Next, we focused on the relationship between *dupA* and *cagA* statuses for *H. pylori*. We measured the *dupA* prevalence of *H. pylori* strains with East Asian-*cagA*, the most dominant *cagA* genotype in Japan, against those with non-East Asian *cagA* status (i.e., the combination of Western-type *cagA*$^+$ and *cagA*$^-$ strains). The 2 strains with hybrid-type *cagA* were excluded from this analysis due to the association between *dupA* and *cagA*. In Okinawa, only 1 *cagA*$^-$ strain (3.8%) of the 26 Western-type *cagA* or *cagA*$^-$ strains was *dupA*$^+$. In contrast, 37 (33.6%) of the 110 Okinawan strains with the East Asian-type *cagA* carried *dupA* (p=0.0013)(**Table 4**). The relationship of *dupA* and *cagA* statuses could not be assessed in Fukui because of the extremely small number of *H. pylori* strains with non-East Asian *cagA* status.

In the 246 tested Japanese strains isolated from both Fukui and Okinawa (excluding F80 and OK204), the associations of *dupA* prevalence with clinical diagnosis, geographic locations, and *cagA* genotypes were analyzed (**Table 5**). In the univariate analysis, only East Asian-*cagA* positivity was related to the presence of dupA (26.5% vs. 3.7%, p=0.0073). In the multivariate analysis, strains isolated from Okinawa and those with East Asian-type *cagA* positivity were significantly associated with an increased probability for *dupA* positive status (odds ratio [OR]=2.128, 95% confidence interval [CI]=1.146-3.949 and OR=12.924, 95% CI=1.689-98.901, respectively). No significant association was found between any tested clinical diagnosis and *dupA* status in both statistical analyses.

Comparison of dupA *sequences and the upstream region between genome-sequenced reference strains*

We first compared *dupA* sequences (corresponding to *jhp0917* and *jhp0918*) and the upstream region between the released genome sequences from 3 *dupA*$^+$ *H. pylori* strains (Shi470, G27, and J99). In strain Shi470, the sequence corresponding to *dupA* (*jhp0917* and *jhp0918*) was part of a continuous 2499 bp gene, *HP_04615. HPG27_963* is a 2500 bp sequence from strain G27, which was isolated from Italy and was homologous to *HP_04615*; however, *HPG27_963* contained a stop codon, resulting in the truncation of this gene product. In J99, the upstream region of *dupA* was entirely different from those of the other 2 strains. In the genome sequences of J99, the direction of *jhp0917*, *jhp0918*, and the downstream genes was opposite to that of *jhp0916*, *jhp0915*, and *jhp0914*, while the series of genes centered by *HP_04615* and *HPG27_963* were aligned in the same direction in the partial genome sequences of Shi470 and G27, respectively (**Fig. 1**). Thus, we hypothesized the "original" *dupA* reported by Lu et al. may be the legacy of a truncated gene that resulted from the deletion of the 5′ region of the full-length *dupA*. Here, we sub-classified *dupA* into 2 genotypes by defining *jhp0917* and *jhp0918* as J99-type *dupA* and *HPSH_04615* as Shi470-type *dupA*. On the basis of these results, a chromosomal alignment was performed from 12 released *H. pylori* genome sequences available in the public database GenBank as of February 2012. Seven strains (Shi470, G27, Cuz20, SouthAfrica7, F57, Puno135, and SNT49) were of the Shi470 type, and 5 strains (J99, HP 908, Gambia 94/24, HP 2017, and HP 2018), of the J99 type. Strains HP 2017 and HP 2018 were the chronological subclones of HP 908.[49] With the exception of G27, the Shi470-type strains carried intact full-length *dupA* consisting of 2499 bp.

Sub-classification of dupA *into 2 genotypes*

On the basis of the published sequences, 3 primer pairs were designed for PCR to distinguish the 2 *dupA* genotypes (the Shi470 and J99 types) (**Table 1** & **Fig. 1**). All 60 *dupA*$^+$ strains in this study (22 from Fukui and 38 from Okinawa) were examined: 58 (96.7%) were of the Shi470 type, 57 were positive for both 960F/963R and 962F/Ra and were of the Shi470 type, 1 strain (OK329) was positive for 962F/Ra but was negative for 960F/963R and was assigned as the Shi470 type, and 2 strains (F80 and OK317) were positive for only Fa/Ra and were classified as the J99 type (**Table 2** & **Table 6**).

Sequence analysis of full-length dupA *from clinical isolates in Japan*

We next examined the sequence diversity of 15 full-length *dupA* from Japanese clinical isolates. We compared 13 Shi470-type Japanese strains (6 from Fukui and 7 from Okinawa), including F57, whose complete genome sequence was recently released. Twelve of these strains (92.3%) carried intact full-length *dupA* (**Table 2**). Alignments of the nucleotide and amino acid sequences showed these strains were highly homologous (98.9%, standard deviation [SD] ± 0.4) irrespective of the clinical outcome and the area of isolation (Fukui or Okinawa). The remote Amazonian strain Shi470 was also highly homologous to those 13 Japanese strains (98.5%, SD±0.2)(**Table 7**). Sequences from the remaining 2 Japanese strains (F80 and OK317), which carried the J99-type *dupA*, were also compared with the corresponding J99 sequences (1838 bp). These strains were highly homologous to each other (99.9%) but less homologous to J99 (95.7%). A G/T insertion at position 1633, resulting in a null mutation, was found in both strains (**Table 2**); this mutation was previously reported in a strain from Argentina.[44] All 15 Japanese strains possessed the 1 bp insertion of a C or T in the 3′ region of *jhp0917*, which was initially reported by Lu et al. Recently, Hussein et al. also classified *dupA* into 2 main groups. These investigators reported that *dupA1* was a 1884 bp ORF with a longer 3′ end than that initially described by Lu et al. and that *dupA2* was a truncated gene.[45] To evaluate the correspondence of the Shi470-type *dupA* to *dupA1*, we compared the 13 Shi470-type *dupA* sequences of the Japanese isolates included in the present study, with the *dupA1* sequence of strain AB21 released by Hussein et al. We found all of these sequences included an intact *dupA1* at the 3′ end of the gene. OK108, a strain with an incomplete Shi470-type *dupA*, also possessed intact *dupA1* sequences, as the stop codon causing a truncation of the 2499 bp ORF was located upstream of the beginning of the *dupA1* lesion.

Discussion

In this study, we found no significant association between *dupA* and certain diseases in the 2 distant areas in Japan (Fukui and Okinawa) and could confirm no relationship between the *dupA* gene and the lower incidence of GC in Okinawa. However, our results indicated patients infected with *dupA*$^+$ strains were prone to DU in Fukui, as opposed to the high frequency of *dupA*$^+$ strains

Table 3 Comparison of *dupA* prevalence among *H. pylori* groups with various clinical outcomes in the 2 different areas in Japan

Clinical outcome	*dupA* prevalence (%)	
	Fukui (n=111)	Okinawa (n=137)
Duodenal ulcer	7/20 (35.0)	5/26 (19.2)
Gastric ulcer	3/20 (15.0)	5/18 (27.8)
Chronic gastritis	6/39 (15.4)	14/60 (23.3)
Gastric cancer	6/32 (18.8)	14/33 (42.4)
Total	22/111 (19.8)	38/137 (27.7)

H. pylori, *Helicobacter pylori*; *dupA*, duodenal ulcer-promoting gene A.

Table 4 Relationship between dupA prevalence and the cagA genotype

cagA genotype	*dupA* prevalence (%)	
	Fukui	Okinawa
East Asian	21/109 (19.3)	37/110 (33.6)
Western and *cagA*$^-$	0/1 (0.0)	1/26 (3.8)

In Okinawa, *dupA* positivity was significantly higher in the East Asian-type cagA$^+$ strains than in the Western-type *cagA*$^+$ and *cagA*$^-$ strains (P=0.0013, Fisher's exact probability test). Two stains isolated from duodenal ulcer hosts with hybrid-type *cagA*, F80 (*dupA*$^+$) and OK204 (*dupA*$^-$), were excluded. *cagA*, cytotoxin-associated gene A; *dupA*, duodenal ulcer-promoting gene A.

Table 5 The associations of *dupA* prevalence with clinical diagnosis, geographic locations, and *cagA* genotypes

	dupA positivity (%)	Univariate[a] p value	Multivariate[b]	
			OR (95% CI)	p value
Disease				
Chronic gastriti	20/99 (20.2)	Referent	–	0.472
Gastric ulcer	8/38 (21.1)	1.0000	–	0.272
Duodenal ulcer	11/44 (25.0)	0.5177	–	0.295
Gastric cancer	20/65 (30.8)	0.1395	–	0.167
Geography				
Okinawa	38/136 (27.9)	0.1330	2.128 (1.146–3.949)	0.017
Fukui	21/110 (19.1)			
cagA genotype				
East Asian	58/219 (26.5)	0.0073	12.924 (1.689–98.901)	0.014
Non-East Asian	1/27 (3.7)			

Two stains isolated from duodenal ulcer hosts with hybrid-type *cagA*, F80 (*dupA*$^+$) and OK204 (*dupA*$^-$), were excluded. *dupA*, duodenal ulcer-promoting gene A; OR, odds ratio; CI, confidence interval; *cagA*, cytotoxin-associated gene A; a Fisher's exact probability test; b multivariable logistic regression.

Table 6 Distribution of *dupA* genotypes in clinical strains

	Prevalence (%)		
	Fukui (n=22)	Okinawa (n=38)	Total (n=60)
J99-type	1(4.5)	1 (2.6)	2 (3.3)
Shi470-type	21 (95.5)	37 (97.4)	58 (96.7)

dupA, duodenal ulcer-promoting gene A.

among patients with GC in Okinawa. In a systematic review, Hussein reported *dupA* promotes DU in some populations and GU and GC in others.[50] Meanwhile, based on a meta-analysis, Shiota et al. reported the importance of *dupA* positivity in DU pathogenesis, especially in Asian countries.[51] These conflicting results probably result from differences in the circulating *H. pylori* strains between East Asia and the West. Although the mechanism by which *H. pylori* is involved in 2 extremely different gastroduodenal diseases (DU and GC) remains unclear, our discrepant results in 2 different areas in Japan suggest *dupA* may be one of the virulence determinants that causes severe diseases, while other virulence factors, host differences, and environmental factors might affect the clinical outcome of the infection. Fukui is a typical rural prefecture located on the central Japanese mainland (Honshu), while Okinawa consists of islands in the southwestern part of Japan. Historically, Okinawa has had more active international communication than the rest of the country. Because these geographical and historical factors may affect the development of gastroduodenal diseases in Okinawan hosts, the pathogenesis of *H. pylori* isolated from Okinawa needs to be analyzed more comprehensively.

Seven studies have investigated the association of *dupA* with *cagA*, one of the most widely studied virulence genes, but the results have been inconsistent. A positive association between the *dupA* and *cagA* statuses was reported in a study investigating strains isolated from patients with chronic gastritis or DU in North India,[31] whereas 3 other studies refuted the presence of a relationship with any clinical outcomes in China,[37] Iraq, and Iran.[35,42] Douraghi et al. reported *dupA* was associated with *cagA* only in patients with DU in Iran.[34] A Brazilian study also reported the association of these 2 genes was limited to a group of strains from adult hosts with DU.[33] Argent et al. investigated the

Table 7 Analysis of divergence of the Shi470-type *dupA*

	Nucleotide and amino acid identity[a] (%)													
Strain	Shi470	F51	F57	F58	F64	F77	F228	OK99	OK108[b]	OK165	OK169	OK203	OK303	OK309
Shi470		98.2	98.8	99.0	99.0	98.3	98.9	98.3	–	98.1	98.1	98.4	98.8	98.2
F51	98.3		98.2	98.2	98.2	98.0	98.3	97.7	–	97.7	97.6	97.8	98.2	97.6
F57	98.6	98.4		99.0	99.0	98.8	99.4	98.8	–	98.8	98.8	99.2	99.3	98.9
F58	98.8	98.4	98.7		100	98.6	99.2	98.8	–	98.6	98.3	98.7	99.0	98.4
F64	98.8	98.4	98.7	100		98.6	99.2	98.8	–	98.6	98.3	98.7	99.0	98.4
F77	98.7	98.6	99.0	98.8	98.8		98.7	99.0	–	98.3	98.1	98.4	98.6	98.2
F228	98.8	98.4	99.4	98.8	98.8	98.9		98.7	–	98.7	98.7	99.0	99.6	98.8
OK99	98.5	98.2	98.8	98.9	98.9	99.1	98.8		–	98.6	98.1	98.4	98.4	98.2
OK108[b]	98.5	98.2	99.2	99.1	99.1	98.9	99.2	98.4		–	–	–	–	–
OK165	98.5	98.5	98.9	98.9	98.9	99.0	98.9	99.3	98.6		99.3	99.4	98.6	99.4
OK169	98.2	98.2	99.0	98.3	98.3	98.6	99.1	98.6	98.7	99.2		99.6	98.6	99.9
OK203	98.3	98.3	99.1	98.5	98.5	98.8	99.2	98.7	98.9	99.2	99.8		98.9	99.8
OK303	98.7	98.4	99.4	98.8	98.8	98.9	98.8	98.8	99.2	98.9	99.1	99.2		98.7
OK309	98.2	98.2	99.0	98.4	98.4	98.7	99.1	98.6	98.8	99.2	100	99.8	99.1	

dupA, duodenal ulcer-promoting gene A; a values below and above the diagonal represent nucleotide and amino acid sequence, respectively; b a stop codon is present in the *dupA* nucleotide sequence.

dupA prevalence in *H. pylori* strains from Belgium, South Africa, China, and the United States. These investigators reported a significant association between *dupA* positivity with GC, independent of *cagA* status.[32] We previously showed that more than 20% of Okinawan strains were either Western-type *cagA*$^+$ or *cagA*$^-$, both of which were rare in East Asia.[23] We also found these Western-type strains were isolated among patients with DU more frequently than the East Asian strains,[26,27] whereas the East Asian-type strains in Okinawa were associated with GC.[27] Therefore, we examined the association between *dupA* and *cagA* status in Okinawa. Intriguingly, we found a low prevalence of the *dupA*$^+$ strain among strains with the non-East Asian *cagA* (the Western or hybrid type) in Okinawa. Moreover, both univariate and multivariate analyses showed that the presence of East Asian-type *cagA* was significantly associated with *dupA* positivity. Although the reason why *dupA*$^+$ strains are rare among non-East Asian *cagA*$^+$ strains is unclear, the variety in *cagA* toxicity may partially, if not completely, explain this mystery. Hussein et al. reported that strains with *cagA* genes encoding more than 3 tyrosine phosphorylation motifs of the Src homology phosphatase 2 (SHP-2) binding site were significantly associated with *dupA* positivity when compared to strains with only 3 phosphorylation motifs in Iran.[35] The virulence of *cagA*, which is determined by the degree of CagA SHP-2 binding activity, depends on the genotypes of the SHP-2 binding site (the East Asian-type is more toxic than the Western-type) and the number of its phosphorylation motifs (the greater the number of motifs, the stronger the binding).[22,25,27,52] Together, the results of Hussein et al. and those of our present study suggest the possible association of *dupA* with "toxic" *cagA*.

We found that the *dupA* prevalence in Okinawa was significantly higher than in Fukui by using multivariable logistic regression. This result seems partially consistent with those of recent meta-analyses showing that the prevalence of *dupA* is higher in Western countries than in Asian countries,[50,51] as our previous reports suggested *H. pylori* strains in Okinawa may have had a greater opportunity for the transfer of DNA from Western countries than *H. pylori* strains in other Japanese areas.[23,26] Since this question has not been sufficiently studied, determining the genetic origin of *H. pylori* strains circulating in Okinawa is required in examining the roles of *H. pylori* virulent factors, including *dupA*, in this area.

In this study, we analyzed the upstream nucleotide sequences of *dupA* in Japanese clinical *H. pylori* isolates, which have not been sufficiently examined in Japan thus far. A comparison between the genome sequences of 12 representative *dupA*$^+$ strains of *H. pylori* in GenBank, including our strain (F57), revealed the existence of 2 major distinct chromosomal arrangements (Shi470 and J99 types). Regardless of the host's clinical outcome, we showed that most *dupA* sequences of the Japanese *H. pylori* strains were of the intact Shi470-type gene, which encodes a protein of 832 amino acids. Although this finding made it difficult to determine if *dupA* was a specific virulence factor in Japan, we demonstrated a strong association between intact *dupA* and *H. pylori* strains circulating in this country. When discussing the virulence of a particular *H. pylori* gene, it is important to consider not only the presence of the gene but also whether the gene is intact. We previously showed *vacA* from half of the Japanese non-cytotoxic strains contained null mutations resulting in a lack of VacA activity.[53] In terms of *dupA*, Queiroz et al. reported an inverse association between the presence of *dupA* without particular null mutations (a deletion of adenine at position 1311 and insertion of adenine at position 1426) and the development of GC in Brazil.[41]

Notably, Hussein et al. had classified *dupA* into 2 main groups (*dupA1* and *dupA2*) before us. This group reported *dupA1* is the most common genotype, as well as the active form that induces host secretion of IL-12 from cluster of differentiation 14 (CD14) positive mononuclear cells. Among the 25 *dupA1*$^+$ isolates studied by Hussein et al., additional sequencing was performed for 2 isolates. Surprisingly, these isolates possessed the *dupA* gene with an extended 5′ end, giving a total length of 2499 bp.[45] Because all 13 Shi470-type *dupA* strains sequenced

in this study included the 1884bp dupA1 defined by Hussein et al., *dupA1* may be longer than initially reported, corresponding to the Shi470-type *dupA* in the present study. On the other hand, we cannot deny the possibility the Shi470-type *dupA* is a third novel genotype of *dupA*, different from both *dupA1* and *dupA2*. Following Hussein et al., Queiroz et al. sequenced the complete *dupA* gene from 6 *H. pylori* isolates in Brazil. Though all these genes were reported to be identical to *dupA1*, the report did not specify whether the 5′ end was extensive or not.[46] For assessing the correspondence of the Shi470-type *dupA* to *dupA1*, further investigations on the upstream region of *dupA* are required. Investigating the *dupA* genotype might provide insight into the potential relationship between intact *dupA* and the strong virulence of *H. pylori* strains.

It should be noted that *dupA* gene expression has not been sufficiently analyzed thus far. Only 2 of the previous studies on *dupA* have investigated its expression in various isolates, as determined by reverse transcription PCR (RT-PCR). In Japan, Nguyen et al. confirmed *dupA* expression in all 10 randomly selected *H. pylori* strains among 72 *dupA*$^+$ isolates.[39] Only recently, Alam et al. reported a significant association of *dupA* with DU development in a South Indian population.[43] They also reported the detection of *dupA* transcription in 28 of 35 *dupA*$^+$ *H. pylori* strains. The discovery of strains with "expressive *dupA*" by RT-PCR and an assessment of their relationship with clinical outcomes would reveal the true role of the DupA protein in the disease development of the host. Therefore, we should next assess the *dupA* expression of the Japanese isolates used in the present study, especially the Shi470-type *dupA*$^+$ isolates, by using the methods of Alam et al. Furthermore, the detection of DupA protein still remains an unsolved problem.[30]

Further exploration of the surrounding genes of *dupA* may also be promising for understanding *H. pylori* virulence. Previous studies have shown that *dupA* is present in the plasticity zone. This zone is characterized by a low G+C content and an abundance of strain-specific ORFs, including TFSSs.[11,28,29] Recently, Kersulyte et al. described a putative T4SS-containing *dupA* as a type IV secretion 3a (*tfs3a*) gene, and a putative T4SS-containing a *virB4* sequence, but not *dupA* as *tfs3b*.[29] Jung et al. defined the presence of *dupA* and all 6 adjacent virulence (*vir*) gene homologs (*virB8*, *B9*, *B10*, *B11*, *virD4*, and *D2*) as a complete *dupA* cluster.[30] They reported that the presence of a complete *dupA* cluster but not *dupA* alone is associated with DU development in the United States population. Because we could not find a particular association between *dupA* and any clinical outcomes, the *vir* genes around *dupA* in the Japanese *H. pylori* isolates studied in the present study will need to be researched further.

In conclusion, we were unable to confirm a close association between *dupA* and clinical outcomes in 2 areas in Japan with different GC risks. However, we found intact *dupA* consists of a 2499 bp sequence and most Japanese *dupA*$^+$ strains possess the intact genotype. We also discovered a significant relation between the presence of *dupA* and the East Asian-type *cagA* in Japan. In addition, *dupA* positivity was independently associated with strains isolated from Okinawa.

Further global analyses of the genetic structure of *dupA* and the association between the *H. pylori* strain with a *dupA*-positive state and clinical outcomes are required.

Contributors

Hidetaka Matsuda, Yoshiyuki Ito, Hiroyuki Suto, Satoko Satomi, Masahiro Ohtani, Yukinao Yamazaki, Yoshiki Shimabukuro, Kaoru Kikuchi, Yoshihide Keida, Takeshi Azum, and Yasunari Nakamoto were involved in conceiving and designing this study. Hidetaka Matsuda, Yoshiyuki Ito, and Akiyo Yamakawa analyzed and interpreted the data. Yukinori Kusaka supervised and revised the statistics. This manuscript was drafted by Hidetaka Matsuda, revised critically by Yoshiyuki Ito for important intellectual content, and finalized by Yasunari Nakamoto with the assistance of all authors.

Conflicts of interest

The authors declared no conflicts of interest.

Summary Box

What is already known:

- The association between duodenal ulcer-promoting gene A (*dupA*) of *Helicobacter pylori* (*H. pylori*) and various clinical outcomes or other virulence factors remains controversial.
- In Japan, Okinawa has lowest incidence of gastric cancer (GC). The diversity of cytotoxin-associated gene A (*cagA*) detected in Okinawa includes both East Asian and Western genotypes and is an important factor for the low prevalence of GC.
- *dupA* is reported to be polymorphic; *dupA1* (1884 bp) is the active form, while *dupA2* is the truncated version. Several *H. pylori* strains are reported to possess long *dupA1* sequences (2499 bp) because of an extended 5′ end.

What the new findings are:

- We found *dupA* could be classified into 2 genotypes—a 2499 bp genotype and the initially reported 1839 bp genotype. These genotypes were designated Shi470-type *dupA* and J99-type *dupA*, respectively, after the *H. pylori* strains whose complete genomes have been released.
- In Japan, most *dupA*$^+$ *H. pylori* strains possess intact Shi470-type *dupA*.
- In Japan, *dupA* appeared to be independently associated with the East Asian-type *cagA* and with host residence in Okinawa.

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NS5A Sequence Heterogeneity of Hepatitis C Virus Genotype 4a Predicts Clinical Outcome of Pegylated-Interferon–Ribavirin Therapy in Egyptian Patients

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Hepatitis C virus genotype 4 (HCV-4) is the cause of approximately 20% of the 180 million cases of chronic hepatitis C in the world. HCV-4 infection is common in the Middle East and Africa, with an extraordinarily high prevalence in Egypt. Viral genetic polymorphisms, especially within core and NS5A regions, have been implicated in influencing the response to pegylated-interferon and ribavirin (PEG-IFN/RBV) combination therapy in HCV-1 infection. However, this has not been confirmed in HCV-4 infection. Here, we investigated the impact of heterogeneity of NS5A and core proteins of HCV-4, mostly subtype HCV-4a, on the clinical outcomes of 43 Egyptian patients treated with PEG-IFN/RBV. Sliding window analysis over the carboxy terminus of NS5A protein identified the IFN/RBV resistance-determining region (IRRDR) as the most prominent region associated with sustained virological response (SVR). Indeed, 21 (84%) of 25 patients with SVR, but only 5 (28%) of 18 patients with non-SVR, were infected with HCV having IRRDR with 4 or more mutations (IRRDR ≥ 4) ($P = 0.0004$). Multivariate analysis identified IRRDR ≥ 4 as an independent SVR predictor. The positive predictive value of IRRDR ≥ 4 for SVR was 81% (21/26; $P = 0.002$), while its negative predictive value for non-SVR was 76% (13/17; $P = 0.02$). On the other hand, there was no significant correlation between core protein polymorphisms, either at residue 70 or at residue 91, and treatment outcome. In conclusion, the present results demonstrate for the first time that IRRDR ≥ 4, a viral genetic heterogeneity, would be a useful predictive marker for SVR in HCV-4 infection when treated with PEG-IFN/RBV.

Hepatitis C virus (HCV) is a major cause of chronic liver disease, hepatocellular carcinoma, and deaths from liver disease and is the most common indication for liver transplantation (7, 26–28, 38). HCV has been classified into seven major genotypes and a series of subtypes (35, 36). In general, HCV genotype 4 (HCV-4) is common in the Middle East and Africa, where it is responsible for more than 80% of HCV infections (23). Although HCV-4 is the cause of approximately 20% of the 180 million cases of chronic hepatitis C in the world, it has not been a major subject of research.

Egypt has the highest prevalence of HCV worldwide (15%) and the highest prevalence of HCV-4, which is responsible for 90% of the total HCV infections, with a predominance of the subtype 4a (HCV-4a) (1, 32). This extraordinarily high prevalence results in an increasing incidence of hepatocellular carcinoma in Egypt, which is now the second most frequent cause of cancer and cancer mortality among men (17, 21). More than 2 decades have passed since the discovery of HCV, and yet therapeutic options remain limited. Up to 2011, the standard treatment for chronic hepatitis C consisted of pegylated alpha interferon (PEG-IFN) and ribavirin (RBV) (19); however, by May 2011 two protease inhibitors (telaprevir and boceprevir) were approved by the Food and Drug Administration (FDA) for use in combination with PEG-IFN/RBV for adult chronic hepatitis C patients with HCV genotype 1 (24, 34). Since the approval of these new protease inhibitors for treatment of HCV-1 infection, the response of HCV-4 to the standard regimen of treatment (PEG-IFN/RBV) has lagged behind other genotypes and HCV-4 has become the most resistant genotype to treat. As PEG-IFN/RBV still remains to be used to treat HCV-4-infected patients, exploring the factors that predict the outcome of PEG-IFN/RBV treatment, such as sustained virological response (SVR), for HCV-4 infections is needed to assess more accurately the likelihood of SVR and thus to make more informed treatment decisions.

While the SVR rate for PEG-IFN/RBV treatment hovers at 50 to 60% in HCV-1 and -4 infection, it is up to 80% in HCV-2 and -3 infections (19, 33). This difference in responses among patients infected with different HCV genotypes suggests that viral genetic heterogeneity could affect, at least to some extent, the sensitivity to IFN-based therapy. In this context, the correlation between IFN-based therapy outcome and sequence polymorphisms within the viral core and NS5A proteins has been widely discussed, in particular in regard to Japanese patients with HCV-1b infection. Initially, in the era of IFN monotherapy, it was proposed that sequence variations within a region in NS5A of HCV-1b, called the IFN sensitivity-determining region (ISDR), were correlated with IFN responsiveness (18). Subsequently, in the era of PEG-IFN/

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RBV combination therapy, we identified a new region near the C terminus of NS5A, referred to as the IFN/RBV resistance-determining region (IRRDR) (13). Recently, we also demonstrated the correlation between IRRDR polymorphism and PEG-IFN/RBV treatment outcome in HCV-2a and -2b infections (15). In addition, HCV core protein polymorphism, in particular at positions 70 and 91, was also proposed as a pretreatment predictor of poor virological response in patients infected with HCV-1b (4–6). To the best of our knowledge, there is no information regarding the correlation between sequence heterogeneity in the NS5A and core proteins of HCV-4 and PEG-IFN/RBV treatment outcome. In the present study, we aimed to investigate this issue in Egyptian patients infected with HCV-4.

MATERIALS AND METHODS

Ethics statement. The study protocol, which conforms to the provisions of the Declaration of Helsinki, was approved beforehand by the Ethic Committees in Cairo University Hospital and in Kobe University, and written informed consent was obtained from each patient prior to the treatment.

Patients. A total of 43 previously untreated patients who were chronically infected with HCV-4a (34 patients), HCV-4m (3 patients), HCV-4n (3 patients), or HCV-4o (3 patients) were consecutively evaluated for antiviral treatment at Cairo University Hospital, Cairo, Egypt, between January 2008 and September 2010. The HCV subtype was determined according to the method of Okamoto et al. (31). The patients were treated with PEG-IFN α-2a (180 μg/week, subcutaneously) and RBV (1,000 to 1,200 mg daily, *per os*) for 48 weeks. The quantification of serum HCV RNA titers was performed as previously reported (14). To minimize the therapeutic burdens, including the high cost and possible side effects, therapy was discontinued if HCV RNA titers at week 12 did not drop by 2 log compared with baseline values or if HCV RNA was still detectable at week 24. These were considered a null response (see Results).

Sequence analysis of the NS5A and core regions of the HCV genome. Blood samples were collected using Vacutainer tubes. The sera were separated within 2 h of blood collection, transferred to sterile cryovials, and kept frozen at −80°C until use. HCV RNA was extracted from 140 μl of serum using a commercially available kit (QIAmp viral RNA kit; Qiagen, Tokyo, Japan). The extracted RNA was reverse transcribed and amplified for the HCV genome encoding a carboxy terminus of NS5A (amino acids [aa] 2193 to 2417) and the core protein (aa 1 to 191) using SuperScript III one-step RT-PCR Platinum *Taq* HiFi (Invitrogen, Tokyo, Japan). The resultant reverse transcription (RT)-PCR product was subjected to a second-round PCR by using Platinum *Taq* DNA polymerase high fidelity III (Invitrogen). Primers used for amplification of the 3′ half of the NS5A region of HCV-4 were as follows: NS5A-4/F1 (5′-CTCAAYTCGTTCGTRGTGGGATC-3′; sense) and NS5A-4/R1 (5′-CGAAGGTCACCTTCTTCTGCCG-3′; antisense) for one-step RT-PCR; and NS5A-4/F2 (5′-ATGCGAGCCYGAGCCGGACGT-3′; sense) and NS5A-4/R2 (5′-GCTCAGGGGGYTRATTGGCAGCT-3′; antisense) for the second-round PCR. Primers for amplification of the core region of HCV-4 were 249-F (5′-GCTAGCCGAGTAGTGTTG-3′; sense) and 984-R (5′-GATGTGRTGRTCGGCCTC-3′; antisense) (40) for one-step RT-PCR; and 319-F (5′-GGAGGTCTCGTAGACCGTGC-3′; sense) (40) and primer-186 (5′-ATGTACCCCATGAGGTCGGC-3′; antisense) (2) for the second-round PCR. RT was performed at 45°C for 30 min and terminated at 94°C for 2 min, followed by the first-round PCR over 35 cycles, with each cycle consisting of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 68°C for 90 s. The second-round PCR was performed under the same conditions. The sequences of the amplified fragments were determined by direct sequencing without subcloning. The amino acid sequences were deduced and aligned using Genetyx Win software version 7.0 (Genetyx Corp., Tokyo, Japan). The numbering of amino acid residues for HCV-4 isolates is according to the polyprotein of ED43 isolate (accession no. Y11604) (10). Consensus sequences of the carboxy terminus of NS5A of a given HCV-4 subtype were inferred by alignment of all sequences obtained in this study as well as all available NS5A sequences of HCV-4a (accession no. Y11604, DQ418782 to DQ418789, DQ516084, and DQ988073 to DQ988079), HCV-4m (FJ462433), HCV-4n (FJ462441), and HCV-4o (FJ462440) from the databases.

Statistical analysis. Numerical data were analyzed by Student's *t* test and categorical data by Fisher's exact probability test. To evaluate the optimal threshold of the number of amino acid mutations in IRRDR for prediction of treatment outcomes, the receiver operating characteristic (ROC) curve was constructed. Univariate and multivariate logistic regression analyses were performed to identify independent predictors for treatment outcomes. All statistical analyses were performed using the SPSS version 16 software (SPSS Inc., Chicago, IL). Unless otherwise stated, a P value of <0.05 was considered statistically significant.

Nucleotide sequence accession numbers. The sequence data reported in this paper have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB725987 through AB726066.

TABLE 1 Virological responses of HCV-4-infected patients treated with PEG-IFN/RBV

Virological response	Proportion (%) of patients with indicated response (no. of patients/total no.)				
	HCV-4[a]	HCV-4a	HCV-4 m	HCV-4n	HCV-4o
SVR	58 (25/43)	56 (19/34)	100 (3/3)	33 (1/3)	67 (2/3)
Non-SVR	42 (18/43)	44 (15/34)	0 (0/3)	67 (2/3)	33 (1/3)
Null response	30 (13/43)	32 (11/34)	0 (0/3)	67 (2/3)	0 (0/3)
Relapse	12 (5/43)	12 (4/34)	0 (0/3)	0 (0/3)	33 (1/3)

[a] Includes all 43 cases with HCV-4 infection (34 cases with HCV-4a and 3 cases each with HCV-4m, -4n, and -4o).

RESULTS

Patients' responses to PEG-IFN/RBV combination therapy. Among 43 patients enrolled in this study, 30 (70%) patients completed the entire course of PEG-IFN/RBV treatment for 48 weeks and follow-up for 24 weeks. On the other hand, the treatment was discontinued for 13 (30%) patients due to poor virological responses at 12 or 24 weeks after initiation of the therapy. Overall, 25 (58%) patients achieved SVR while 18 (42%) patients had non-SVR (Table 1). When analyzed on the basis of the subtype classification, SVR was achieved by 56% (19/34), 100% (3/3), 33% (1/3), and 67% (2/3) of patients infected with HCV-4a, -4m, -4n, and -4o, respectively.

Non-SVR patients are classified into two groups: (i) patients with null response, who did not achieve >2-log reduction of the initial viral load at week 12 or who had detectable viremia at week 24 of the treatment period; and (ii) patients with relapse, who were negative for HCV-RNA at the end of the treatment period (week 48) followed by a rebound viremia at a certain time point during the follow-up period of 24 weeks. Patients with null response represented 30% (13/43) of all the HCV-4-infected subjects analyzed, while those with relapse represented 12% (5/43). A similar tendency was observed for subtype HCV-4a.

Among various patients' demographic characteristics, SVR patients had a significantly lower average age than that of non-SVR patients (Table 2). Furthermore, a tendency for SVR patients to have a lower average titer of initial viral load than that of non-SVR was noted, although the difference was not statistically significant, due possibly to the small number of patients analyzed ($P = 0.07$).

TABLE 2 Demographic characteristics of HCV-4-infected patients with SVR and non-SVR[a]

Factor	SVR	Non-SVR	*P* value
Age	38.47 ± 9.51	45.80 ± 5.65	0.014
Sex (male/female)	18/7	15/3	0.48
BMI	27.36 ± 3.65	27.67 ± 5.28	0.85
Platelets ($\times 10^3/\mu l$)	204.4 ± 40.63	216.7 ± 87.25	0.59
Hemoglobin (g/dl)	14.54 ± 1.38	15.08 ± 1.39	0.25
WBC count	7,041 ± 1,876	7,078 ± 2,977	0.96
Albumin (g/dl)	4.12 ± 0.36	4.328 ± 0.41	0.11
ALT (IU/liter)	78.72 ± 59.68	82.39 ± 41.80	0.83
AST (IU/liter)	64.94 ± 27.63	58.17 ± 23.98	0.44
HCV-RNA (IU/ml)	84,290 ± 186,300	501,800 ± 816,700	0.07

[a] Values are means ± standard deviations. SVR, sustained virological response; BMI, body mass index; WBC, white blood cell; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

Correlation between NS5A sequence heterogeneity and SVR in HCV-4 infection. We and other researchers reported significant correlation between sequence polymorphisms within the C-terminal half of NS5A, including that in ISDR and IRRDR, and PEG-IFN/RBV treatment outcome in HCV-1 and HCV-2 infections (13, 15, 18, 30). However, this information is quite limited in HCV-4 infection. To clarify this issue, part of the HCV-4 genome encoding a carboxy terminus (aa 2193 to 2417) of NS5A in pretreatment sera was amplified and sequenced, and amino acid sequences were deduced. The sequences obtained as well as all available NS5A sequences of HCV-4a, -4m, -4n, and -4o from the databases were aligned, and the consensus sequences for a desired HCV-4 subtype were inferred (see Materials and Methods). Next, to identify an NS5A region(s) that would be significantly correlated with treatment outcome, we carried out a sliding window analysis with a window size of 30 residues over the C-terminal half (aa 2193 to 2417) of NS5A sequences obtained from all SVR (n = 25) and non-SVR (n = 18) patients along with corresponding consensus sequences of each HCV-4 subtype as described previously (30). This analysis revealed that the difference in the overall number of amino acid mutations between SVR and non-SVR isolates exceeded the significant threshold only in a region corresponding to IRRDR of HCV-1b (13), ranging from aa 2331 to 2383, thus being referred to as IRRDR[HCV-4] (Fig. 1). Indeed, the average number of amino acid mutations in IRRDR[HCV-4] was significantly larger in SVR than in non-SVR (P = 0.0005) isolates (Fig. 2A). Sequences of IRRDR of HCV-4a, -4m, -4n, and -4o obtained from SVR and non-SVR patients along with the number of IRRDR mutations of each isolate are shown in Fig. 2B.

Next, we performed ROC curve analysis to estimate the optimal cutoff number of IRRDR[HCV-4] mutations for SVR prediction. This analysis estimated 4 mutations as the optimal number of IRRDR[HCV-4] mutations to predict SVR, since it achieved the highest sensitivity (84%; sensitivity refers to the proportion of SVR patients who were infected with HCV isolates of IRRDR[HCV-4] with 4 or more mutations) and specificity (72%; specificity refers to the proportion of non-SVR patients who were infected with HCV isolates of IRRDR[HCV-4] with 3 or fewer mutations) with an area under the curve (AUC) of 0.82 (Fig. 3). Accordingly, 21 (84%) of 25 patients with SVR, in contrast to only 5 (28%) of 18 patients with non-SVR, had IRRDR[HCV-4] with 4 or more mutations (referred to as IRRDR[HCV-4]≥4), with the difference between the two groups being statistically significant (P = 0.0004) (Table 3). It should be noted that 4 (31%) of 13 patients with null response and only 1 (20%) of 5 patients with relapse had HCV with IRRDR[HCV-4]≥4. These results collectively suggest that IRRDR[HCV-4]≥4 is significantly associated with SVR. In this connection, we also tested the impact of a higher (≥5) and a lower (≥3) degree of IRRDR mutations on treatment outcome. IRRDR[HCV-4]≥5 was significantly associated with SVR, though with a relatively lower sensitivity (64%) than that of IRRDR[HCV-4]≥4 (Table 3). On the other hand, there was no significant correlation between IRRDR[HCV-4]≥3 and SVR.

Correlation between core protein sequence heterogeneity and SVR in HCV-4 infection. A close correlation between core protein sequence patterns at positions 70 and 91 and treatment outcome has been proposed, especially in Japanese patients with HCV-1b infection (4–6). To examine this hypothesis in Egyptian patients infected with HCV-4, core sequences of the viral genome were amplified from the pretreated sera, and the amino acid sequences were deduced. Due to a high degree of sequence homology among core sequences of various HCV-4 subtypes, all sequences obtained were aligned with the prototype sequence, ED43 (10). The residues at positions 70 and 91 were both well conserved among the sequences analyzed, and therefore, no correlation with treatment outcome was observed for these residues (Fig. 4). All but two isolates had arginine at position 70 (Arg^{70}), the residue that has been associated with an IFN-sensitive phenotype as far as the core protein of HCV-1b is concerned (4–6). On the other hand, Pro at position 71 showed a tendency to be more frequent in SVR than in non-SVR patients; however, the frequency was not statistically different between the two groups.

Identification of independent predictive factors for SVR in HCV-4 infection. In order to identify significant independent

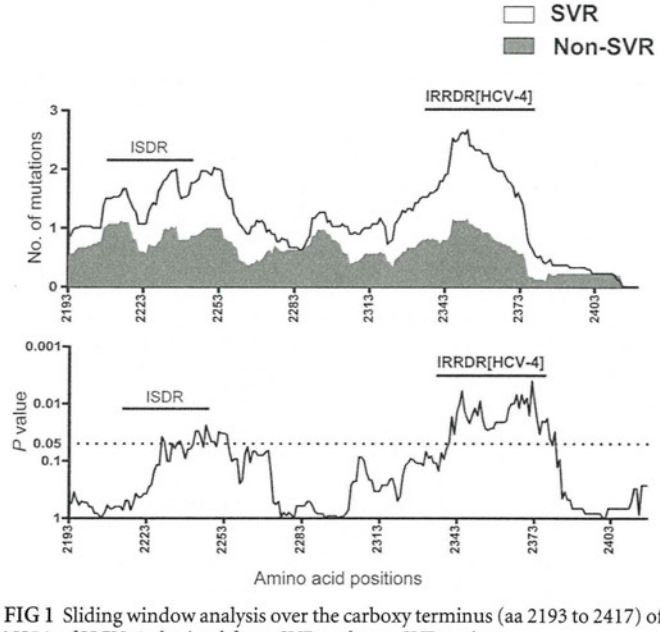

FIG 1 Sliding window analysis over the carboxy terminus (aa 2193 to 2417) of NS5A of HCV-4 obtained from SVR and non-SVR patients.

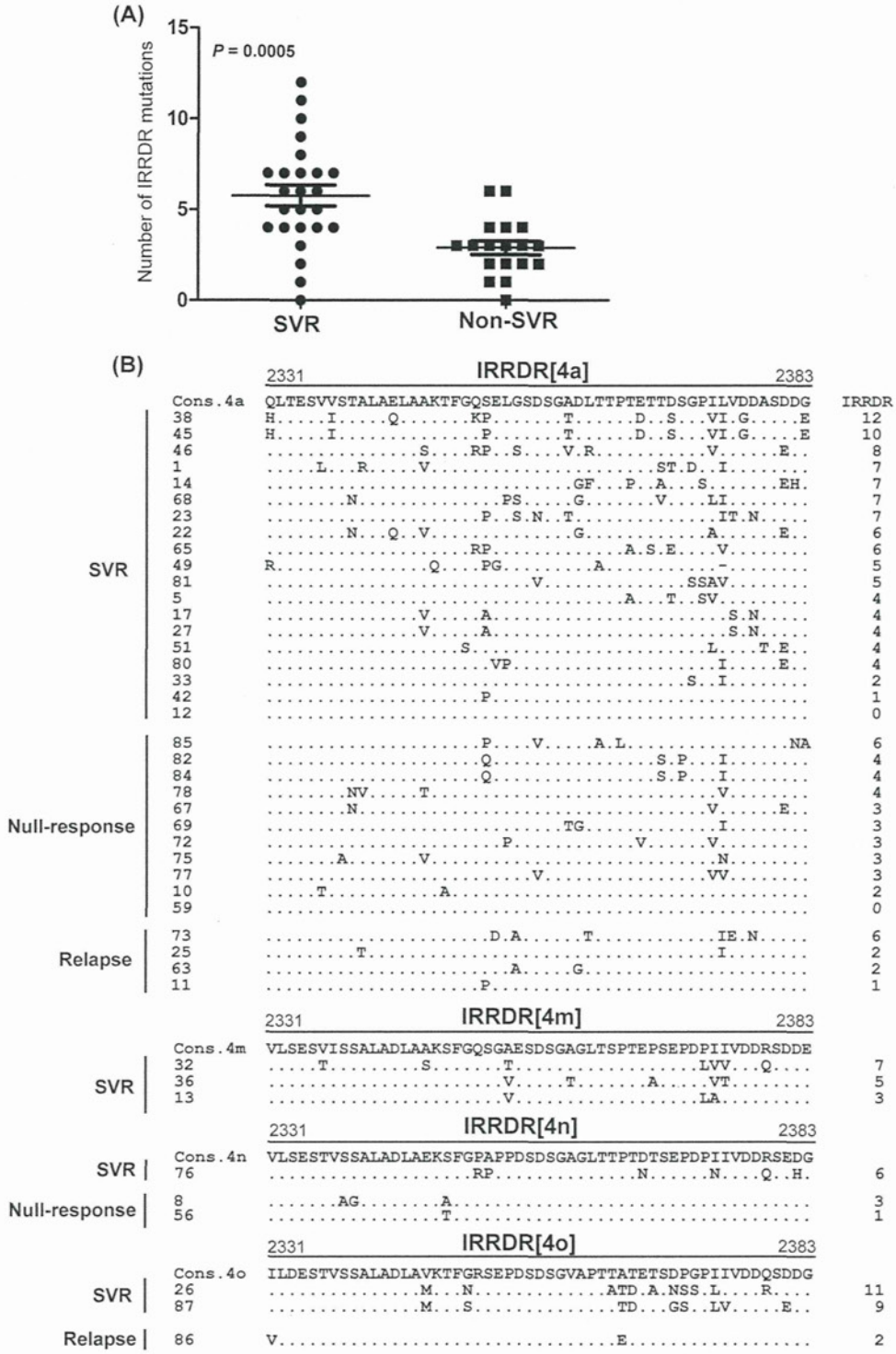

FIG 2 Correlation between IRRDR[HCV-4] sequence variations and treatment outcome. (A) Average number of amino acid mutations in IRRDR[HCV-4] obtained from SVR and non-SVR patients. (B) Alignment of IRRDR[HCV-4] sequences obtained from SVR and non-SVR patients with HCV-4a, -4m, -4n, and -4o. The consensus sequence (Cons) of each subtype is shown on the top. The numbers along the sequence indicate the amino acid positions. Dots indicate residues identical to those of the Cons sequence. The numbers of the mutations in each IRRDR (4a, 4m, 4n, or 4o) are shown on the right.

predictive factors of SVR for PEG-IFN/RBV treatment outcome in HCV-4 infection, first, all available data of baseline patients' parameters and IRRDR[HCV-4] polymorphism were entered in a univariate logistic analysis. This analysis yielded 3 factors that were correlated or nearly correlated with SVR: IRRDR[HCV-4]≥4 ($P = 0.0004$), patient's age (<42 years; $P = 0.03$), and HCV RNA titer (<5,200 IU/ml; $P = 0.08$). Subsequently, these 3 factors were entered in multivariate logistic regression analysis. This anal-

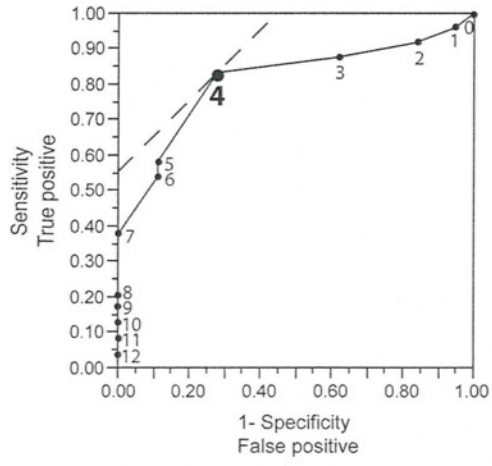

FIG 3 ROC curve analysis of IRRDR[HCV-4] sequence heterogeneity for SVR prediction. The solid line curve shows the AUC. Solid circles with numerals plotted on the curve represent different numbers of IRRDR mutations analyzed. The dashed line in the upper left corner indicates the optimal number of IRRDR[HCV-4] mutations for SVR prediction, which yields the highest sensitivity (84%) and the highest specificity (72%).

ysis revealed that the IRRDR[HCV-4]≥4 was the only independent predictive factor for SVR in HCV-4 infection (Table 4). We then assessed SVR predictability by means of IRRDR[HCV-4]≥4. As shown in Table 5, IRRDR[HCV-4]≥4 would predict SVR with a positive predictive value (PPV) of 81% ($P = 0.002$) and sensitivity of 84%. On the other hand, IRRDR[HCV-4]≤3 would predict non-SVR with a negative predictive value (NPV) of 76% ($P = 0.02$) and specificity of 72%. Thus, the degree of sequence variation in IRRDR[HCV-4] would yield useful positive and negative predictive markers for PEG-IFN/RBV therapy outcome in HCV-4-infected patients.

DISCUSSION

Both host and viral genetic factors have been implicated in influencing the clinical response to PEG-IFN/RBV therapy for HCV infection (22). It has recently been reported that host genetic polymorphisms near or within the IL28B gene on chromosome 19 show a critical impact on the treatment outcome of patients infected with HCV-1 (20, 37, 39). As for the viral factor(s), polymorphisms of NS5A and core regions of a given HCV genotype have been linked to a difference in SVR rates (3, 4, 13, 18, 30). This hypothesis was mostly inferred from studies carried out with Asian populations, in particular Japanese, with HCV-1b infection. However, whether it can be applied to non-Asian populations infected with non-HCV-1 is still unknown. To the best of our knowledge, this is the first study that specifically examines the relationship between HCV genome heterogeneity, in particular in NS5A and core regions, and PEG-IFN/RBV treatment outcome in Egyptian patients infected with HCV-4. In analogy with our previous studies that identified IRRDR as a significant determinant for PEG-IFN/RBV treatment outcome in Japanese patients infected with HCV-1b, -2a, and -2b (12–16), we have demonstrated in the present study that sequence heterogeneity within IRRDR is closely associated with the ultimate treatment outcome in Egyptian patients infected with HCV-4. A high degree of sequence variation in IRRDR[HCV-4], i.e., more than 4 (IRRDR ≥ 4), significantly correlated with SVR, while a low degree of sequence variation in this region (IRRDR ≤ 3) correlated with non-SVR, null response, and relapse. The majority of patients with SVR (84%) had HCV with IRRDR of ≥4. In contrast, nearly two-thirds (72%) of the patients with non-SVR had HCV with IRRDR ≤ 3 ($P = 0.0004$) (Table 3). Notably, 21 of the 26 patients infected with HCV with IRRDR[HCV-4]≥4 achieved SVR. Accordingly, the PPV and NPV of IRRDR[HCV-4]≥4 for SVR and non-SVR patients were 81% ($P = 0.002$) and 76% ($P = 0.02$), respectively (Table 5). Our present results thus strongly suggest that the degree of sequence heterogeneity within IRRDR[HCV-4] would be a useful marker for prediction of treatment outcome in HCV-4 infection.

The molecular mechanism underlying the possible involvement of this region in IFN responsiveness of the virus is still unknown. The significant difference among IRRDR sequence patterns may suggest genetic flexibility of this region. Indeed, the C-terminal portion of NS5A was shown to tolerate sequence insertions and deletions (29). 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 (8, 29, 41). Also, it is worth noting that IRRDR is among the most variable sequences across the different genotypes and subtypes of HCV (25) whereas its upstream and downstream sequences show a higher degree of sequence conservation (15). 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).

A mutation at position 70 of the core protein of HCV-1b has been reported to be correlated with PEG-IFN/RBV treatment out-

TABLE 3 Correlation between NS5A sequence heterogeneity and virological responses in HCV-4 infection

Factor	No. of isolates/total no. (%)				*P* value for SVR versus:		
	SVR	Non-SVR	Null response	Relapse	Non-SVR	Null response	Relapse
IRRDR ≥ 4	21/25 (84)[a]	5/18 (28)	4/13 (31)	1/5 (20)	0.0004	0.003	0.01
IRRDR ≤ 3	4/25 (16)	13/18 (72)[b]	9/13 (69)	4/5 (80)			
IRRDR ≥ 5	16/25 (64)[a]	2/18 (11)	1/13 (8)	1/5 (20)	0.0006	0.002	0.14
IRRDR ≤ 4	9/25 (36)	16/18 (89)[b]	12/13 (92)	4/5 (80)			
IRRDR ≥ 3	22/25 (88)[a]	11/18 (61)	10/13 (77)	1/5 (20)	0.066	0.39	0.006
IRRDR ≤ 2	3/25 (12)	7/18 (39)[b]	3/13 (23)	4/5 (80)			

[a] Sensitivity (proportion of SVR patients with the favorable factor).
[b] Specificity (proportion of non-SVR patients with the unfavorable factor).

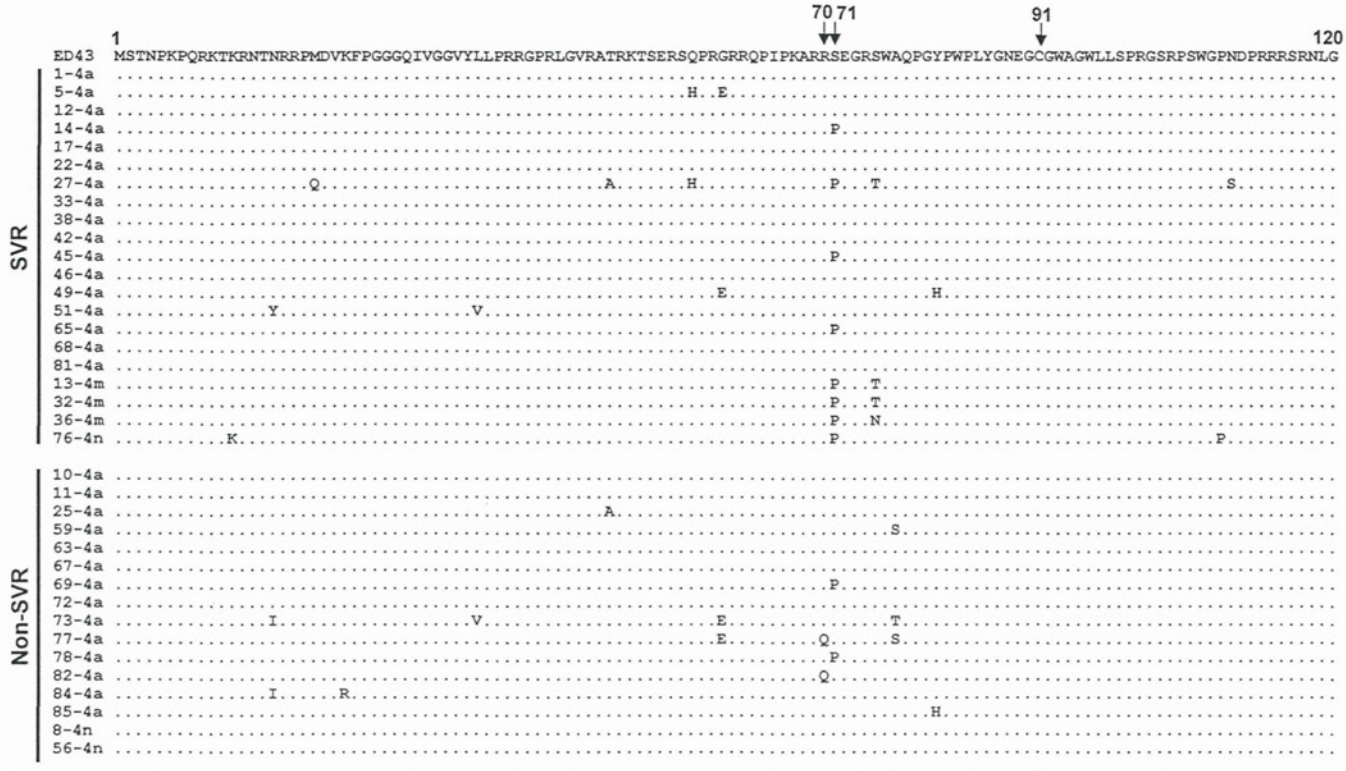

FIG 4 Sequence alignment of the core protein of HCV-4 isolates. Core protein sequences (aa 1 to 120) of HCV-4 obtained from SVR and non-SVR patients are aligned. The prototype sequence of ED43 (10) is shown on the top. The numbers along the sequence indicate the amino acid positions. Dots indicate residues identical to those of the prototype sequence.

come (4, 12). In the present study, however, we found no significant correlation between core protein polymorphism and treatment outcome in HCV-4 infection. The residue at position 70 of the core protein of all but two HCV-4 isolates analyzed in this study was Arg (Fig. 4), which is known to be associated with SVR in HCV-1b infection (4, 12). This high degree of sequence conservation at position 70 might be the reason for the lack of significant correlation between core protein polymorphism and treatment outcome in HCV-4 infection.

Single nucleotide polymorphisms (SNPs) near the IL28B region have been identified as the strongest baseline predictors of SVR to PEG-IFN/RBV in patients with HCV-1 infection. More recently, in two major studies that were carried out exclusively with HCV-4-infected patients (9, 11), the CC genotype of rs12979860 IL28B SNP was also strongly associated with SVR. It is worth noting that although the SVR rate was more than 80% among the patients with the CC genotype, these patients represented only around 40% of total SVR cases in both studies. Furthermore, the CC genotype was found in only 34% of all Egyptian patients analyzed (9). Taken together, those observations support the idea that in addition to IL28B polymorphism, there should be an additional factor(s) that influences SVR. In this context, an interplay between IRRDR and IL28B polymorphisms might explain why some patients with undesirable IL28B genotype achieve SVR and why some patients infected with HCV isolates with IRRDR[HCV-4]≥4 do not achieve SVR. Further comprehensive study is needed to validate the importance of IRRDR and IL28B polymorphisms in predicting the treatment outcome of HCV-4-infected patients.

In conclusion, the present study emphasizes the importance of IRRDR sequence heterogeneity in the prediction of PEG-IFN/RBV treatment outcome for different HCV genotype infections in

TABLE 4 Univariate and multivariate analyses for identification of independent predictive factors for SVR in HCV-4-infected patients treated with PEG-IFN/RBV therapy

Univariate analysis		Multivariate analysis	
Variable	*P* value	Odds ratio (95% CI)	*P* value
IRRDR mutations (IRRDR ≥ 4 versus IRRDR ≤ 3)	0.0004	10.5 (1.12–98.91)	0.04
Age (<42 years)	0.03		
HCV-RNA (<5,200 IU/ml)	0.08		

TABLE 5 PPV, NPV, sensitivity, and specificity of IRRDR sequence heterogeneity on the likelihood of achieving SVR and non-SVR in HCV-4 infection

Factor	PPV	NPV	Sensitivity[c]	Specificity[d]
IRRDR ≥ 4	81% (21/26)[a]		84% (21/25)	
IRRDR ≤ 3		76% (13/17)[b]		72% (13/18)

[a] $P = 0.002$.
[b] $P = 0.02$.
[c] Proportion of SVR patients who were infected with HCV isolates with IRRDR of ≥4.
[d] Proportion of non-SVR patients who were infected with HCV isolates with IRRDR of ≤3.

different ethnic groups, including Egyptian patients infected with HCV-4.

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Mutations in non-structural 5A and rapid viral response to pegylated interferon-α-2b plus ribavirin therapy are associated with therapeutic efficacy in patients with genotype 1b chronic hepatitis C

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Abstract. For patients chronically infected with hepatitis C virus (HCV), mutations in the non-structural 5A (NS5A) gene are important predictive factors for the response to interferon (IFN) therapy. In the present study, factor analysis of the therapeutic response of patients following pegylated IFN and ribavirin combination therapy was assessed in a multicenter study. Chronic HCV-infected patients with genotype 1b and high viral load (n=96, mean age 56.5 years; 59 males, 68 females) treated with pegylated IFN-α-2b and ribavirin combination therapy were enrolled. This study was conducted at Kobe University Hospital and 25 affiliated hospitals in Hyogo prefecture. Sixty-five patients (68%) completed treatment with both pegylated IFN and ribavirin at >80% of the weight-based scheduled dosages. Patients who reduced or terminated therapy were frequently aged women (mean age 60.8 years; 11 males, 17 females). Overall, a sustained viral response (SVR) was achieved in 42 (44%) patients out of 96. Based on per-protocol-based (PPB) analysis, the SVR rate in patients with ≥6 amino acid (aa) mutations in the IFN resistance-determining region (IRRDR) (75%) or ≥1 aa mutation in the IFN sensitivity-determining region (ISDR) (61%) was significantly higher than that in patients with <5 aa mutations in IRRDR (30%) or no mutation in ISDR (29%). Multivariate analysis revealed that rapid viral response (RVR) (odds ratio, 18.1) and mutations of ≥6 in IRRDR (odds ratio, 15.5) were significantly associated with SVR. In conclusion, mutations in the NS5A region, particularly in patients with ≥6 aa mutations in IRRDR were strongly associated with a therapeutic response to pegylated IFN and ribavirin combination therapy.

Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver disease, with an estimated 170 million people infected worldwide. In Japan, the carrier rate is estimated to be approximately 1% of the general population. This rate increases depending on age and reaches approximately 5% in individuals over 70 years of age. The main goal of treatment for chronic hepatitis C is prevention of cirrhosis and hepatocellular carcinoma by eradication of the virus. Interferon (IFN)-based therapy was initiated in 1992, and efficacy of treatment regimens has

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Key words: chronic hepatitis C, pegylated IFN and ribavirin therapy, non-structural 5A

improved year by year. Although the HCV viral eradication rate is approximately 5% following 24 weeks of treatment with conventional IFN therapy, the therapeutic result of combined pegylated IFN and ribavirin is ~55%. However, approximately half of patients treated with pegylated-IFN do not achieve a sustained viral response (1-3).

Due to the numerous side effects and the high cost of treatment, it is important to understand the individual mechanisms involved in non-response to treatment and to predict therapeutic efficacy prior to treatment. It has been reported that various viral and host factors are associated with the therapeutic response.

The role of amino acid (aa) mutations within the functional regions of non-structural 5A (NS5A) in relation to therapeutic response has been reported by several researchers. In 1996, it was reported that a high number of mutations in the IFN-sensitivity-determining region (ISDR) (aa 2209-2248) was strongly related to the sustained viral response (SVR) to IFN monotherapy in genotype 1b Japanese patients (4,5). In 2008, high mutations in the IFN-ribavirin resistance-determining region (IRRDR) (aa 2334-2379) were also related to the SVR to combined pegylated-IFN and ribavirin therapy (6). The significance of these mutations was also confirmed by studies carried out in different populations in different countries (7).

Based on previous studies, factor analysis and determination of NS5A viral mutations in relation to SVR of patients treated with pegylated-IFN and ribavirin combination therapy for HCV genotype 1b and a high viral load was carried out in a collaborative study in Kobe, Japan.

Materials and methods

Sample collection. Serum samples were collected from chronic hepatitis C patients with genotype 1b and a high viral load. A total of 96 patients (age 57.7±8.3 years; 45 males, 51 females) who were treated by subcutaneous injections of pegylated-IFN-α-2b once every week (1.5 μg/kg) (Pegintron; Schering-Plough, Innishannon, Country Cork, Ireland) in combination with oral ribavirin (400-800 mg) daily for 48 weeks between September, 2006 and June, 2008 were enrolled. HCV-RNA in serum samples was examined at 4 weeks, at the end of treatment and 6 months after the end of treatment. Serum samples were collected and stored at -80˚C until virological examination. The rapid virological response (RVR) was defined as undetectable HCV-RNA at 4 weeks. Patients who had persistent undetectable serum HCV-RNA and normal serum alanine aminotransferase (ALT) levels 6 months after the end of treatment were considered to have an SVR.

The standard dosage of PEG-IFN (1.5 μg/kg) and ribavirin (12 mg/kg) was determined depending on the weight-based dose. Patients treated with >80% of the standard dosage were considered as high drug adherence and patients treated with at least one drug at <80% of the standard dosage were categorized as a low drug adherence group.

This study was conducted by Kobe University Hospital and 25 affiliated hospitals in Hyogo prefecture. The study protocol was approved by the Ethics Committee of Kobe University Hospital, and written informed consent was obtained from each patient before treatment.

Table I. Comparison of the base characteristics of the SVR and the non-SVR groups.

Factor	SVR	Non-SVR	P-value
No. of patients (%)	42 (44%)	54 (56%)	
Age, years	55.1±8.6	59.7±7.5	0.005
Males:Females	22:20	23:31	
BMI (kg/m^2)	24.0±3.4	23.2±3.4	0.85
ALT (IU/l)	72.3±69.4	75.8±61.8	0.66
PLT (x10^4/mm^3)	17.7±4.9	17.0±5.3	0.68
RVR	15/38	3/49	<0.001
PPB/ITT	30/41 (73%)	25/54 (46%)	0.03

SRV, sustained viral response; BMI, body mass index; PLT, platelets; ALT, alanine aminotransferase; RVR, rapid viral response; PPB, per-protocol-based analysis; ITT, intention-to-treat analysis.

Table II. Drug adherence of patients to pegylated-interferon and ribavirin therapy.

	High drug adherence	Low drug adherence	P-value
No. of patients (%)	65 (68%)	31 (32%)	
Age, years	57.4±8.2	59.3±7.2	0.25
Male:Female	33:32	13:18	
BMI (kg/m^2)	23.6±2.8	23.5±4.3	NS
ALT (IU/l)	78.2±54.5	72.7±68.5	0.7
PLT (x10^4/mm^3)	16.3±5.6	16.7±4.6	0.8
SVR	30/65 (46%)	11/31 (35%)	NS
ISDR ≥1	26/50 (52%)	12/26 (46%)	NS
IRRDR ≥6	18/50 (36%)	11/26 (42%)	NS

BMI, body mass index; ALT, alanine aminotransferase; PLT, platelets; SRV, sustained viral response; ISDR, IFN sensitivity-determining region; IRRDR, IFN resistance-determining region.

NS5A sequence analysis. HCV-RNA was extracted from 140 μl serum using a commercial kit according to the manufacturer's protocol (QIAmp Viral RNA kit; Qiagen, Tokyo, Japan). The NS5A region of the HCV genome was amplified and sequenced by nested RT-PCR using primer sets (6). The aa sequences were deduced and aligned using GENETYX Win software version 7.0 (Genetyx Corp., Tokyo, Japan).

Statistical analysis. Differences in parameters, including all available patient demographic, biochemical, hematological, and virological data, as well as ISDR and IRRDR sequence variations factors, were determined between the different patient groups by the Student's t-test for numerical variables, and Fisher's exact probability test for categorical variables.

Subsequently, univariate and multivariate logistic analyses were performed to identify variables that independently predict SVR. The odds ratios (OR) and 95% confidence intervals

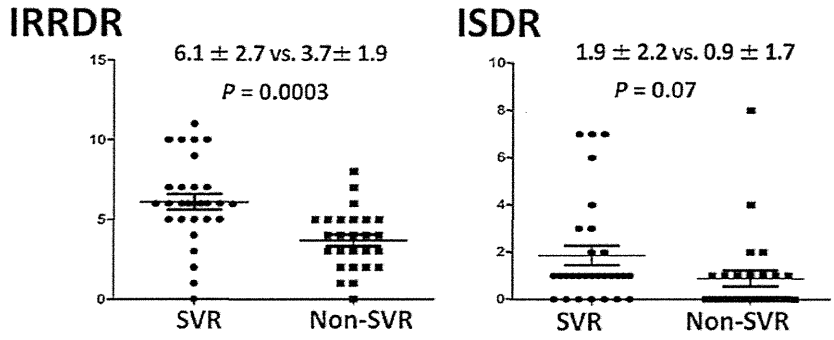

Figure 1. The number of mutations in IRRDR and ISDR. The number of mutations in IRRDR and ISDR was higher in the SVR group than in the non-SVR group.

Table III. Number of mutations in the NS5A region in relation to sustained viral response (SVR).

NS5A	Factor	SVR n (%)	Non-SVR n (%)	P-value
IRRDR	≥6	9/15 (60)[a]	3/17 (18)[a]	0.02[a]
	≥4	13/15 (87)	9/17 (53)	0.05
ISDR	≥4	3/15 (20)	1/17 (6)	0.25
	≥2	5/15 (33)	3/17 (18)	0.22
	≥1	11/15 (73)	7/17 (41)	0.06

[a] Statistically significant result. ISDR, IFN sensitivity-determining region; IRRDR, IFN resistance-determining region.

Table IV. Univariate and multivariate analyses in relation to the sustained viral response (SVR).

Univariate analysis		Multivariate analysis	
Factor	P-value	Odds ratio (95% CI)	P-value
IRRDR (IRRDR ≥6 vs. IRRDR ≤5)	0.000	18.1 (3.5-94.4)	0.001
ISDR (ISDR ≥1 vs. ISDR =0)	0.000		
RVR	0.017	15.5 (1.3-179.1)	0.028
LVR	0.001		
HCV-RNA titer (≥1000 vs. <1000)	0.099		
Age (≥60 vs. <60)	0.072		
Gender (male)	1.000		
PLT (≥15 vs. <15)	0.427		

ISDR, IFN sensitivity-determining region; IRRDR, IFN resistance-determining region; RVR, rapid viral response; LVR, late viral response.

(CIs) were also calculated. Positive and negative predictive values of SVR were computed, and their significance levels were evaluated using the sign test. All statistical analyses were performed using the SPSS version 16 software (SPSS Inc., Chicago, IL). Unless otherwise stated, a P-value of <0.05 was considered to indicate a statistically significant result.

Results

Baseline characteristics and on-treatment response in association with SVR. Baseline characteristics and on-treatment response are summarized in Table I. Overall, 42 cases out of 96 (44%) achieved an SVR. SVR patients were significantly younger in age and had a higher rate of RVR than the non-SVR patients. The prevalence of high drug adherence in SVR patients (73%) was significantly higher than that in non-SVR patients (46%) (P=0.03).

Drug adherence to pegylated interferon and ribavirin therapy. Due to various side effects, 31 patients were not treated with a sufficiently high dosage. Table II summarizes the patient groups with low and high drug adherence. Sixty-five (68%)

patients had high drug adherence to the therapy. Older age women tended to require dose reductions. The SVR rate (35%) in patients with low drug adherence was significantly lower than those (46%) with high drug adherence.

Mutations in the NS5A region and predictive indicators for SVR. Factor analysis in association with the SVR was performed by per-protocol-based (PPB) analysis. The average number of mutations in IRRDR was significantly higher in the SVR group (6.1±2.7) than that in the non-SVR group (3.7±1.9) (P=0.0003). The average number of mutations in ISDR was also higher in the SVR group (1.9±2.2) than that in the non-SVR group (0.9±1.7), but this difference did not achieve statistical significance (Fig. 1). The SVR group and the non-SVR group were compared based on the number of mutations in the NS5A region. The prevalence of patients with ≥6 aa mutations within IRRDR in the SVR group (60%) was significantly higher than that in the non-SVR group (18%) (P=0.02). Similarly, the prevalence of patients with ≥1 aa mutation within ISDR in the SVR group (73%) was higher than that in the non-SVR group (41%), but this difference was not statistically significant (P=0.06). All patients with ≥6 aa mutations in IRRDR and ≥1 aa mutation in ISDR achieved an SVR (Table III). The positive predictive values of SVR in patients with ≥6 aa mutations in IRRDR was 78%. The sensitivity and specificity were 64 and 86%, respectively.

Factor analysis in association with the SVR. Univariate and multivariate analyses are summarized in Table IV. Univariate analysis showed that ≥6 aa mutations in IRRDR and ≥1 aa mutation in ISDR were strongly associated with an SVR. In addition, RVR and LVR were also significant between the two groups. Multivariate analysis revealed that ≥6 aa mutations in IRRDR (odds ratio 18.1) and RVR (odds ratio 15.5) were significantly related to the SVR.

Discussion

Pegylated-IFN and ribavirin combination therapy has been a standard treatment for patients with chronic hepatitis C. However, HCV genotype 1 is more resistant to IFN treatment than genotypes 2 or 3. In Japan, genotype 1b is the most prevalent and it is important to predict the therapeutic response for these patients prior to therapy (7-9). In general, approximately 50% of patients with genotype 1b do not achieve SVR even when using a combination of pegylated-IFN plus ribavirin treatment (10). In the present study, the overall SVR rate was 44% and this value was slightly lower than that in a previous study (8). The reason for this is possibly related to the patient age and drug adherence. The present study showed that age, drug adherence and RVR in the SVR group were significantly different than these values in the non-SVR group. The SVR rate in patients younger than 65 years was 52% and was significantly higher than that in patients over 65. In addition, the SVR rate (46%) in patients with high drug adherence was higher than that (35%) in patients with low drug adherence. There is no doubt that elder patients have difficulties continuing therapy and are forced to reduce the dosage or terminate treatment because of side effects. In the present study, the percentage of patients having low drug adherence was 32%, and the majority of patients in this group were aged women. Physically and mentally, it is frequently difficult to continue therapy for elder patients. The average age of patients in Japan is older than that in most other European countries and this is one of the important reasons for the therapeutic difference among Japanese studies and those carried out in other countries.

On-treatment response is an important factor for predicting SVR; RVR 4 weeks following the initiation of treatment has been reported to be a good predictor of SVR (11-13). In this study, RVR was an important factor for predicting SVR by multivariate analysis. The positive predictive value was 82% and RVR was confirmed to be a good predictor in this study. However, even when patients are predicted as good responders for IFN/RBV therapy, they do not always achieve SVR as side effects result in dose reduction or termination of the planned IFN/RBV treatment. It was also reported that drug adherence is related to SVR (14). In this study, 3 patients relapsed after achieving RVR. The first case was over 65 years of age, the second case had low drug adherence, and the third was an older patient over 65 years with low drug adherence. Incomplete treatment is an important factor contributing to the failure of achieving SVR. This result suggests the necessity for prolonged therapy or therapeutic modification in patients with RVR receiving a dosage reduction.

Mutations in several amino acids in the NS5A protein have been described and are thought to play an important role in response to IFN treatment. It has been reported that a high number of mutations in ISDR and IRRDR are significantly associated with SVR (6). In the present study, patients with ≥1 aa mutation in ISDR and ≥6 aa mutations in IRRDR tended to achieve SVR, which was supported by previous data (6). For ISDR, the mutation results are similar to previous studies (4,5). Compared with ISDR, IRRDR was more strongly associated with SVR in this study. Based on the multivariate analysis, only IRRDR was associated with an SVR. Patients with more than 6 IRRDR mutations had a higher SVR rate and it was the same as previous studies (6). The positive predictive value and sensitivity was >80%, suggesting it to be a good predictive marker. All patients with ≥6 aa mutations and ≥1 aa mutation in ISDR achieved SVR following pegylated-IFN and ribavirin combination therapy. The importance of the NS5A mutation is still controversial. It has been reported that a mutation in NS5A is not related to the IFN response in European and American HCV strains (15-18). However, the importance of NS5A was reported in Asian HCV strains including Taiwan and Chinese strains (19,20). To date, this inconsistency is unclear but is partly related to the fact that HCV strains are different depending on geographic distribution (21). Meta-analysis revealed that the prevalence of a mutation in ISDR was 44.1% in Japanese and 24.8% in European patients, respectively (21). Mutational studies are sometimes inconsistent even among Japanese studies, suggesting that mutations in the NS5A region vary based on different geographical regions even in Japan.

The NS5A protein has a transcriptional activation function and represses IFN-induced gene expression (22). In addition, the NS5A protein interacts with antiviral protein PKR resulting in suppressed PKR activity (23). It is possible that mutations in the NS5A protein may affect the structural and/or biological functions of NS5A and inhibit IFN activity (23,24).

Mutations in E2-PePHD (aa 659-670), PKRBD (aa 2209-2274) and NS5A-V3 (aa 2356-2379) are also reported to be associated with IFN sensitivity (24,25).

Recent studies have shown that SNPs in the IL28B region are strongly associated with response to IFN therapy (26). In this study, genomic factors in the host were not analyzed due to the pre-treatment study design and informed consent. Therapeutic prediction can be more accurate upon examination of host factors as well as viral factors. In the near future, new drug therapies such as protease and polymerase inhibitors called new direct-acting antivirals (DAAs) will become available (27). Standard therapy for hepatitis C virus will include combination therapies using DAAs and pegylated-IFN plus ribavirin. However, the SVR rate by telaprevir-based pegylated-IFN plus ribavirin combination therapy (REALIZE study; phase III, randomized, double blind, placebo-controlled study) was found to be as high as 31% in patients who were non-responders to prior treatment (28). The viral response to pegylated-IFN and ribavirin combination therapy is important for the development of future combination therapies.

In conclusion, mutations in the NS5A region, particularly in patients with more than 6 aa mutations in the IRRDR region are strongly associated with the therapeutic response to pegylated-IFN and ribavirin combination therapy.

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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

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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 $\geq$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 $\geq$6 and age <60 years improved SVR predictability (93.3 %), and that of IRRDR $\leq$5 and age $\geq$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 $\geq$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

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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

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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