Table 7. Uni- and multivariate logistic regression analyses to identify independent predictive factors for success of PEG-IFN/RBV combination therapy

Univariate		Multivariate	
variable	p value	odds ratio (95% CI)	p value
IRRDR mutations (IRRDR≥6 vs. IRRDR≤5)	<0.0001	14.33 (2.24–91.65)	0.005
Ala^{2360}	0.01	1.75 (0.19–15.36)	0.62
Core polymorphism (wild-core vs. non-wild-core)	0.06	0.41 (0.05–3.28)	0.34
Gln^{70}	0.04		
RVR	<0.0001		
EVR	<0.0001	41.83 (6.12–285.68)	0.0001
ETR	<0.0001		
HCV core antigen, fmol/l	0.05		
Age	0.01	0.91 (0.84–0.99)	0.02
Platelets, $\times 10^4/mm^3$	0.07		
Hemoglobin, g/dl	0.006		

IRRDR = Interferon/ribavirin resistance-determining region; Ala^{2360} = alanine at position 2360; Gln^{70} = glutamine at position 70; RVR = rapid virological response; EVR = early virological response; ETR = end-of-treatment response.

subgenomic RNA replicon containing NS5A of HCV-1b exerted more profound inhibitory effects on IFN activity 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-terminus region including IRRDR resulted in a transfer of their anti-IFN activity. Since the C-terminal region of NS5A is among the most variable sequences across the different genotypes and subtypes of HCV [22], the difference in IFN responsiveness among different strains of a given HCV subtype could also be attributable, at least partly, to the genetic polymorphism within this region. The molecular mechanism underling the possible involvement of IRRDR in IFN responsiveness of the virus is still unknown. The significant difference in IRRDR sequence pattern may suggest genetic flexibility of this region and, indeed, the C-terminal portion of NS5A was shown to tolerate sequence insertions and deletions [23, 24]. This means that the C-terminal portion of NS5A is not essential for virus replication in cultured cells. It does not exclude the possibility, however, that the same region plays an important role in modulating the interaction with various host systems, including IFN responsiveness. It is also possible that the genetic flexibility of this region, especially IRRDR, is accompanied by compensatory changes elsewhere in the viral genome and that these compensatory changes affect overall viral fitness and responses to IFN therapy [25].

While we observed significant correlation between the overall number of mutations in IRRDR and PEG-IFN/RBV responsiveness, we also found a particular aa mutation, Ala^{2360}, that was significantly associated with SVR (tables 3, 7; fig. 1). It is possible that Ala or Val at this position confers a certain advantage for interaction between NS5A and the other viral or host proteins, which might affect IFN-induced antiviral responses. This issue needs to be elucidated in further studies.

The ISDR polymorphism was the only virological factor examined that showed a significant correlation with RVR (table 4), with the result being consistent with a recent report by other investigators [26]. This significant correlation, however, disappeared as the treatment went on. In contrast, the IRRDR polymorphism did not correlate significantly with RVR, however, it was the dominant viral genetic factor that was correlated with SVR (tables 3, 7). Interestingly, the combination of IRRDR and ISDR polymorphisms (IRRDR≥6 *plus* ISDR≥2) was significantly correlated with RVR and SVR (p = 0.0001 and 0.01, respectively; data not shown). This suggests a possible integrated influence of IRRDR and ISDR polymorphisms, or NS5A as a whole, on the treatment outcome. Further study is needed to clarify the issue.

The core protein polymorphisms (wild-core vs. non-wild-core, and Gln^{70} and non-Gln^{70}) were significantly correlated with the on-treatment HCV clearance kinetics

(fig. 2c, d; tables 4, 6). However, this significant correlation became blurred thereafter and eventually no significant correlation was observed between wild-core (Arg^{70}/Leu^{91}) and the final treatment outcomes (table 3). On the other hand, Gln^{70} was significantly associated with null-response, and almost significantly with non-SVR. This result is consistent, at least partly, with previous reports, including a recent multicenter study in Japan, that identified Gln^{70} as a predictive factor for poor responses to PEG-IFN/RBV treatment [8, 9, 14].

Recently, it was reported that the C-terminal region of NS5A plays a critical role in regulating the early phase of HCV particle formation [27, 28]. Moreover, sequence alteration within this region affected the degree of interaction between NS5A and core protein, which in turn affected the efficiency of progeny virus production [29]. In the present study, we observed a significant correlation between the degree of IRRDR mutations (IRRDR≥6) and the core polymorphism (table 5). Therefore, it would be interesting to investigate the degree of interaction between NS5A with IRRDR of high or low degrees of sequence variation and the wild-type (Arg^{70}/Leu^{91}) or non-wild-type of core protein, and also the impact of these interactions on progeny virus production and IFN sensitivity of the virus.

The present study identified the IRRDR polymorphism as the only viral genetic factor that independently predicted PEG-IFN/RBV treatment outcome (table 7). On the other hand, HCV is likely to utilize an alternative mechanism(s) by which to escape IFN actions through its various structural and non-structural proteins [30]. Also, a different lineage(s) of HCV-1b strains that relies more on the alternative mechanism than on IRRDR may prevail in other regions of the world. It is possible, therefore, that the impact of the IRRDR polymorphism differs with different cohorts. Analysis in a large-scale multicenter study is needed to clarify this issue.

In conclusion, NS5A (IRRDR and ISDR) and core protein polymorphisms are useful viral markers for predicting the outcome of PEG-IFN/RBV therapy for chronic hepatitis C. In particular, IRRDR≥6 is a useful marker for prediction of SVR.

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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.

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).

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.

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

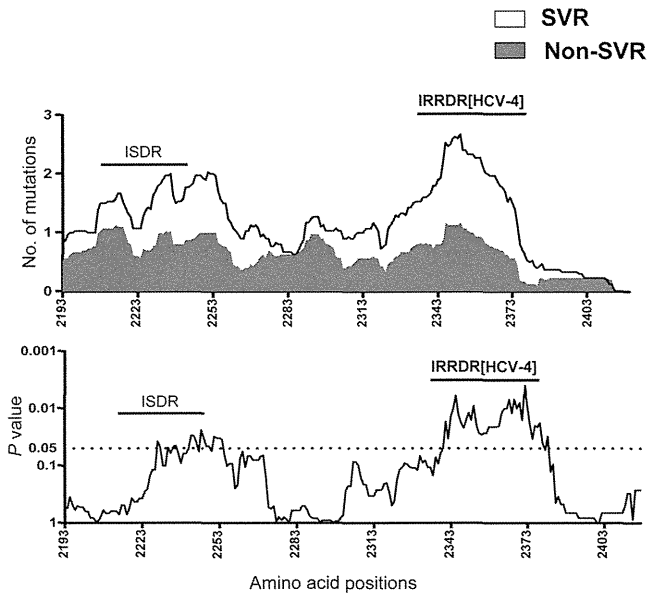

FIG 1 Sliding window analysis over the carboxy terminus (aa 2193 to 2417) of NS5A of HCV-4 obtained from SVR and non-SVR patients.

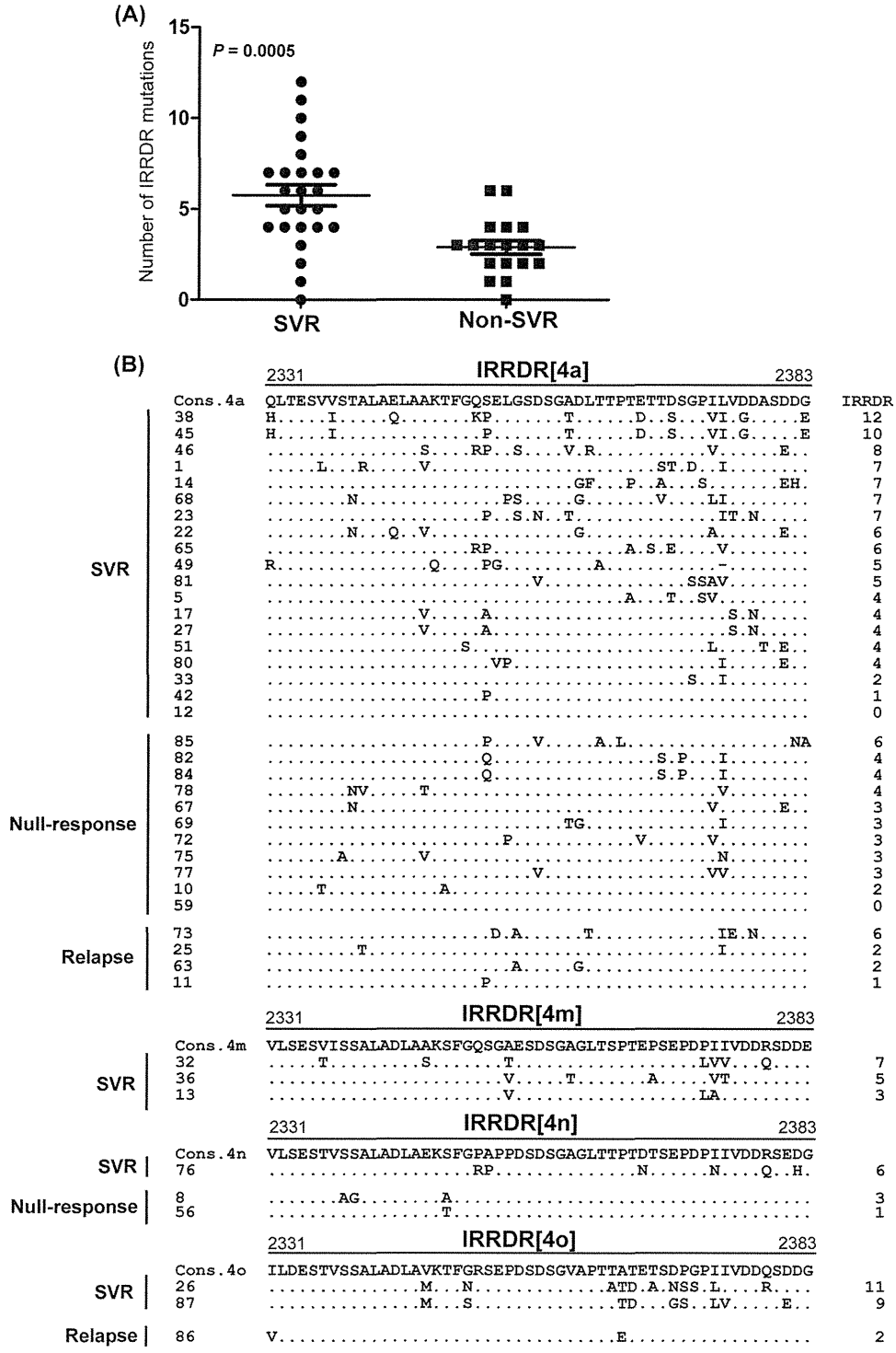

FIG 2 Correlation between IRRDR[HCV-4] sequence variations and treatment outcome. (A) Average number of amino acid mutations in IRRDR[HCV-4] obtained from SVR and non-SVR patients. (B) Alignment of IRRDR[HCV-4] sequences obtained from SVR and non-SVR patients with HCV-4a, -4m, -4n, and -4o. The consensus 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-

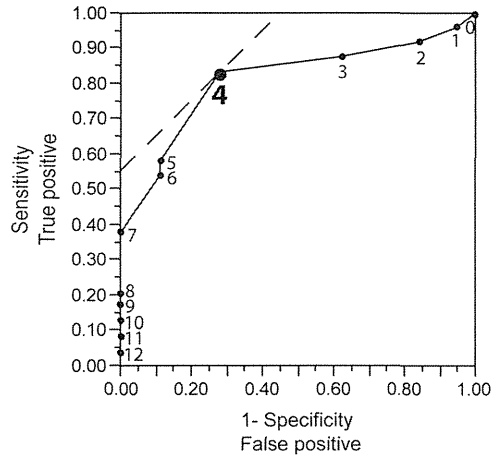

FIG 3 ROC curve analysis of IRRDR[HCV-4] sequence heterogeneity for SVR prediction. The solid line curve shows the AUC. Solid circles with numerals plotted on the curve represent different numbers of IRRDR mutations analyzed. The dashed line in the upper left corner indicates the optimal number of IRRDR[HCV-4]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

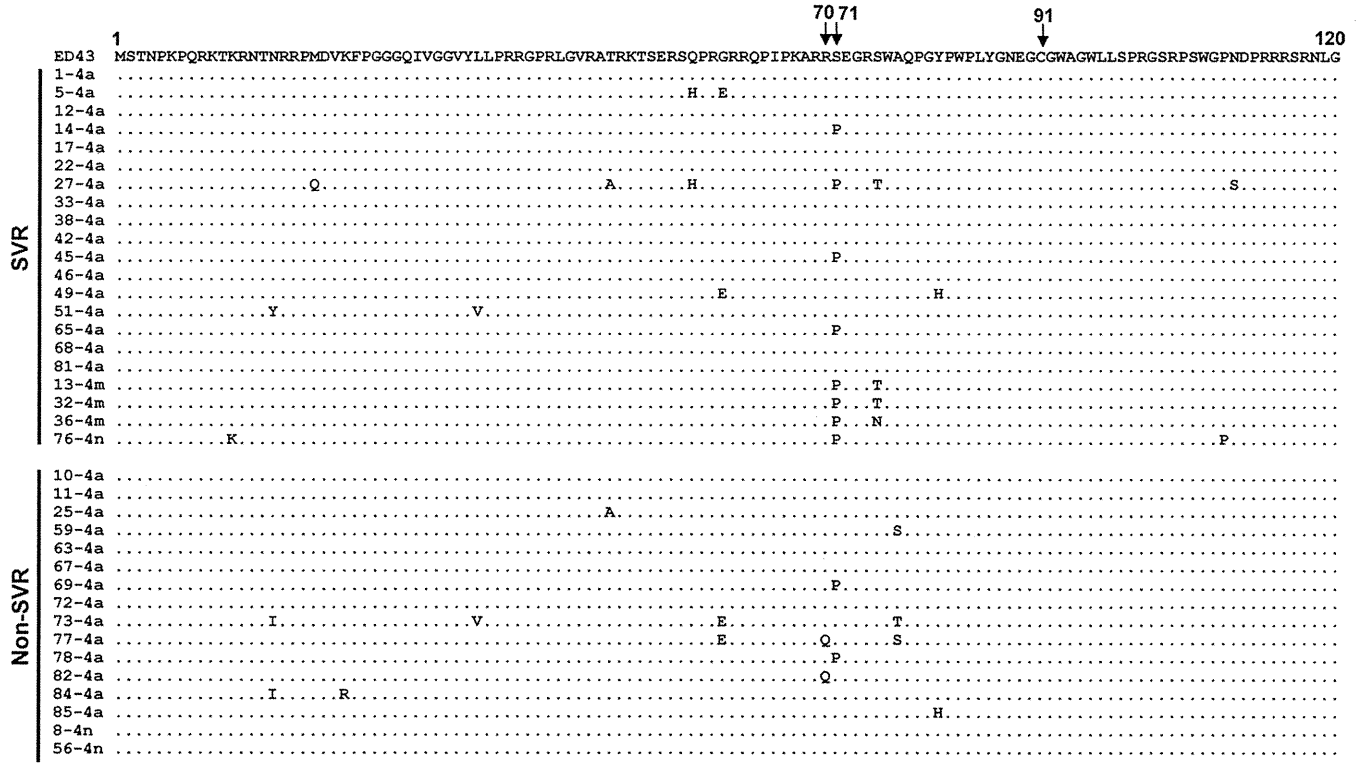

FIG 4 Sequence alignment of the core protein of HCV-4 isolates. Core protein sequences (aa 1 to 120) of HCV-4 obtained from SVR and non-SVR patients are aligned. The prototype sequence of ED43 (10) is shown on the top. The numbers along the sequence indicate the amino acid positions. Dots indicate residues 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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No conflicts of interest exist.

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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

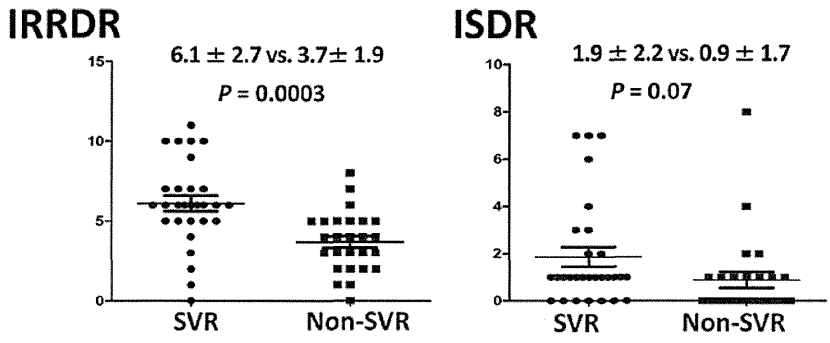

Figure 1. The number of mutations in IRRDR and ISDR. The number of mutations in IRRDR and ISDR was higher in the SVR group than in the non-SVR group.

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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PLOS ONE

HCV NS5A Protein Containing Potential Ligands for Both Src Homology 2 and 3 Domains Enhances Autophosphorylation of Src Family Kinase Fyn in B Cells

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Abstract

Hepatitis C virus (HCV) infects B lymphocytes and induces mixed cryoglobulinemia and B cell non-Hodgkin's lymphoma. The molecular mechanism for the pathogenesis of HCV infection-mediated B cell disorders remains obscure. To identify the possible role for HCV nonstructural 5A (NS5A) protein in B cells, we generated the stable B cell lines expressing Myc-His tagged NS5A. Immunoprecipitation study in the presence or absence of pervanadate (PV) implied that NS5A was tyrosine phosphorylated by pervanadate (PV) treatment of the cells. Therefore we examined pull-down assay by using glutathione S-transferase (GST)-fusion proteins of various Src homology 2 (SH2) domains, which associates with phosphotyrosine within a specific amino acid sequence. The results showed that NS5A specifically bound to SH2 domain of Fyn from PV-treated B cells in addition to Src homology 3 (SH3) domain. Substitution of Arg^{176} to Lys in the SH2 domain of Fyn abrogated this interaction. Deletion mutational analysis demonstrated that N-terminal region of NS5A was not required for the interaction with the SH2 domain of Fyn. Tyr^{334} was identified as a tyrosine phosphorylation site in NS5A. Far-western analysis revealed that SH2 domain of Fyn directly bound to NS5A. Fyn and NS5A were colocalized in the lipid raft. These results suggest that NS5A directly binds to the SH2 domain of Fyn in a tyrosine phosphorylation-dependent manner. Lastly, we showed that the expression of NS5A in B cells increased phosphorylation of activation loop tyrosine in the kinase domain of Fyn. NS5A containing ligand for both SH2 and SH3 domains enhances an aberrant autophosphorylation and kinase activity of Fyn in B cells.

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Introduction

HCV is a small enveloped positive-sense RNA virus classified within the family *Flaviviridae* [1,2]. In addition to liver cells, HCV infects B cells, leading to mixed cryoglobulinemia and B cell non-Hodgkin's lymphoma [3–5]. HCV infection in B cells enhances the expression of lymphomagenesis-related genes, such as activation-induced cytidine deaminase (AID) [6,7]. However, the molecular mechanisms of HCV infection-mediated B cell disorders remain elusive.

Non-receptor type of protein-tyrosine kinase Fyn is a member of the Src family kinases, and has regulatory roles in immune receptor signaling. Recently, Fyn has been recognized as an important mediator of mitogenic signaling and regulator of cell cycle entry, growth and proliferation. As for pathological aspects, Fyn is overexpressed in various cancers, and overexpression of Fyn in cultured cells resulted in cancer-like phenotypes [8].

The Src family kinases all share a common structure and pattern of activation. The domains of these proteins include SH2, SH3, and kinase domains followed by a short C-terminal regulatory tail. The SH2 and SH3 domains are highly conserved regions and mediate protein-protein interactions: the SH2 domain binds to phosphotyrosine residue within the specific amino acid sequence, while the SH3 domain recognizes proline rich regions. HCV NS5A was shown to interact with various SH3 domains of intracellular signaling molecules, and the kinase activity of Fyn was upregulated in liver cell lines harboring HCV replicon [9]. Binding of ligands to both the SH2 and SH3 domains disrupts autoinhibitory intramolecular interactions and leads to the opened conformation. Then autophosphorylation of the activation loop tyrosine (Tyr^{420} in Fyn) and dephosphorylation of the C-terminal tail (Tyr^{531} in Fyn) by protein-tyrosine phosphatases lead to the activation of kinase activity [10].

Previously, we reported that Syk, another non-receptor type of protein-tyrosine kinase interacts with transiently expressed NS5A in PV treated BJAB B cells [11]. This suggested that protein-tyrosine phosphorylation is required for the association of NS5A with Syk, because PV is a nonspecific inhibitor of protein-tyrosine phosphatases and treatment of cells with PV causes increase in

protein-tyrosine phosphorylation in whole cells. Recently Pfannkuche *et al.* reported that NS5A binds to the SH2 domain of Src [12]. However, molecular mechanism of their interaction and effect of NS5A on the kinase activity of Src remain unclear.

In this study, we investigated the interaction between NS5A and the SH2 domain of Fyn in B cells.

Materials and Methods

Antibodies and cDNAs

Anti-NS5A and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mAbs were purchased from Millipore (Bedford, MA, USA). Anti-Myc mAb and anti-Fyn antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phosphotyrosine (pTyr) (PY20) and human anti-IgM mAbs were from Zymed (South San Francisco, CA, USA). Anti-GST mAb was from Nacalai (Kyoto, Japan). Anti-phospho-Src family (Tyr416) antibody, which detects phosphorylated amount of Tyr^{420} in Fyn, was from Cell Signaling Technology (Danvers, MA, USA). The pEF1A-NS5A(Con1)-Myc-His plasmid and its deletion or substitution mutants were described previously [11]. Deletion of NS5A 127–146 (NS5A Δ127–146) was generated by the PCR-based method using four primers, 5′-TTGGTACCATGTCCGGCTCGTGGCTAAGAG-3′, 5′-GCTCTAGAGCAGCAGACGACGTCCTCA-3′, 5′-GGTTACGCGGGTGGGGGATCCCGAATTCTTCACAGAAGTG-3′, and 5′-CACTTCTGTGAAGAATTCGGGATCCCCCACCCGCGTAACC-3′, using NS5A cDNA as a template. Substitution of Tyr^{129} to Phe (Y129F) of NS5A 1–146 was generated by the site-directed mutagenesis using two primers, 5′-GGGGATTTCCACTTCGTGACGGGCA-3′ and 5′-TGCCCGTCACGAAGTGGAAATCCCC-3′, using NS5A 1–146 cDNA as a template. Substitutions of Tyr^{182} to Phe (Y182F), Tyr^{321} to Phe (Y321F), and Tyr^{334} to Phe (Y334F) of NS5A 147–447 were generated by the site-directed mutagenesis using two specific primers designed by QuikChange Primer Design Program (www.genomics.agilent.com), using NS5A 147–447 as a template. The resulted mutations were confirmed by the DNA sequencing.

Cell culture and transfection

B-lymphoid leukemia BJAB cells were kindly provided from Dr. Satoshi Ishido (RIKEN, Yokohama, Japan) [13] and maintained as described previously [14]. For the stable transfection of BJAB cells, 6 μg of linearized pEF1A-NS5A(Con1)-Myc-His was transfected into 5×10^6 cells/500 μl of cells by electroporation (240 V, 950 μF). Stably transfected cell lines were selected with 0.4 mg/ml of active G418 (Wako, Osaka, Japan) [15]. Cell lines were screened by level of protein expression by immunoblotting of detergent soluble lysates with anti-NS5A and anti-GAPDH mAbs as an internal control. Two positive cloned lines were selected for further analysis. For control cells, linearized empty vector was transfected by electroporation, and pooled clones resistant to 0.4 mg/ml of active G418 were utilized as control cells. COS cells were obtained from American Type Culture Collection (Manassas, VA, USA) and Ramos-T cells were kindly provided from Dr. Hamid Band (Nebraska Medical Center, NE, USA) [16]. Transient transfection of COS cells and Ramos-T cells were described previously [17]. Huh-7.5 cells were kindly provided from Dr. Charles M. Rice (The Rockefeller University, NY, USA) [18] and stably harboring an HCV replicon (pFK5B/2884 Gly) were described previously [11].

Cell activation, immunoprecipitation and immunoblotting

BJAB cells (10^8) were washed twice with serum free medium and treated with 100 μM PV or 10 μg/ml of anti-IgM mAb for 3 min at 37°C in the same medium. Either unstimulated or stimulated cells were washed twice with ice-cold PBS and then solubilized in the lysis buffer (1% Triton X-100, 50 mM Tris, pH7.4, 150 mM NaCl, 10 mM EDTA, 100 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride and 2 μg/ml aprotinin) on ice. In some experiments, 0.5% Nonidet P-40 was used instead of 1% Triton. Precleared cell lysates were incubated with the indicated antibodies prebound to protein A-agarose beads (Sigma, St. Louis, MO, USA). After rotation for 90 min at 4°C, the beads were washed 4 times with the lysis buffer, and the immunoprecipitated proteins were eluted by the heat treatment for 5 min at 100°C with 2×sampling buffer. Precipitated proteins or cell lysates were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore). After blocking in 5% milk in TBST (25 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Tween 20), the blots were incubated with the primary antibodies and then horseradish peroxidase-conjugated goat anti-rabbit IgG, goat anti-mouse IgG antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), or horseradish peroxidase-conjugated protein G (Sigma) in TBST. To enhance the signals, Immuno-enhancer Reagent A (Wako) was utilized in the reaction with anti-pTyr (pY20) mAb. Finally, proteins were visualized by the enhanced chemiluminescence (ECL) reagent (Western Lightning, PerkinElmer Life Sciences, Boston, MA) [19]. Immunoblot quantification was performed using the program Scion Image (Scion, Frederick, MD, USA).

Pull-down assay

The cDNA for Fyn-SH2 (Trp^{149}-Ala^{257}) and –SH3 (Thr^{82}-Glu^{148}) were amplified by PCR using paired primers 5′-GGAATTCATGGTACTTTGGAAAACTTGGC-3′ and 5′-CCGCTCGAGATCTTTAGCCAATCCAGAAGT-3′ for –SH2, 5′-GGAATTCAACAGGAGTGACACTGTTTGTG-3′ and 5′-CCGCTCGAGCTCTTCTGCCTGGATGGAGTC-3′ for –SH3, using mouse Fyn(T) cDNA (a gift from Dr. Yasuhiro Minami, Kobe University, Kobe, Japan) as a template. The cDNA for c-Abl-SH2 (Typ^{146}-His^{221}) and -SH3 (Leu^{84}-Val^{138}) were amplified by PCR using paired primers 5′-CGGAATTCCTGGTATCATGGCCCTGTATCT-3′ and 5′-ATAGTTTAGCGGCCGCTAGCTGGGTAGTGGAGTGTGGT-3′ for -SH2, 5′-CGGAATTCCCTTTTTGTGGCACTCTATGAT-3′ and 5′-TAGTTTAGCGGCCGCTGACGGGGGTGATGTAGTTGCT-3′ for -SH3, using mouse c-Abl cDNA (a gift from Dr. David Baltimore, California Institute of Technology, CA, USA) as a template. The cDNA for Cbl-b N-terminal region containing SH2 domain (Ala^2-Pro^{349}) was amplified by PCR using 5′-CGGAATTCCGCAAACTCAATGAATGGCAGA-3′ and 5′-CCGCTCGAGCTAAGGTGTAGGTTCACATAATCC-3′, using human Cbl-b cDNA (a gift form Dr. Stanley Lipkowitz, National Naval Cancer Center, MD, USA) as a template. Resulted PCR fragments were subcloned into pGEX-4T.3 (GE Healthcare, Piscataway, NJ, USA) to make domain in-frame with the downstream of GST and verified by DNA sequencing. The GST-rat Lyn-SH2 and Syk-SH2 (N+C) expression constructs were provided by Dr. Reuben P. Siraganian (National Institutes of Health, MD, USA). Preparation of GST-rat Vav1-SH2, mouse c-Abl SH3 domain-binding protein-2 (3BP2)-SH2, human phospholipase C (PLC)-γ2-SH2 (N+C), and rat Lyn-SH3 domain expression constructs were described elsewhere [17,20,21]. Substitution of Arg^{176} to Lys (R176K) by a point mutation of pGEX-

4T.3-Fyn-SH2 was generated by the site-directed mutagenesis using two primers 5′-TCAAAGAGAGCCAAACCACCAAAGG-3′ and 5′-TAAGAAAGGTACCTCTTGGGTTTCC-3′, using Fyn-SH2 cDNA wild type as a template. The resulted point mutation was confirmed by the DNA sequencing. All these SH2 and SH3 domains were fused downstream of GST. The GST fusion proteins were affinity-purified with glutathione Sepharose 4B beads (GE Healthcare). Extraction of GST-fusion proteins from bacteria was confirmed by the SDS-PAGE and Coomassie brilliant blue staining [22].

BJAB cells (10^8), Huh-7.5 cells stably harboring an HCV replicon (3×10^6), COS cells (10^6) or Ramos B cells expressing SV40 T antigen (Ramos-T cells) (10^7) were washed twice with serum free medium and stimulated with 100 μM PV for 3 min at 37°C. Either unstimulated or stimulated cells were solubilized in the binding buffer (1% NP-40, 50 mM Tris, pH7.4, 150 mM NaCl, 10 mM EDTA, 100 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride and 2 μg/ml aprotinin). After centrifugation, the resulted supernatants were reacted with 20 μg of GST-fusion proteins prebound to glutathione Sepharose 4B beads for 90 min at 4°C. The beads were washed 4 times with the binding buffer. Proteins interacting with GST-fusion proteins were eluted by heat treatment for 5 min at 100°C with 2×sampling buffer, separated by SDS-PAGE, and analyzed by immunoblotting.

Far-western

Anti-NS5A immunoprecipitates from BJAB cells (3×10^7) were separated by SDS-PAGE and transferred to PVDF membrane. After blocking, the membranes were incubated with 2.5 μg/ml of GST or GST-Fyn-SH2 for 1 h at 4°C. After extensive washing, membranes were reacted with anti-GST mAb, subsequently reacted with horseradish peroxidase conjugated goat anti-mouse IgG antibody, and then subjected to ECL detection [17].

Subcellular fractionation

The low density detergent-insoluble fractions were prepared by sucrose density gradient centrifugation as described [23]. BJAB cells (10^8) were solubilized in 2.5 ml of 0.05% Triton in MNEV buffer (150 mM NaCl, 25 mM Mes, pH 6.5, 5 mM EDTA, 1 mM Na_3VO_4, and protease inhibitors) and dounced 10 times. Homogenates were cleared of intact cells by centrifugation for 10 minutes at 200 *g*. The resultant supernatants (2.4 ml) were mixed with equal volumes of 80% sucrose in MNEV buffer (final, 40% sucrose and 0.025% Triton), overlayered by 4.8 ml 30% and 2.4 ml 5% sucrose in MNEV buffer, and then centrifuged for 20 hours at 200 000 *g* (P40ST rotor, Himac CP80WX, Hitachi, Tokyo, Japan). After sucrose density gradient centrifugation, 9 fractions were collected from the top of the gradient and analyzed by the immunoblotting.

Statistical analysis

Quantification of Fyn was analyzed by ImageJ software. The two-tailed Student t-test was applied to evaluate the statistical significance of differences found. A P value of <0.05 was considered statistically significant.

In vitro kinase assay

Unstimulated BJAB cells were washed twice with ice-cold PBS and then solubilized in the lysis buffer. Precleared cell lysates were incubated with anti-Fyn antibody prebound to protein A-agarose beads. After rotation for 90 min at 4°C, the beads were washed 4 times with the lysis buffer, 2 times with the kinase buffer without ATP, then incubated with 20 μl of the kinase buffer (40 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 4 μM ATP, 4 μCi [γ-^{32}P] ATP) and 2.5 μg of acid-treated enolase (Sigma) for 30 min at room temperature. Reaction was terminated and proteins were eluted by the heat treatment for 5 min at 100°C with 2×sampling buffer. Proteins were separated by SDS-PAGE and gel was incubated with 1N KOH for 1 h at 56°C to remove phosphoserine and most of phosphothreonine. After fixation, the gel was dried and radiolabeled proteins were visualized by autoradiography. Immunoprecipitation of Fyn was confirmed by the immunoblotting.

Results

HCV NS5A associates with the SH2 domain of Fyn

To identify HCV NS5A-interacting protein in B cells, we generated the stable B cell lines in which Myc-His tagged NS5A protein is constitutively expressed. Since we confirmed that the parental cells did not express NS5A, we choose the clones in which the level of NS5A expression was highest (Fig. 1A). In the following experiments, two cloned lines (clone 3 and 7) were examined, although some figures present the results from only one representative cell line. For control, vector plasmid was transfected into the same parental cells and G418-resistant clones were pooled and utilized as control cells.

Next, we performed immunoprecipitation study using anti-Myc mAb and found tyrosine phosphorylated proteins (Fig. 1B). This suggests that NS5A was tyrosine phosphorylated by PV treatment or another protein with similar size that associates with NS5A (Fig. 1B). Another experiment by affinity tag purification using Nickel column which could react with His-tag (His-Accept kit, Nacalai) also showed some tyrosine phosphorylated proteins in NS5A protein complex (data not shown). These findings suggest the possible involvement of protein-tyrosine phosphorylation associating with NS5A. Therefore, we tried to identify the protein which associates with NS5A through SH2 domain, which recognizes specific phosphotyrosine-containing amino acid sequence.

Then we carried out pull-down assay using GST-fusion proteins of various SH2 domains (Fig. 1C). Among these, the SH2 domain of Fyn dramatically bound to NS5A from PV-treated B cells. The SH2 domains of PLC-γ2 weakly bound to NS5A. The SH2 domains of Lyn, Abl, Vav, 3BP2, Syk or Cbl-b interacted with NS5A at very low level (long exposure, data not shown). GST-Lyn-SH3 was utilized as positive control because it was reported to interact with NS5A [9]. Therefore, this data demonstrated that HCV NS5A selectively binds to the SH2 domains of Fyn and PLC-γ2 in B cells. GST-human Fyn-SH2 also interacted with NS5A (Fig. S1). Moreover, the NS5A interaction with GST-Fyn-SH2 was observed even in the context of HCV RNA replication (Fig. 1D). Thus, HCV NS5A selectively associates with the SH2 domain of Fyn.

NS5A binds to the SH2 domain of Fyn in a tyrosine phosphorylation-dependent manner

PV treatment of cells dramatically enhances the binding of NS5A to the SH2 domain of Fyn, but not with that of Lyn or Abl (Fig. 2A). Substitution of Arg^{176} to Lys in the SH2 domain of Fyn, which caused the loss of association with phosphotyrosine residue, abrogated the binding of the SH2 domain of Fyn to NS5A (Fig. 2B). Arg^{176} is located in the consensus sequence within the SH2 domains to interact with phosphotyrosine residue. On the other hand, the SH3 domain of these kinases associated with NS5A to a comparable level (Fig. 2C). These results suggest that

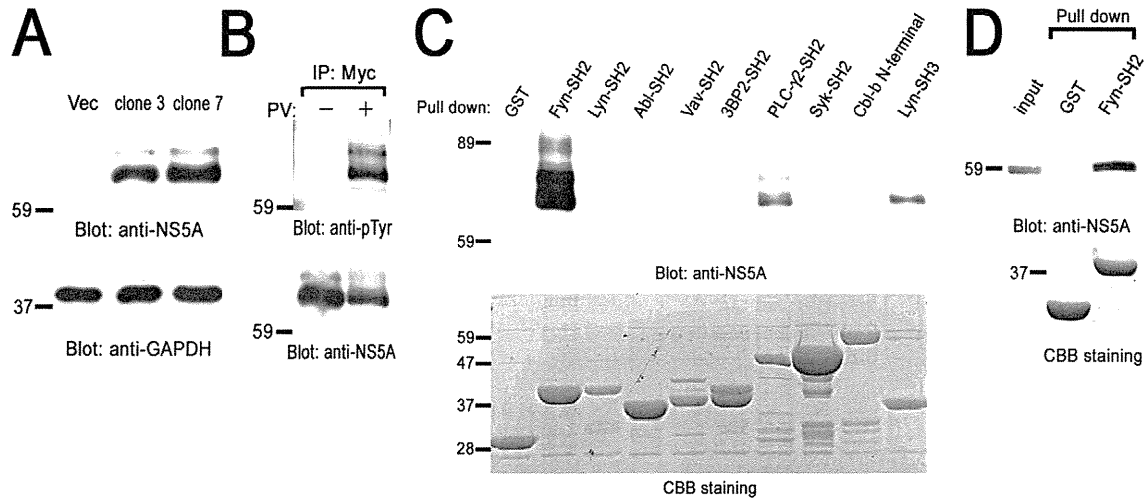

Figure 1. Identification of HCV NS5A-interacting proteins in B cells. (A) Generation of stable B cell lines expressing HCV NS5A. Detergent soluble cell lysates from vector cells (Vec) and Myc-His-NS5A expressing clones (clones 3 and 7) were separated by SDS-PAGE and analyzed with immunoblotting with anti-NS5A and anti-GAPDH mAbs. (B) BJAB cells expressing Myc-His-NS5A (clone 7) were treated without (−) or with (+) PV and solubilized in the lysis buffer. Cell lysates were immunoprecipitated with anti-Myc mAb and immunoprecipitated proteins were separated by SDS-PAGE and analyzed with immunoblotting with anti-pTyr (PY20) and anti-NS5A mAbs. PV-treated cells expressing Myc-His-NS5A (clone 7) (C) or Huh-7.5 cells stably harboring an HCV subgenomic replicon (D) were solubilized in the binding buffer. Precleared lysates were reacted with the indicated GST-fusion proteins and binding proteins were separated by SDS-PAGE and analyzed with immunoblotting with anti-NS5A mAb. The amount of GST-fusion proteins was confirmed by Coomassie brilliant blue (CBB) staining (C and D). Molecular sizing markers are indicated at left in kilodalton. The results were representative of three independent experiments. Similar results were obtained when another line was examined (B and C).
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increase in tyrosine phosphorylation of NS5A itself, or other associating proteins, allow the interaction with the SH2 domain of Fyn.

Central and/or C-terminal regions of NS5A binds to the SH2 domain of Fyn

To map the Fyn-SH2-binding region in NS5A, a series of deletion mutants were examined (Fig. 3). The results obtained reveals that N-terminal region (amino acids number 1–126) is not required for the interaction with the SH2 domain of Fyn in COS cells, although this region contains the region to interact with another kinase Syk (Fig. 3A) [11]. NS5A 127–146 and 147–447 could interact with the SH2 domain of Fyn. This demonstrates that deletion of the Fyn-binding region in the context of the full-length protein leads to loss of function. Similar results were obtained when HCV NS5A proteins were transiently expressed in Ramos B cells expressing SV40 T antigen (Ramos-T cells), and examined by pull-down assay (Fig. 3B). Deletion of 127–146 (NS5A Δ127–146) still allowed binding of NS5A to the SH2

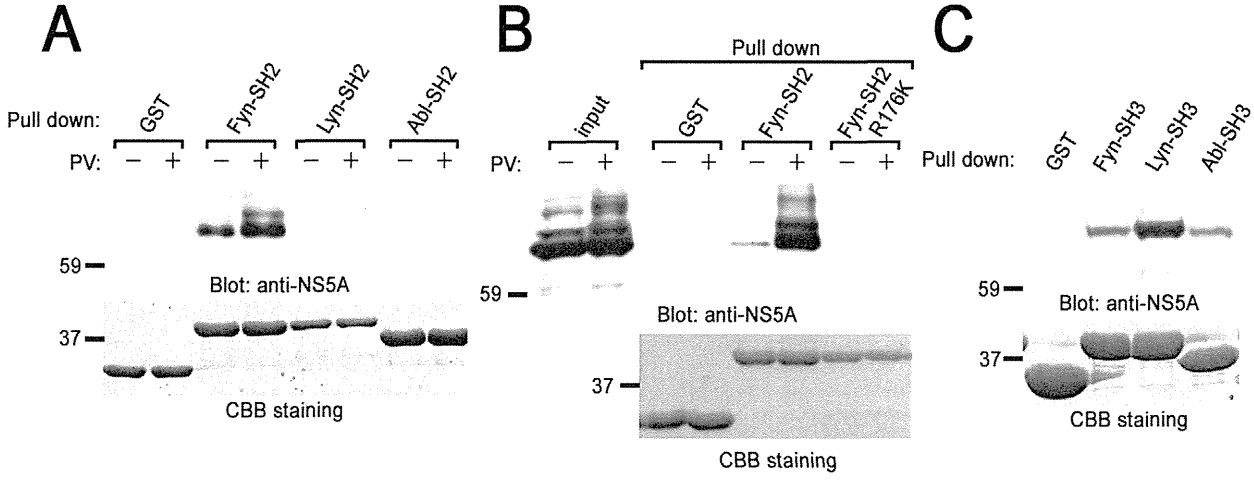

Figure 2. Pervanadate treatment of cells stimulates the binding of NS5A to the SH2 domain of Fyn in B cells. Either nontreated or PV-pretreated cells expressing Myc-His-NS5A (clone 7) were reacted with GST-fusion proteins of SH2 domains of various protein-tyrosine kinases (PTKs) (A), GST-Fyn-SH2 or GST-Fyn-SH2 R176K (B), or SH3 domains of various PTKs (C). Binding proteins were separated by SDS-PAGE and analyzed with immunoblotting with anti-NS5A mAb. The amount of GST-fusion proteins was confirmed by CBB staining. Molecular sizing markers are indicated at left in kilodalton. The results were representative of three independent experiments. Similar results were obtained when another line was examined.
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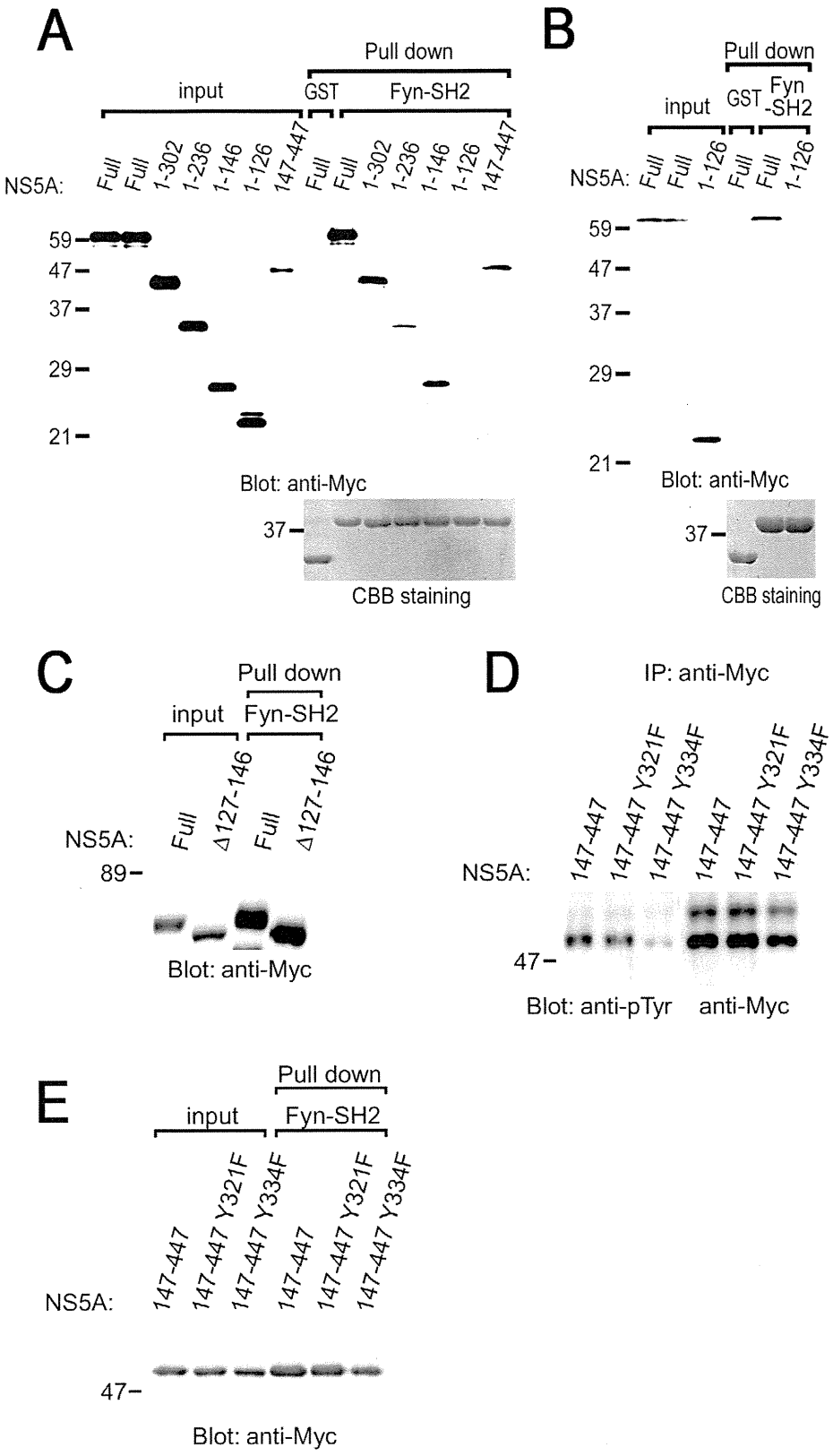

Figure 3. Structural analysis of the association of NS5A with the SH2 domain of Fyn in B cells. COS cells (A, C, E) or B cells (Ramos-T) (B) expressing different kinds of NS5A were stimulated with PV and subjected to pull-down assay using GST-Fyn-SH2. Binding proteins were separated by SDS-PAGE and analyzed with immunoblotting with anti-Myc mAb. The amount of GST-fusion proteins was confirmed by CBB staining. (D) COS cells expressing different kinds of NS5A mutants were stimulated with PV and subjected to immunoprecipitation. Precipitated proteins were separated by SDS-PAGE and analyzed with immunoblotting with anti-pTyr (PY20) and anti-Myc mAbs. Molecular sizing markers are indicated at left in kilodalton. The results were representative of three independent experiments.
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