analysis, sex, BMI, HCV RNA, ALT, total cholesterol levels, and drug doses of PEG-IFN and RBV showed no significant difference between SVR and non-SVR (relapse *plus* NVR) patients. SVR patients were significantly younger ($p = 0.0018$) with a higher level of hemoglobin ($p = 0.0049$) than non-SVR patients. Relapse patients were significantly older ($p = 0.0071$) than SVR patients. NVR patients had a significantly higher level of γ-GTP ($p = 0.07$) and lower level of hemoglobin ($p = 0.0020$) with fewer platelets ($p = 0.0016$) than response (SVR plus relapse) patients (Table 1).

Correlation between the number of NS5A mutations and treatment responses

Using receiver operating characteristic curve analysis, the optimal cutoff number of mutations in IRRDR for predicting SVR has been estimated at 6 [12, 13]. By univariate analysis, examination of a possible correlation between IRRDR mutations and treatment responses revealed that among 30 patients infected with HCV isolates involving 6 or more IRRDR mutations (IRRDR ≥6), SVR was achieved by 80 % (24/30), relapse was shown by 10 % (3/30), and NVR was shown by 10 % (3/30). By contrast, among 45 patients infected with HCV isolates involving 5 or fewer mutations (IRRDR ≤5), SVR was achieved by 29 % (13/45), relapse was shown by 38 % (17/45), and NVR was shown by 33 % (15/45). There was a significant difference in the proportion of HCV isolates involving IRRDR ≥6 and those involving IRRDR ≤5 between SVR and non-SVR patients ($p = 0.00002$), between SVR and relapse patients ($p = 0.00035$), and between response and NVR patients ($p = 0.027$) (Table 1). Notably, among the 30 patients infected with HCV isolates of IRRDR ≥6, 24 (80 %) achieved SVR, suggesting that IRRDR ≥6 could predict SVR with a positive predictive value of 80 %.

Examination of the possible correlation between treatment response and ISDR mutation at a cutoff point of 2 mutations, a newly proposed ISDR criterion for PEG-IFN/RBV responsiveness [14], revealed that among 18 patients infected with HCV isolates involving 2 or more ISDR mutations (ISDR ≥2), SVR was achieved by 56 % (10/18), relapse was shown by 11 % (2/18), and NVR was shown by 33 % (6/18). By contrast, among 57 patients infected with HCV isolates involving ISDR ≤1, SVR was achieved

Table 1 Univariate analysis of factors associated with SVR, relapse, and NVR

Factor	All	SVR	Relapse	NVR	*p* value		
					SVR versus non-SVR	SVR versus relapse	Response versus NVR
n	75	37	20	18	–	–	–
Age (years)	60 (30–74)	57 (33–70)	63 (30–74)	63 (40–71)	**0.0018**	**0.0071**	0.111
Sex: male/female	43/32	23/14	11/9	9/9	0.486	0.778	0.587
BMI (kg/m^2)	22.2 (15.7–37.6)	22.1 (18.3–37.6)	21.9 (15.7–30.7)	23.0 (16.6–31.3)	0.844	0.357	0.298
HCV RNA (Log IU/mL)	6.2 (5.0–7.1)	6.2 (5.0–7.1)	6.2 (5.3–6.7)	6.2 (5.3–7.1)	0.727	0.913	0.606
ALT (U/L)	38 (8–265)	37 (11–174)	37 (10–265)	41 (8–148)	0.618	0.493	0.896
γ-GTP (U/L)	32 (9–406)	32 (9–406)	25 (9–127)	44 (20–151)	0.614	0.503	**0.07**
Hemoglobin (g/dL)	14.0 (11.0–18.6)	14.4 (11.9–18.6)	14.3 (11.0–16.1)	13.2 (12.0–14.5)	**0.0049**	0.213	**0.0020**
Platelets ($\times 10^4$/mm^3)	16.3 (9.1–30.9)	16.9 (9.1–30.9)	18.9 (9.8–25.2)	12.1 (9.1–21.8)	0.124	0.802	**0.0016**
Total cholesterol (mg/dL)	176 (99–248)	181 (106–248)	164 (100–230)	182 (99–237)	0.572	0.243	0.617
PEG-IFN (μg/kg/week): $<1.4/\geq 1.4$	23/52	14/23	5/15	4/14	0.184	0.326	0.373
Ribavirin (mg/kg/day): $<11.0/\geq 11.0$	46/29	21/16	13/7	12/6	0.422	0.545	0.594
IRRDR mutations: $\leq 5/\geq 6$	45/30	13/24	17/3	15/3	**0.00002**	**0.00035**	**0.027**
ISDR mutations: $\leq 1/\geq 2$	57/18	27/10	18/2	12/6	0.597	0.182	0.346
HCV core aa 70: wild/mutant	51/24	30/7	12/8	9/9	**0.025**	0.117	**0.083**
HCV core aa 91: wild/mutant	53/22	27/10	14/6	12/6	0.801	1.000	0.768
IL28B genotype: major/minor	57/18	34/3	16/4	7/11	**0.0024**	0.226	**0.0000095**

Values in bold are significant

SVR sustained virological response, *NVR* null virological response, *non-SVR* relapse *plus* NVR, *Response* non-NVR (SVR *plus* relapse), *BMI* body mass index, *ALT* alanine aminotransferase, *γ-GTP* gamma-glutamyl transpeptidase, *IRRDR* interferon/ribavirin resistance-determining region, *ISDR* interferon sensitivity-determining region, *IL* interleukin, *HCV* hepatitis C virus, *PEG-IFN* pegylated interferon

by 47 % (27/57), relapse was shown by 32 % (18/57), and NVR was shown by 21 % (12/57). Although a significant correlation was observed between ISDR heterogeneity and early virological response such as RVR ($p = 0.028$) (data not shown), no significant correlation was observed between ISDR heterogeneity and late virological response such as SVR, relapse, and NVR (Table 1). In this connection, ISDR heterogeneity at a cutoff point of one mutation (ISDR ≥ 1 vs. ISDR $= 0$) was also not significantly associated with treatment outcome (data not shown).

Correlation between core mutations and treatment responses

Examination of the possible correlation of either arginine at position 70 (Arg^{70}) or leucine at position 91 (Leu^{91}) of the core protein of HCV with treatment responses [15] revealed that among 51 patients infected with HCV core aa 70 wild (Arg^{70}), SVR was achieved by 59 % (30/51), relapse was shown by 24 % (12/51), and NVR was shown by 18 % (9/51). By contrast, among 24 patients infected with HCV core aa 70 mutant (non-Arg^{70}), SVR was achieved by 29 % (7/24), relapse was shown by 33 % (8/24), and NVR was shown by 38 % (9/24). There was a significant difference in the proportion of HCV core aa 70 wild and mutant between SVR and non-SVR patients ($p = 0.025$), and between response and NVR patients ($p = 0.083$). No significant correlation was observed between HCV core aa 91 heterogeneity and virological responses (Table 1).

Correlation between the genetic variation near the IL28B gene and treatment responses

The frequency of allele rs8099917 among the patients was 76 % for TT (57/75), 4 % for TG (3/75), and 20 % for GG (15/75). Univariate analysis revealed that among patients with genotype TT (IL28B major), SVR was achieved by 60 % (34/57), relapse was shown by 28 % (16/57), and NVR was shown by 12 % (7/57). By contrast, among patients with TG or GG (IL28B minor), SVR was achieved by 17 % (3/18), relapse was shown by 22 % (4/18), and NVR was shown by 61 % (11/18). There was a significant

difference in the proportion of IL28B major and minor between SVR and non-SVR patients ($p = 0.0024$), and between response and NVR patients ($p = 0.0000095$) (Table 1).

Identification of independent predictive factors for SVR, relapse, and NVR by multivariate logistic regression analysis

Factors significantly associated with certain virological responses were identified by multivariate analysis: IRRDR ≥6 [odds ratio (OR) 11.906, $p < 0.0001$] and age <60 years (OR 0.228, $p = 0.015$) were significantly associated with SVR; IRRDR ≤5 (OR 0.070, $p = 0.0008$) and age ≥60 years (OR 5.825, $p = 0.015$) with relapse; and IL28B minor (OR 14.618, $p = 0.0019$) and platelets $<15 \times 10^4/mm^3$ (OR 0.113, $p = 0.0096$) with NVR (Table 2).

Positive predictive values of combinations of IRRDR mutation and age for SVR

As stated above, IRRDR ≥6 predicted SVR with a positive value of 80 % (24/30) (Table 1). Assessment of the predictability of SVR by combinations of IRRDR mutation and age, the two most potent factors identified by multivariate analysis, revealed that IRRDR ≥6 and age <60 years predicted SVR with a positive value of 93.3 % (14/15) and that IRRDR ≤5 and age ≥60 years predicted non-SVR with a value of 84.0 % (21/25) (Table 3).

Positive predictive values of combinations of IL28B and platelets for NVR

Based on their significant correlation with NVR as demonstrated by multivariate analysis, combinations of IL28B genotype and platelets were examined for their positive predictive values for NVR. IL28B minor and platelets $<15 \times 10^4/mm^3$ predicted NVR with a positive value of 85.7 % (6/7). On the other hand, IL28B major and platelets $\geq 15 \times 10^4/mm^3$ predicted viral disappearance either transiently (relapse) or sustainably (SVR), referred to as response, with a value of 97.1 % (34/35) (Table 4).

Positive predictive values of combinations of IRRDR mutation and IL28B for SVR and non-NVR (response)

Significant correlation was observed between IRRDR and IL28B ($p = 0.003768$) (data not shown). The combination of IRRDR ≥6 and IL28B major predicted SVR with a positive value of 82.1 % (23/28), and predicted non-NVR (response) with a value of 92.9 % (26/28). On the other hand, IRRDR ≤5 and IL28B minor predicted non-SVR with a value of 87.5 % (14/16) (Table 5).

Positive predictive values of combinations of IRRDR mutation and HCV core aa 70 for SVR and non-NVR (response)

Combinations of IRRDR ≥6 and HCV core aa 70 wild predicted SVR with a positive value of 82.6 % (19/23), and predicted non-NVR (response) with a value of 91.3 %

Table 2 Multivariate analysis of factors associated with SVR, relapse, and NVR

Factor	Category	SVR		Relapse		NVR	
		Odds ratio (95 % CI)	*p* value	Odds ratio (95 % CI)	*p* value	Odds ratio (95 % CI)	*p* value
IRRDR mutations	≤5	1	**<0.0001**	1	**0.0008**	NA	NA
	≥6	11.906 (3.421–41.440)		0.070 (0.015–0.331)			
Age (years)	<60	1	**0.015**	1	**0.015**	NA	NA
	≥60	0.228 (0.069–0.749)		5.825 (1.415–23.980)			
HCV core aa 70	Wild	1	0.112	NA	NA	NA	NA
	Mutant	0.358 (0.101–1.270)					
IL28B genotype	Major	NA	NA	NA	NA	1	**0.0019**
	Minor					14.618 (2.699–79.173)	
Platelets ($\times 10^4/mm^3$)	<15	NA	NA	NA	NA	1	**0.0096**
	≥15					0.113(0.022–0.588)	
γ-GTP (U/L)	<80	NA	NA	NA	NA	NA	NA
	≥80						
Hemoglobin (g/dL)	<14	NA	NA	NA	NA	1	0.105
	≥14					0.274 (0.057–1.309)	

SVR sustained virological response, *NVR* null virological response, *95 % CI* 95 % confidence interval, *γ-GTP* gamma-glutamyl transpeptidase, *IRRDR* interferon/ribavirin resistance-determining region, *NA* not applicable

Table 3 Positive predictive values of combinations of IRRDR mutation and age for SVR

IRRDR mutations	Age (years)	SVR	Non-SVR	Odds ratio (95 % CI)	*p* value
≥6	**<60**	**14/15 (93.3 %)**	1/15 (6.7 %)	73.481 (7.418–727.850)	0.0002
≥6	≥60	10/15 (66.7 %)	5/15 (33.3 %)	10.500 (2.308–47.777)	0.0024
≤5	<60	9/20 (45.0 %)	11/20 (55.0 %)	4.295 (1.075–17.167)	0.0392
≤5	**≥60**	4/25 (16.0 %)	**21/25 (84.0 %)**	1	–

SVR sustained virological response, *IRRDR* interferon/ribavirin resistance-determining region, *95 % CI* 95 % confidence interval

Table 4 Positive predictive values of combinations of IL28B genotype and baseline platelets for NVR

IL28B genotype	Platelets ($\times 10^4/mm^3$)	NVR	Response	Odds ratio (95 % CI)	*p* value
Major	**≥15**	1/35 (2.8 %)	**34/35 (97.1 %)**	1	–
Major	<15	6/22 (27.3 %)	16/22 (72.7 %)	12.750 (1.414 to 114.931)	0.023
Minor	≥15	5/11 (45.5 %)	6/11 (54.5 %)	28.333 (2.796 to 287.103)	0.0047
Minor	**<15**	**6/7 (85.7 %)**	1/7 (14.3 %)	203.999 (11.174 to >999.999)	0.0003

NVR null virological response, *Response* non-NVR (SVR *plus* relapse), *95 % CI* 95 % confidence interval

(21/23). On the other hand, IRRDR ≤5 and HCV core aa 70 mutant predicted non-SVR with a value of 88.2 % (15/17) (Table 6).

Discussion

Host factors (such as age, sex, ethnicity, platelets, liver fibrosis, and obesity) and viral factors (genotype and viral load) have been associated with the outcome of PEG-IFN/RBV therapy [6]. To date, few studies have compared the impact of viral genetic polymorphisms, such as IRRDR, ISDR, and core mutations, and IL28B polymorphisms as host genetic factors on the clinical outcome of PEG-IFN/RBV therapy. Recently, viral genetic polymorphisms including double-wild in the core region, IRRDR ≥6, and ISDR ≥2 have been described as significant predictors of SVR to PEG-IFN/RBV therapy for 48 weeks [13, 19]. IL28B major genotype (TT) and core aa 70 substitutions are independent predictors of SVR, and IL28B minor genotype is an independent predictor of NVR [20]. Also, IL28B polymorphisms and mutations in the ISDR of HCV are significant pretreatment predictors of response to PEG-IFN/RBV therapy [21]. Nonetheless, IRRDR polymorphism, which is a potent viral determiner of SVR [11–13], was not examined in these studies.

In the present study we compared the impact of IRRDR, ISDR, and core mutations as viral genetic polymorphisms, and IL28B genotype as a host genetic factor, on the clinical outcome of PEG-IFN/RBV therapy—SVR, relapse, and NVR—for CHC-1b with a high viral load. IRRDR ≥6 was identified as a viral genetic polymorphism that independently predicted SVR to PEG-IFN/RBV treatment (Tables 1, 2). Moreover, IRRDR ≤5 was identified as a viral genetic polymorphism that most effectively predicted relapse, and IL28B minor genotype (TG or GG) was identified as a host genetic factor that most effectively predicted NVR.

On the other hand, ISDR ≥2 was not significantly associated with treatment outcome in the present cohort, although it is considered a viral determiner of SVR [19, 21]. ISDR was identified as a factor showing significant correlation with RVR ($p = 0.028$) by univariate analysis (data not shown). In other words, ISDR was a factor related to only early viral dynamics.

The C-terminal region of NS5A such as IRRDR is among the most variable sequences across the different genotypes and subtypes of HCV [22, 23]. The correlation observed between IRRDR heterogeneity and PEG-IFN/RBV responsiveness might be linked to experimental observations that an HCV subgenomic RNA replicon containing NS5A of HCV-1b exerts more profound inhibitory effects on IFN activities than does its original HCV-2a replicon, and that domain swapping of a C-terminal region of NS5A including IRRDR results in a transfer of their anti-IFN activities [24]. Moreover, the C-terminal region of NS5A has been implicated as playing important roles in viral replication and particle formation [25, 26]. These clinical and experimental data thus support our hypothesis that IRRDR is involved, at least partly, in the viral strategy of evading IFN-mediated antiviral host defense mechanisms. Similarly, the aa substitutions in the core region are associated with proteins involved in resistance to IFN monotherapy, such as SOCS, which are

Table 5 Positive predictive values of combinations of IRRDR mutation and IL28B genotype for SVR and non-NVR (response)

IRRDR mutations	IL28B genotype	SVR	Non-SVR	SVR versus non-SVR		NVR	Response	NVR versus response	
				Odds ratio (95 % CI)	*p* value			Odds ratio (95 % CI)	*p* value
≥6	**Major**	**23/28 (82.1 %)**	5/28 (17.9 %)	32.200 (5.489–188.909)	0.0001	2/28 (7.1 %)	**26/28 (92.9 %)**	1	–
≥6	Minor	1/2 (50.0 %)	1/2 (50.0 %)	7.000 (0.302–162.202)	0.225	1/2 (50.0 %)	1/2 (50.0 %)	13.000 (0.572–295.204)	0.107
≤5	Major	11/29 (37.9 %)	18/29 (62.1 %)	4.278 (0.813–22.513)	0.0863	5/29 (17.2 %)	24/29 (82.8 %)	2.708 (0.480–15.294)	0.259
≤5	Minor	2/16 (12.5 %)	**14/16 (87.5 %)**	1	–	10/16 (62.5 %)	6/16 (37.5 %)	21.667 (3.733–125.766)	0.0006

SVR sustained virological response, *NVR* null virological response, *Response* non-NVR (SVR *plus* relapse), *IRRDR* interferon/ribavirin resistance-determining region, *95 % CI* 95 % confidence interval

Table 6 Positive predictive values of combinations of IRRDR mutation and HCV core aa 70 for SVR and non-NVR (response)

IRRDR mutations	HCV core aa 70	SVR	Non-SVR	SVR versus non-SVR		NVR	Response	NVR versus response	
				Odds ratio (95 % CI)	*p* value			Odds ratio (95 % CI)	*p* value
≥6	**Wild**	**19/23 (82.6 %)**	4/23 (17.4 %)	35.625 (5.730–221.504)	0.0001	2/23 (8.7 %)	**21/23 (91.3 %)**	1	–
≥6	Mutant	5/7 (71.4 %)	2/7 (28.6 %)	18.750 (2.065–170.214)	0.0092	1/7 (14.3 %)	6/7 (85.7 %)	1.750 (0.134–22.778)	0.669
≤5	Wild	11/28 (39.3 %)	17/28 (60.7 %)	4.853 (0.924–25.496)	0.062	7/28 (25.0 %)	21/28 (75.0 %)	3.500 (0.650–18.852)	0.145
≤5	**Mutant**	2/17 (11.8 %)	**15/17 (88.2 %)**	1	–	8/17 (47.1 %)	9/17 (52.9 %)	9.333 (1.6346–52.917)	0.012

SVR sustained virological response, *NVR* null virological response, *Response* non-NVR (SVR *plus* relapse), *IRRDR* interferon/ribavirin resistance-determining region, *95 % CI* 95 % confidence interval

known to inhibit IFN-α-induced activation of the Jak-STAT pathway and the expression of the antiviral proteins 2′,5′-OAS and MxA [27].

The IL28B gene encodes a cytokine distantly related to type I (α and β) IFN and to the IL10 family. IL28B, IL28A, and IL29 are three closely related cytokine genes that encode proteins known as type III IFN (IFN-λs) and form a cytokine gene cluster at chromosomal region 19q13 [28]. The three cytokines IFN-λ1, -λ2, and -λ3 are induced by viral infection and have antiviral activities [29, 30]: IFN-λ induces a steady increase in the expression of a subset of IFN-stimulated genes, whereas IFN-α induces the same genes with more rapid and transient kinetics [31].

In the present study, the prediction of response to PEG-IFN/RBV combination therapy based on these concurrent factors was highly positive: SVR was positively predicted in 93.3 % of patients with IRRDR ≥6 and age <60 years (Table 3), in 82.1 % of those with IRRDR ≥6 and IL28B major (Table 5), and in 82.6 % of those with IRRDR ≥6 and HCV core aa 70 wild (Table 6). Relapse was positively predicted in 73.3 % of patients with IRRDR ≤5 and age ≥60 years, and in 77.8 % of those with IRRDR ≤5 and HCV core aa 70 mutant (data not shown). NVR was positively predicted in 85.7 % of patients with IL28B minor and platelets $<15 \times 10^4/mm^3$ (Table 4). On the basis of these observations, new therapeutic strategies could be designed for treating chronic HCV-1b infection: patients predicted to achieve an SVR would be most eligible for standard PEG-IFN/RBV therapy for 48 weeks, those predicted to relapse could be advised to adopt an extended 72-week therapy instead of the 48-week standard therapy [30], and those predicted to have NVR could be advised to wait for a future therapy such as a combination of protease inhibitors [32, 33].

In conclusion, viral genetic polymorphisms in IRRDR (≥6 or ≤5 mutations) and HCV core aa 70 (wild or mutant), host factors such as IL28B genotype (major or minor), age (<60 or ≥60 years), and platelet counts ($\geq 15 \times 10^4/mm^3$ or less), and combinations of these factors could be used to design therapeutic strategies for patients infected with HCV-1b with high viral loads. Further prospective study is needed to verify this hypothesis.

Acknowledgments This work was supported in part by Grants-in-Aid for Research on Hepatitis from the Ministry of Health, Labor and Welfare, Japan, and a SATREPS Grant from JST and JICA. The study was also carried out as part of the J-GRID Program, Ministry of Education, Culture, Sports, Science and Technology, Japan, and the G-COE Program at Kobe University Graduate School of Medicine. We are indebted to Ms. Yoshiko Kawamura of Kobe Asahi Hospital for assistance in the preparation of the manuscript.

Conflict of interest None of the authors has any conflict of interest.

References

1. Bialek SR, Terrault NA. The changing epidemiology and natural history of hepatitis C virus infection. Clin Liver Dis. 2006;10:697–715.
2. Williams R. Global challenges in liver disease. Hepatology. 2006;44:521–6.
3. Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med. 2002;347:975–82.
4. Backus LI, Boothroyd DB, Phillips BR, Mole LA. Predictors of response of US veterans to treatment for the hepatitis C virus. Hepatology. 2007;46:37–47.
5. Davis GL, Wong JB, McHutchison JG, Manns MP, Harvey J, Albrecht J. Early virologic response to treatment with peginterferon alfa-2b plus ribavirin in patients with chronic hepatitis C. Hepatology. 2003;38:645–52.
6. Kurosaki M, Sakamoto N, Iwasaki M, Sakamoto M, Suzuki Y, Hiramatsu N, et al. Pretreatment prediction of response to peginterferon plus ribavirin therapy in genotype 1 chronic hepatitis C using data mining analysis. J Gastroenterol. 2011;46:401–9.
7. Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, et al. Genome-wide association of IL28B with response to pegylated interferon-α and ribavirin therapy for chronic hepatitis C. Nat Genet. 2009;41:1105–9.
8. Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature. 2009;461:399–401.
9. Suppiah V, Moldovan M, Ahlenstiel G, Ber T, Weltman M, Abate ML, et al. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet. 2009;41:1105–9.
10. Rauch A, Kutalik Z, Descombes P, Cai T, Di Iulio J, Mueller T, et al. Genetic variation in IL28B is associated with chronic hepatitis C and treatment failure—a genome-wide association study. Gastroenterology. 2010;138:1338–45.
11. El-Shamy A, Sasayama M, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, et al. Prediction of efficient virological response to pegylated interferon/ribavirin combination therapy by NS5A sequences of hepatitis C virus and anti-NS5A antibodies in pretreatment sera. Microbiol Immunol. 2007;51:471–82.
12. El-Shamy A, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, Hotta H. Sequence variation in hepatitis C virus nonstructural protein 5A predicts clinical outcome of pegylated interferon/ribavirin combination therapy. Hepatology. 2008;48:38–47.
13. El-Shamy A, Kim SR, Ide YH, Sasase N, Imoto S, Deng L, et al. Polymorphisms of hepatitis C virus non-structural protein 5A and core protein and clinical outcome of pegylated-interferon/ribavirin combination therapy. Intervirology. 2012;55:1–11.
14. Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, et al. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. N Engl J Med. 1996;11(334):77–81.
15. Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, et al. Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b: amino acid substitutions in the core region and low-density lipoprotein cholesterol levels. J Hepatol. 2007;6:403–10.
16. Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, et al. Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and

non-virological response to interferon–ribavirin combination therapy. Intervirology. 2005;48:372–80.
17. Okamoto H, Sugiyama Y, Okada S, Kurai K, Akahane Y, Sugai Y, et al. Typing hepatitis C virus by polymerase chain reaction with type-specific primers: application to clinical surveys and tracing infectious sources. J Gen Virol. 1992;73:673–9.
18. Ogata S, Nagano-Fujii M, Ku Y, Yoon S, Hotta H. Comparative sequence analysis of the core protein and its frameshift product, the F protein, of hepatitis C virus subtype 1b strains obtained from patients with and without hepatocellular carcinoma. J Clin Microbiol. 2002;40:3625–30.
19. Fukuhara T, Taketomi A, Okano S, Ikegami T, Soejima Y, Shirabe K, et al. Mutations in hepatitis C virus genotype 1b and the sensitivity of interferon–ribavirin therapy after liver transplantation. J Hepatol. 2010;52:672–80.
20. Hayes CN, Kobayashi M, Akuta N, Suzuki F, Kumada H, Abe H, et al. HCV substitutions and IL28B polymorphisms on outcome of peg-interferon plus ribavirin combination therapy. Gut. 2011; 60:261–7.
21. Kurosaki M, Tanaka Y, Nishida N, Sakamoto N, Enomoto N, Honda M, et al. Pre-treatment prediction of response to pegylated-interferon plus ribavirin for chronic hepatitis C using genetic polymorphism in IL28B and viral factors. J Hepatol. 2011;54:439–48.
22. Macdonald A, Harris M. Hepatitis C virus NS5A: tales of a promiscuous protein. J Gen Virol. 2004;85:2485–502.
23. El-Shamy A, Shoji I, Kim SR, Ide Y, Imoto S, Deng L, et al. Sequence heterogeneity in NS5A of hepatitis C virus genotypes 2a and 2b and clinical outcome of pegylated-interferon/ribavirin therapy. PLoS One. 2012;7(2):e30513.
24. Tsai YH, Kuang WF, Lu TY, Kao JH, Lai MY, Liu CJ, et al. The non-structural 5A protein of hepatitis C virus exhibits genotypic differences in interferon antagonism. J Hepatol. 2008;49:899–907.
25. Hughes M, Gretton S, Shelton H, Brown DD, McCormick CJ, Angus AG, et al. A conserved proline between domains II and III of hepatitis C virus NS5A influences both RNA replication and virus assembly. J Virol. 2009;83:10788–96.
26. Tellinghuisen TL, Foss KL, Treadaway J. Regulation of hepatitis C virion production via phosphorylation of the NS5A protein. PLoS Pathog. 2008;4:e1000032.
27. Vlotides G, Sorensen AS, Kopp F, Zitzmann K, Cengic N, Brand S, et al. SOCS-1 and SOCS-3 inhibit IFN-α-induced expression of the antiviral proteins 2,5-OAS and MxA. Biochem Biophys Res Commun. 2004;320:1007–14.
28. Kotenko SV, Gallagher G, Baurin VV, Lewis-Antes A, Shen M, Shah NK, et al. IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. Nat Immunol. 2003; 4:69–77.
29. Sheppard P, Kindsvogel W, Xu W, Henderson K, Schlutsmeyer S, Whitmore TE, et al. IL-28, IL-29 and their class II cytokine receptor IL-28R. Nat Immunol. 2003;4:63–8.
30. Marcello T, Grakoui A, Barba-Spaeth G, Machlin ES, Kotenko SV, MacDonald MR, et al. Interferons alpha and lambda inhibit hepatitis C virus replication with distinct signal transduction and gene regulation kinetics. Gastroenterology. 2006;131:1887–98.
31. Berg T, von Wagner M, Nasser S, Sarrazin C, Heintges T, Gerlach T, et al. Extended treatment duration for hepatitis C virus type 1: comparing 48 versus 72 weeks of peginterferon-alfa-2a plus ribavirin. Gastroenterology. 2006;130:1086–97.
32. McHutchison JG, Everson GT, Gordon SC, Jacobson IM, Sulkowski M, Kauffman R, et al. Telaprevir with peginterferon and ribavirin for chronic HCV genotype 1 infection. N Engl J Med. 2009;360:1827–38.
33. Hézode C, Forestier N, Dusheiko G, Ferenci P, Plo S, Goeser T, et al. Telaprevir and peginterferon with or without ribavirin for chronic HCV infection. N Engl J Med. 2009;360:1839–50.

J Gastroenterol
DOI 10.1007/s00535-009-0042-x

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Down-regulation of hepatic stearoyl-CoA desaturase 1 expression by angiotensin II receptor blocker in the obese *fa/fa* Zucker rat: possible role in amelioration of insulin resistance and hepatic steatosis

Junji Yokozawa · Takashi Sasaki · Kumiko Ohwada · Yayoi Sasaki · Jun-Itsu Ito · Takafumi Saito · Sumio Kawata

Received: 30 June 2008 / Accepted: 5 January 2009

Abstract
Background It has been reported that angiotensin II type 1 receptor blocker (ARB) can ameliorate hepatic steatosis and insulin resistance. Stearoyl-CoA desaturase 1 (SCD-1), which catalyzes the cellular synthesis of monounsaturated fatty acids, affects lipid metabolism. In this study, we investigated whether SCD-1 gene expression is affected by ARB treatment.
Methods Obese *fa/fa* Zucker rats fed a high-fat diet were treated with a potent ARB and olmesartan, and the resulting changes in the components of serum and liver were studied. Gene expression of hepatic SCD-1 was assayed using real-time PCR.
Results The serum glucose and insulin levels and hepatic TG content of the obese Zucker rats fed a high-fat diet were reduced after olmesartan administration, while the serum adiponectin level was increased. Real-time PCR revealed an increase of SCD-1 gene expression in the liver of these rats, followed by a reduction after olmesartan administration. The ratio of stearic acid (C18:0) to oleic acid (C18:1) in the liver was increased by olmesartan, indicating a reduction in the in vivo activity of SCD-1.
Conclusions ARB ameliorates hepatic steatosis and insulin resistance in obese *fa/fa* Zucker rats fed a high-fat diet. Gene expression of SCD-1 is decreased by olmesartan, suggesting that the beneficial effect is due partly to suppression of the key enzyme for hepatic lipid metabolism by ARB.

J. Yokozawa · T. Sasaki · K. Ohwada · Y. Sasaki · J.-I. Ito · T. Saito · S. Kawata (✉)
Department of Gastroenterology, Faculty of Medicine, Yamagata University, 2-2-2 Iida-Nishi, Yamagata 990-9585, Japan
e-mail: kawata@med.id.yamagata-u.ac.jp

Keywords Hepatic steatosis · Insulin resistance · Stearoyl-CoA desaturase 1 · Adiponectin · Angiotensin II type 1 receptor blocker

Introduction

Metabolic syndrome is a cluster of metabolic alterations whose landmarks include visceral obesity, hyperlipidemia, hepatic steatosis, and insulin resistance [1]. A diet with a high carbohydrate and fat content is considered to be a causative factor in the development of insulin resistance in animals and humans [2–6].

Several lines of evidence have suggested that the renin–angiotensin system (RAS) participates in insulin resistance. Adipocytes are known to secrete angiotensinogen and angiotensin II (Ang II) as adipocytokines [7, 8]. Ang II induces insulin resistance via suppression of intracellular signal transduction of insulin and dysregulation of adipocytokines, including TNF-α and adiponectin [9–12].

Recently, blockade of the Ang II signal by Ang II type 1 receptor blocker (ARB) was reported to ameliorate insulin sensitivity in experimental animals and hypertensive patients [13–15], thereby possibly suppressing TNF-α production by skeletal muscle. Also, ARB can ameliorate insulin resistance in patients with essential hypertension by increasing the level of serum adiponectin [16]. On the other hand, large-scale randomized control studies have shown that ARB can prevent the development of diabetes mellitus in patients with essential hypertension [17–19].

Hepatic steatosis is associated with visceral obesity and insulin resistance, and may progress to nonalcoholic steatohepatitis (NASH) under certain circumstances. A low level of serum adiponectin and decreased sensitivity to

Published online: 14 April 2009

leptin are common in hepatic steatosis and NASH. Recently, it was reported that ARB is able to suppress hepatic fibrosis by suppressing the activation of stellate cells, which play a major role in production of the extracellular matrix [20]. However, little is known about how Ang II participates in insulin resistance and hepatic steatosis and how ARB is able to ameliorate these conditions.

Stearoyl-CoA desaturase-1 (SCD-1) is an enzyme that desaturates palmitate, the saturated end-product of de novo fatty acid synthesis. SCD-1 expression and monounsaturated fatty acid levels are markedly increased in livers of leptin-deficient *ob/ob* mice [21] and leptin receptor-deficient (*fa/fa*) Zucker diabetic fatty (ZDF) rats [22]. Leptin treatment reduces expression of the SCD-1 gene in these animals, indicating that it has a regulatory role in SCD-1 gene expression. While elucidation of leptin's role has permitted a detailed view of the biology underlying energy homeostasis, most obese individuals are leptin-resistant [23]. The *ob/ob* mice lacking SCD-1 are significantly less obese than *ob/ob* controls and have histologically normal liver with a significant reduction of both triglyceride (TG) storage and production of very low density lipoprotein (VLDL) [21]. Pharmacologic manipulation of SCD-1 may be of benefit in the treatment of obesity, diabetes, hepatic steatosis, and other components of metabolic syndrome. However, no study has yet investigated whether Ang II can regulate SCD-1 gene expression.

In this study, we investigated whether a potent ARB, olmesartan, is able to ameliorate hepatic steatosis and insulin resistance in obese *fa/fa* Zucker rats, which have a defect in the leptin receptor, fed a high-fat diet, and whether expression of the SCD-1 gene in the liver is affected by olmesartan. The SCD-1 gene was found to be overexpressed in the liver of obese rats fed a high-fat diet relative to the level in obese rats fed a standard diet and showed reduced expression following exposure to olmesartan for 4 weeks.

Materials and methods

Animals

Five-week-old male obese (*fa/fa*) Zucker rats were purchased from Charles River Laboratories Japan Inc. (Kanagawa, Japan). All rats were housed in a temperature-controlled room (20–23°C) with a 12-h light/dark cycle (light on 0600–1800 hours), and had free access to a laboratory standard diet and water. All animal studies were done according to a protocol approved by the Animal Experimentation Committee of Yamagata University Faculty of Medicine, Japan.

Experimental design

At 6 weeks of age, the rats were divided into two groups: obese rats fed a standard diet ($n = 5$) and obese rats fed a high-fat diet ($n = 15$). These diets had the following compositions (as a percentage of total calories): standard diet (CRF-1, Charles River Laboratories Japan Inc., 10% fat, 20% protein, and 70% carbohydrate); high-fat diet [no. D12450B, Research Diets Inc., New Brunswick, NJ, 45% fat (predominantly from lard), 20% protein, and 35% carbohydrate]. All animals were fed standard or high-fat diets for 8 weeks until the end of the experiment. After 4 weeks on the diets, the high-fat diet-fed obese rats ($n = 15$) were further divided into three experimental groups ($n = 5$ rats per group) treated with olmesartan at 1 or 10 mg/kg body/day and treated with vehicle (0.5% carboxymethyl cellulose) alone as the control. Olmesartan (CS-866), a potent ARB, was kindly provided by Daiichi-Sankyo Co. Ltd. (Tokyo, Japan). Standard diet-fed obese rats ($n = 5$ rats per group) were treated with vehicle alone. The drug was administered once daily by oral gavage for 4 weeks.

Blood and liver tissue sampling

After the end of drug treatment, all 14-week-old rats were fasted overnight (13–15 h, food removed at 1800 h) and then killed under ether anesthesia. Blood was rapidly collected from the inferior vena cava, and serum was prepared by centrifugation (3,000 rpm, 10 min, 4°C) of the blood and stored at −20°C until further analysis. The liver tissue was immediately removed and snap-frozen in liquid nitrogen, and stored at −80°C until further study.

Biochemical assay of serum components

Serum glucose (Glu), triglyceride (TG), and free fatty acid (FFA) were measured using enzymatic assay kits (Shino-Test Co., Tokyo; Wako Pure Chemical Industries Ltd., Osaka; Eiken Chemical Co. Ltd., Tokyo, Japan, respectively) on a Hitachi Autoanalyzer 7181 (Hitachi High-Technologies Inc., Tokyo, Japan). Serum levels of insulin and adiponectin were respectively measured using a rat insulin ELISA kit (Shibayagi Co. Ltd., Gunma, Japan) and a rat adiponectin ELISA kit (Otsuka Pharmaceutical Co. Ltd., Tokushima, Japan).

Measurement of triglyceride in liver

Lipid extraction was performed by a modified version of the method described previously by Folch [24]. Liver tissues were homogenized with methanol/chloroform (1/2 v/v, 20 ml/g tissue), and aliquots of the organic phase were evaporated under nitrogen gas. The dried lipid extracts

were dissolved in isopropyl alcohol. TG content within the lipid extracts was measured using an enzymatic assay kit (Wako Pure Chemical Industries) on a Hitachi Autoanalyzer 7181 (Hitachi High-Technologies).

Analysis of liver fatty acid

The procedure used for lipid extraction was the same as that for liver TG measurement [24]. Fatty acids in lipids were analyzed using a modification of the method described previously [25]. The dried lipid extracts were treated with 5% KOH–ethanol/water (9/1 v/v) solution. The hydrolyzed lipids were then mixed with *n*-hexane and water, and extracted into the aqueous phase. Pentadecane acid as an internal control was then added to the aqueous phase. The aqueous phase was homogenized with 6 M HCl and *n*-hexane, and the fatty acids were extracted into *n*-hexane, then dried under nitrogen gas and transmethylated with BF_3–methanol/benzene/methanol (7/6/7 v/v/v) solvent at 100°C. Fatty acid methyl esters were extracted into *n*-pentane and analyzed on a gas chromatograph (HP6890 series; Agilent Technologies Japan Ltd., Tokyo, Japan) equipped with a capillary column (DB-WAX; 30 m × 0.32 mm, 0.25 μm film, Agilent Technologies, Japan). Fatty acid methyl esters were identified by comparison with the internal control. SCD-1 activity index was calculated from the precursor-to-product ratio as stearic acid to oleic acid (C18:0/C18:1).

Expression analysis of SCD-1 mRNA in liver

Liver mRNA levels of SCD-1 were measured by real-time PCR with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. Two-step real-time PCR was performed as described previously [26, 27]. Total RNAs were isolated from liver tissues using an RNeasy Plus Mini Kit (QIAGEN Inc., Hilden, Germany). A cDNA was synthesized from 1 μg of total RNA using a random primer (Takara Bio Inc., Mie, Japan) and SuperScript® II RNase H⁻ Reverse Transcriptase (Invitrogen) in accordance with the manufacturer's instructions. Real-time PCR reactions were performed using a Fast Start DNA Master SYBR I Kit (Roche Diagnostic AG, Basel, Switzerland) on a LightCycler® 2.0 System (Roche Diagnostic). Specific primers were designed using a Perfect Real Time Support System (Takara Bio) and were as follows: SCD-1 (NM1 39192), GCTTGTGGAGCCACAGGACTTAC (forward), ATCCCGGGCCCATTCATATAC (reverse); GAPDH (NM017008), GACAACTTTGGCATCGTGGA (forward), ATGCAGGGATGATGTTCTGG (reverse). PCR reactions for all samples were run in triplicate. Data were analyzed using the LightCycler Software program version 3.5 (Roche Diagnostic). The amounts of all mRNAs were calculated using a standard curve constructed using serial dilutions of a concentrated cDNA sample. The expression level of SCD-1 was normalized with that of GAPDH.

Data analysis and statistics

All data in figures are expressed as mean ± standard error of the mean (SEM). For comparisons between two groups, statistical analysis was performed using unpaired Student's or Welch's *t* test. Mann–Whitney *U* test was used when appropriate. For comparisons among three groups, data were analyzed by one-way ANOVA with the Tukey–Kramer multiple comparisons test. Differences were considered significant at $P < 0.05$.

Results

Amelioration of hyperglycemia, hyperinsulinemia, and insulin resistance by ARB administration

Obese *fa/fa* Zucker rats fed a high-fat diet showed severe hyperglycemia and hyperinsulinemia. Administration of olmesartan at a dose of 10 mg/kg/day for 4 weeks ameliorated the hyperglycemia and hyperinsulinemia in comparison with vehicle treatment in obese rats fed a high-fat diet (glucose: 291.0 ± 20.6 vs. 434.8 ± 52.2 mg/dl, $P < 0.05$; insulin: 29.0 ± 5.3 vs. 49.0 ± 5.6 ng/ml, $P < 0.05$; Fig. 1a, b). These data suggested that ARB is able to ameliorate insulin resistance in obese Zucker rats fed a high-fat diet. The serum concentrations of glucose and insulin in vehicle-treated obese Zucker rats fed a standard diet were 283.8 ± 19.4 mg/dl and 21.3 ± 4.1 ng/ml, respectively.

Decrease in serum FFA level, serum TG level, and hepatic TG content by ARB administration

Olmesartan administration at a dose of 10 mg/kg/day decreased the serum level of FFA in comparison with vehicle treatment in obese rats fed a high-fat diet (697.2 ± 47.5 vs. 1,004.0 ± 215.4 μEQ/l; Fig. 2a), although the difference was not statistically significant. On the other hand, olmesartan administration did not change the serum level of TG (vehicle: 154.6 ± 16.0 vs. olmesartan: 189.6 ± 28.3 and 213.4 ± 31.7 mg/dl; Fig. 2b). Histologically, the hepatocytes of obese rats fed a high-fat diet contained fat droplets in three zones of all hepatic lobules (data not shown). The hepatic TG content was decreased dose-dependently by olmesartan administration at 1 and 10 mg/kg/day (vehicle: 362.9 ± 27.8 vs. olmesartan: 252.8 ± 25.3 and 215.1 ± 21.2 mg/g liver, $P < 0.05$ and $P < 0.005$, respectively; Fig. 2c), suggesting that ARB worked to ameliorate fatty

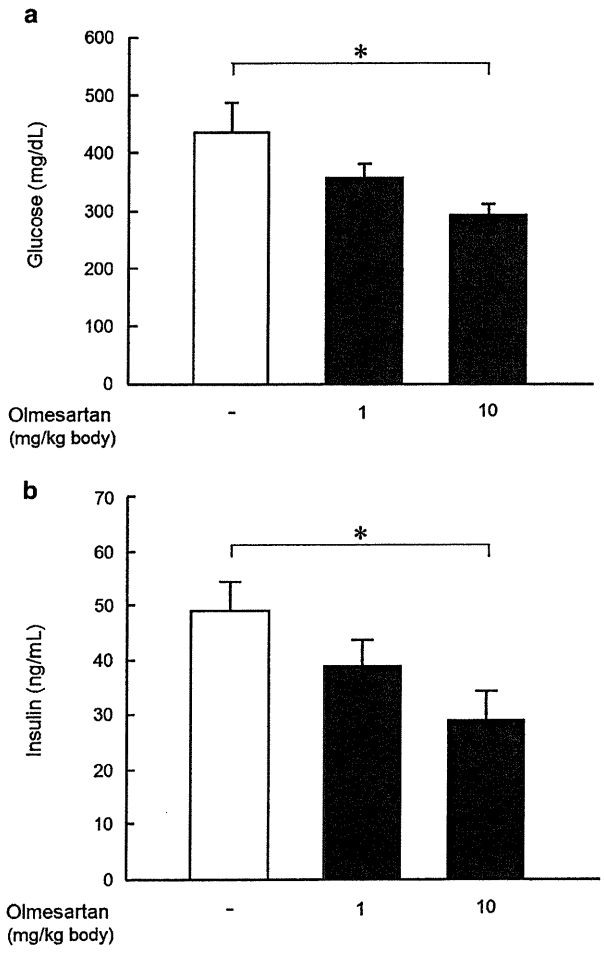


Fig. 1 Effect of olmesartan on serum levels of glucose and insulin in obese Zucker rats fed a high-fat diet. Serum levels of (**a**) glucose and (**b**) insulin in high-fat diet-fed obese Zucker rats treated with olmesartan (1 or 10 mg/kg body/day) for 4 weeks and treated with vehicle alone as the control. High-fat diet was fed to rats for 8 weeks from 6 to 14 weeks of age. Values are mean ± SEM ($n = 5$) for these separate experiments. *$P < 0.05$

liver in obese rats fed a high-fat diet. The serum concentrations of FFA and TG, and the hepatic TG content in obese Zucker rats fed a standard diet were 650.4 ± 80.7 μEQ/l, 554.2 ± 43.8 mg/dl, and 95.1 ± 15.9 mg/g liver, respectively.

Increase of serum adiponectin level by ARB administration

Olmesartan administration at a dose of 10 mg/kg/day increased the serum adiponectin level in comparison with vehicle treatment in obese rats fed a high-fat diet (8.2 ± 0.9 vs. 5.1 ± 0.5 μg/ml, $P < 0.05$; Fig. 3), suggesting a mechanism for improvement of glucose and fat

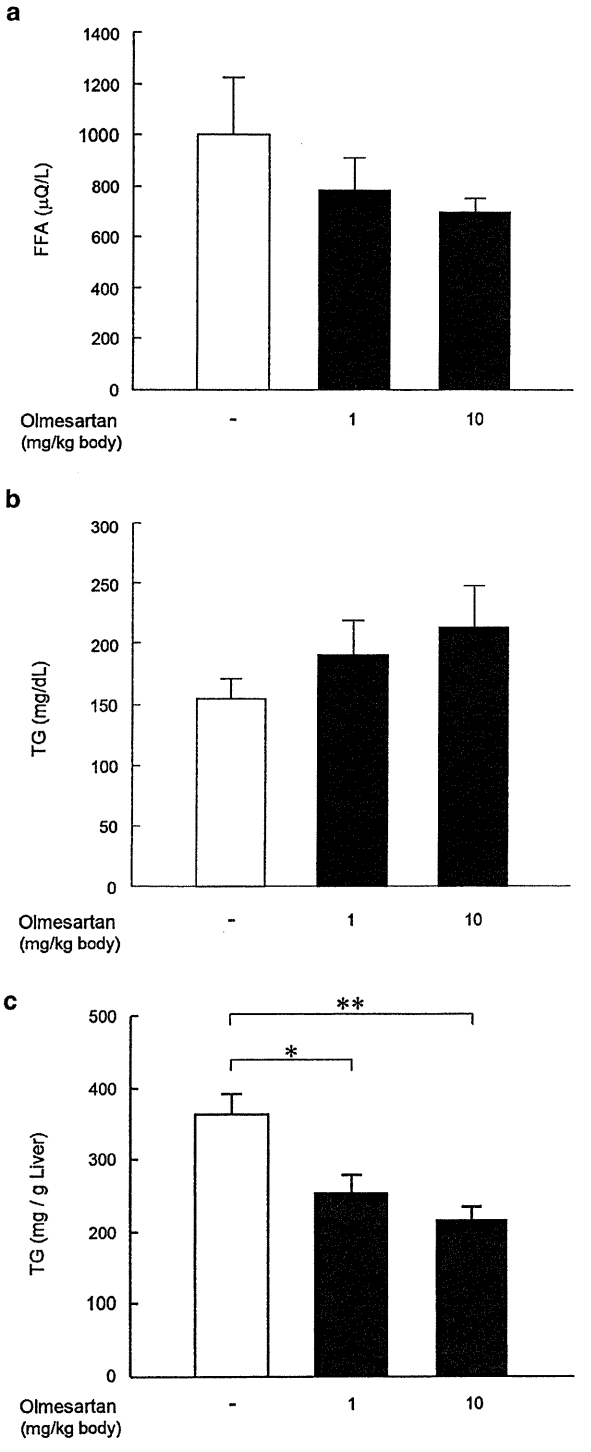


Fig. 2 Effect of olmesartan on serum triglyceride, serum free fatty acid, and liver triglyceride in obese Zucker rats fed a high-fat diet. (**a**) Serum free fatty acid (FFA) level and triglyceride (TG) level in serum (**b**) and liver (**c**) are shown, respectively, in groups of high-fat diet-fed obese rats treated with olmesartan (1 or 10 mg/kg body/day) for 4 weeks and treated with vehicle alone as the control. High-fat diet was fed to rats for 8 weeks from 6 to 14 weeks of age. Values are mean ± SEM ($n = 5$) for these separate experiments. *$P < 0.05$, **$P < 0.01$