solution and then with anti-Syk rabbit polyclonal antibody (N-19; Santa Cruz Biotech). Normal rabbit IgG served as a control. The sections were then incubated with horseradish peroxidase-labelled polymer-conjugated secondary antibody. The sections were counter-stained with haematoxylin and examined under a light microscope. To confirm the specificity of immunostaining, anti-Syk antibody was pre-incubated with a 1000-fold excess of blocking peptide (Santa Cruz Biotech) for 2 h at room temperature prior to staining.

Detection of HCV RNA by *in situ* RT-PCR. *In situ* RT-PCR was performed as described previously (Maeda *et al.*, 2004) with some modifications. Briefly, OCT-embedded frozen liver biopsy sections were fixed with 10% formaldehyde and treated with proteinase K. The samples were subjected to *in situ* reverse transcription using Moloney murine leukemia virus reverse transcriptase with an antisense primer for HCV (nt 290–272; 5′-AGTACCACAA GGCCTTTCG-3′), followed by *in situ* PCR using an *in situ* PCR System 1000 (Applied Biosystems) in the reaction mixture containing the antisense and a sense primer (nt 129–147; 5′-CCGGGAGAG CCATAGTGGT-3′). After being fixed in 4% paraformaldehyde, the PCR products were detected by *in situ* hybridization using a digoxigenin (DIG)-labelled oligonucleotide probe, 5′-(DIG)-ATTTGGGCTGTGCCCCCGCGAGACTGCTAGCCGAGTAGTGTT-GGGT-$(DIG)_n$-3′ (nt 225–270). Anti-DIG antibody conjugated with alkaline phosphatase (Roche) was used to detect the probe. The slides were incubated in a dye solution containing nitro blue tetrazolium, 5-bromo-4-chloro-3-indolylphosphate and levamizole to yield a purplish-blue precipitate.

Immunoprecipitation and Western blotting. Cultured cells were lysed with a buffer containing 1% Triton X-100, 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 10 mM EDTA, 1 mM NaF, 1 mM Na_3VO_4 and 1 mM PMSF. The lysate was centrifuged at 12 000 ***g*** for 20 min at 4 °C and the supernatant was immunoprecipitated with appropriate antibodies. In the case of liver tissue, each tissue sample was placed in a tube containing glass beads (1 mm diameter; BioSpec Products) to which 1 ml lysis buffer was added. The tube was then shaken at 4 °C for 3 min using a Mini-BeadBeater (BioSpec Products) to homogenize the tissues. After centrifugation at 80 ***g*** for 3 min, the supernatant was collected for immunoprecipitation analysis.

Immunoprecipitation and Western blot analyses were performed as described previously (Deng *et al.*, 2006). In brief, the supernatants of the lysates were pre-cleared with control IgG and protein A–Sepharose 4 Fast Flow (GE Healthcare) and incubated with appropriate antibodies at 4 °C for 1 h, followed by incubation with protein A–Sepharose 4 Fast Flow for another 1 h. After six washes with lysis buffer, the immunoprecipitates were analysed by Western blotting.

Antibodies used were as follows: anti-FLAG rabbit polyclonal antibody (Sigma); anti-Myc polyclonal and monoclonal antibodies (Santa Cruz Biotech); anti-Syk monoclonal antibody (4D10; Santa Cruz Biotech); anti-phospho Syk(Tyr^{352}) and Syk($Tyr^{525/526}$) rabbit polyclonal antibodies (Cell Signaling Technology); anti-PLC-γ1 monoclonal antibody (BD Biosciences); mouse monoclonal antibodies against core (Yasui *et al.*, 1998), NS3, NS4A and NS5A (kind gifts from Dr I. Fuke, Osaka University, Japan); anti-NS5A rabbit polyclonal antibody (NS5ACL1; a kind gift from Dr K. Shimotohno, Kyoto University, Japan; Miyanari *et al.*, 2007); and anti-NS5B goat polyclonal antibody (sc-17532; Santa Cruz Biotech). Normal IgG served as a control.

***In vitro* protein kinase assay.** An *in vitro* protein kinase assay was performed as reported previously (Miah *et al.*, 2004; Sada *et al.*, 2000, 2001). In brief, immunoprecipitates obtained with anti-Syk antibody from differentially transfected cells were incubated with 10 μg H2B histone (Sigma) as substrate in 20 μl kinase buffer, composed of 30 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 2 mM $MnCl_2$, 4 μM ATP and 4 μCi (148 kBq) [γ-^{32}P]ATP, for 30 min at room temperature. Reactions were terminated by boiling for 5 min in 2 × sample buffer.

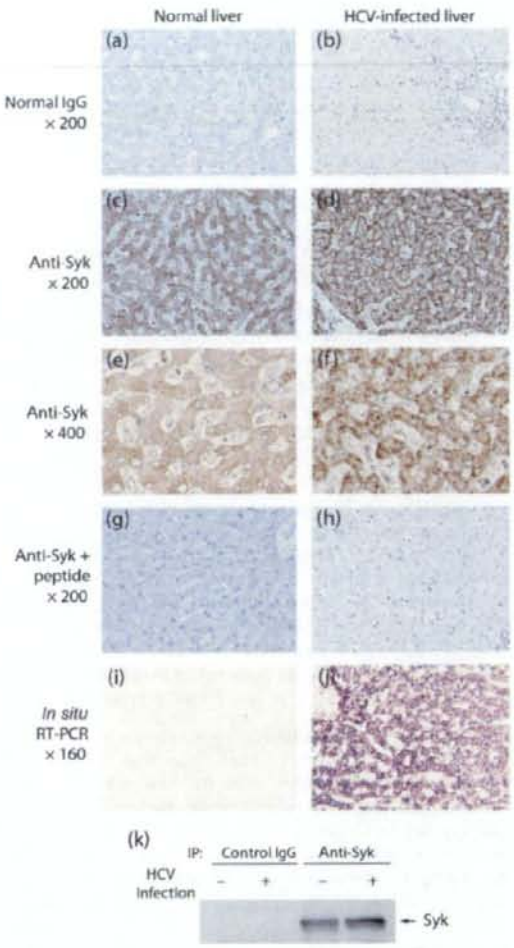

Fig. 1. Endogenous Syk expression in human liver tissues *in vivo*. Normal liver tissues (a, c, e, g, i) and HCV-infected non-cancerous liver tissues (b, d, f, h, j) were analysed. Formalin-fixed samples were stained with control IgG (a, b) or anti-Syk polyclonal antibody without (c–f) or with (g, h) pre-incubation with an excess amount of the immunogenic peptides. Frozen tissues were sectioned and examined for the presence of HCV RNA by *in situ* RT-PCR (i, j). Representative results are shown from four normal livers and ten HCV-infected livers. (k) Western blot analysis of normal human liver and HCV-infected non-cancerous liver. Supernatants of liver tissue homogenates (1.75 mg protein equivalent) were immunoprecipitated with anti-Syk monoclonal antibody (4D10) and probed with the same antibody or with control IgG.

Proteins were separated by SDS-PAGE. The gels were treated with 1 M KOH for 1 h at 56 °C to remove phosphoserine and most of the phosphothreonine. After gel drying, radiolabelled proteins were visualized by autoradiography. For quantitative analysis, γ-^{32}P incorporation was measured using a PhosphorImager (BAS2000; Fuji) and protein amounts with an LAS1000 image analyser (Fuji).

RESULTS

Different expression patterns of endogenous Syk in normal and HCV-infected liver tissues

We first examined whether Syk was expressed in human liver tissues. Immunohistochemical analysis revealed that Syk was indeed expressed and rather diffusely distributed throughout the cytoplasm of normal adult hepatocytes (Fig. 1c, e). This pattern was observed with four out of four normal liver tissues (100 %; data not shown). The specificity of the staining was verified by pre-incubating the antibody with an excess amount of the immunogenic peptides (Fig. 1g, h). We then examined Syk expression in non-cancerous liver tissue obtained from patients with HCV-associated HCC. Interestingly, Syk was detected near the plasma membrane with a patchy pattern in hepatocytes of eight out of ten HCV-infected patients (80 %; Fig. 1d, f, and data not shown). All of the specimens stained with normal rabbit IgG were negative (Fig. 1a, b). We confirmed that almost all of the hepatocytes in the tissue samples were infected with HCV using *in situ* RT-PCR (Fig. 1i, j).

Western blot analysis confirmed Syk expression in human liver tissue, irrespective of HCV infection (Fig. 1k). It should be noted, however, that the Syk expression was rather weak, as we could achieve successful Western blotting only after the tissue lysates were concentrated by immunoprecipitation with specific antibody. Also, possibly due to the low level of expression and comparatively low sensitivity of the antibodies used for Western blotting, we could not detect the phosphorylated forms of Syk in the liver tissue (data not shown).

Identification of Syk as a novel NS5A-interacting protein

We then examined the possible interaction between HCV proteins and Syk in cultured cells. For this purpose, various HCV proteins and Syk were expressed ectopically in Huh-7 cells, as these cells do not express endogenous Syk. Co-immunoprecipitation analysis revealed that NS5A associated with Syk, whereas the other HCV proteins associated with Syk very weakly or not at all (Fig. 2a, b). A specific interaction of NS5A with Syk was also observed when NS5A was expressed as part of an NS3–NS5B polyprotein (Fig. 2c). These results collectively suggested that NS5A interacts specifically with Syk.

Next, we examined the possible interaction of NS5A with endogenously expressed Syk. As human hepatoma-derived cell lines, such as Huh-7, HepG2 and FLC4, are negative for

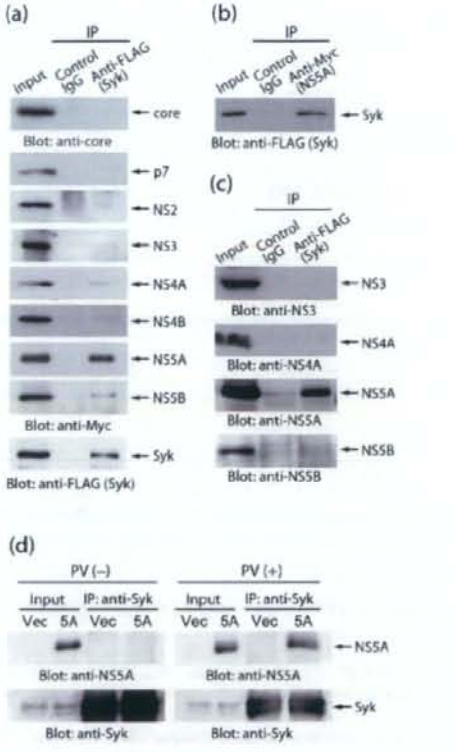

Fig. 2. NS5A specifically interacts with Syk in Huh-7 cells. (a) Each of the Myc-tagged HCV proteins was expressed with FLAG-tagged full-length Syk. Cell lysates were immunoprecipitated using anti-FLAG antibody or control IgG. Cell lysates (input) and the immunoprecipitates were probed with anti-core or anti-Myc antibodies. A representative result verifying efficient immunoprecipitation is shown at the bottom. (b) Myc-tagged NS5A was expressed with FLAG-tagged full-length Syk. Cell lysates were immunoprecipitated using anti-Myc antibody or control IgG, and probed with anti-FLAG antibody. (c) A polyprotein consisting of NS3–NS5B was expressed with FLAG-tagged Syk. Cell lysates were immunoprecipitated with anti-FLAG antibody or control IgG, and probed with the indicated antibodies. (d) NS5A was expressed in BJAB cells expressing endogenous Syk. The cells were treated with pervanadate (PV) or left untreated. Cell lysates were immunoprecipitated with anti-Syk monoclonal antibody and probed with anti-NS5A or anti-Syk monoclonal antibody. Vec, control using empty vector.

endogenous Syk expression, we used BJAB cells endogenously expressing Syk. Unlike ectopically expressed Syk, endogenous Syk in BJAB cells is not tyrosine phosphorylated. Therefore, we treated the cells with pervanadate to induce tyrosine phosphorylation of Syk. Co-immunoprecipitation experiments clearly demonstrated that NS5A

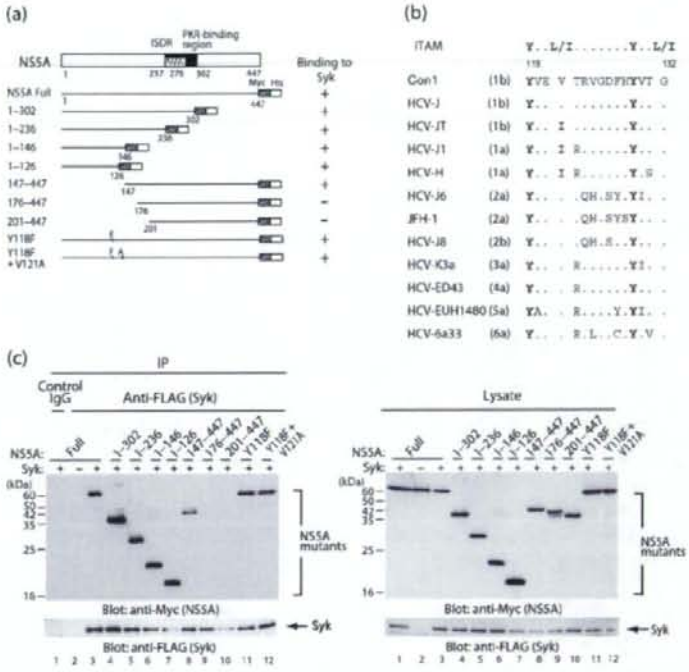

Fig. 3. Determination of the Syk-binding region(s) of NS5A. (a) Schematic diagram of various deletion mutants of NS5A and their Syk-binding capacity. (b) Alignment of amino acid sequences surrounding the ITAM-related sequence in NS5A of various HCV strains. The genotype is indicated in parentheses. Residues identical to those of HCV strain Con1 are shown by a dot. Residues identical to ITAM are shown in bold. (c) Full-length (Full) and a series of deletion mutants of Myc-tagged NS5A were expressed in Huh-7 cells with or without FLAG-tagged full-length Syk. Cell lysates were immunoprecipitated using anti-FLAG antibody and probed with anti-Myc antibody (left panel). Efficient immunoprecipitation was verified (bottom). Cell lysates were probed directly with anti-Myc and anti-FLAG antibodies to verify comparable expression levels of the NS5A mutants and Syk, respectively (right panels).

interacted with endogenous Syk when the cells were treated with pervanadate, but not when the cells were left untreated (Fig. 2d).

The N-terminal region of NS5A is required for interaction with Syk

To map a Syk-interacting region(s) of NS5A, interaction between various deletion mutants of NS5A and Syk was tested. C-terminally deleted mutants of NS5A up to aa 126, as well as the full-length NS5A, were co-immunoprecipitated with Syk (Fig. 3a, c). This result suggested that neither the PKR-binding region nor the interferon sensitivity-determining region (ISDR) of NS5A was required for the interaction with Syk. A proline-rich region of NS5A (aa 343–356), which is reported to bind to the Src family kinases (Macdonald & Harris, 2004; Macdonald *et al.*, 2004), was not involved in the Syk interaction either. In contrast, the N-terminally truncated mutant of NS5A(147–447), but not the further truncated mutants NS5A(176–447) or NS5A(201–447), was co-immunoprecipitated with Syk, suggesting that a region of NS5A between aa 147 and 175 is also involved in the interaction with Syk. We also observed that NS5A(1–126) and NS5A(174–447), but not NS5A(201–447), interacted with Syk(1–261) or Syk(379–635) (data not shown). These results collectively suggested that NS5A interacts with Syk through two independent regions of NS5A (aa 1–126 and 147–175).

Syk is activated by interaction with a diphosphorylated ITAM of immune receptors (Sada *et al.*, 2001; Turner *et al.*, 2000; Weiss & Littman, 1994). NS5A from HCV strain Con1 possesses a sequence (AEE**Y**^{118}VE**V**^{121}-TRVGDFH**Y**^{129}VTG) that resembles an ITAM (Fig. 3b). We found that the two tyrosine residues at positions 118 and 129 are highly conserved across different genotypes and subtypes. The tyrosine at position 118 is exposed on

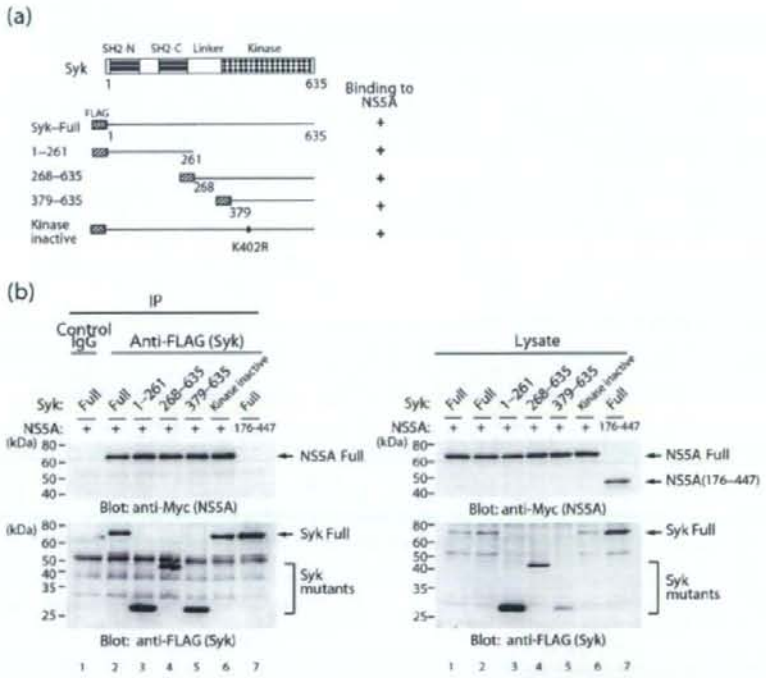

Fig. 4. NS5A interacts with both N-terminal and C-terminal regions of Syk. (a) Schematic diagram of the deletion mutants of Syk and their NS5A-binding capacity. (b) Full-length (Full) and a series of domain-deletion mutants of FLAG-tagged Syk was expressed in Huh-7 cells with Myc-tagged full-length NS5A (lanes 1–6) or NS5A(176–447) (lane 7). Cell lysates were immunoprecipitated using anti-FLAG antibody and probed with anti-Myc antibody (left upper panel). Efficient immunoprecipitation of Syk deletion mutants was verified (bottom). Cell lysates were probed directly with anti-Myc and anti-FLAG antibodies to verify comparable expression levels of the NS5A and Syk mutants, respectively (right panels).

the surface of the NS5A molecule (Tellinghuisen *et al.*, 2005). We examined whether this sequence motif was involved in the interaction with Syk. A single point mutation of Tyr^{118} (Y118F) or double mutations of Tyr^{118} and Val^{121} (Y118F and V121A) in NS5A did not affect the interaction with Syk (Fig. 3c, lanes 11 and 12). Thus, it is unlikely that NS5A binds to Syk through its ITAM-related sequence in the same manner as that observed for immune receptors.

To map the NS5A-binding region in Syk, a series of domain-deleted mutants of Syk was examined. The results obtained revealed that both N-terminal (tandem SH2 domains) and C-terminal halves (linker and the kinase domain) interacted with NS5A (Fig. 4). The kinase domain alone and a kinase-inactive form of Syk were also co-immunoprecipitated with NS5A. These results suggested that the NS5A–Syk interaction occurs through the N- and C-terminal regions of Syk and that the catalytic activity of Syk is not necessary for the interaction.

NS5A expression downregulates the kinase activity of Syk

Next, we tested the possible effect of NS5A expression on Syk kinase activity. An *in vitro* kinase assay revealed that full-length NS5A and a C-terminally deleted NS5A(1–302) mutant significantly inhibited Syk kinase activity (Fig. 5, lanes 2–4). In contrast, NS5A(1–236), which lacked both the PKR-binding region (aa 237–302) and ISDR (aa 237–276), failed to inhibit Syk kinase activity, although it could interact with Syk. NS5A(176–447), which contained the PKR-binding region and ISDR but lacked the Syk-binding region, did not affect Syk kinase activity. These results collectively suggested that NS5A requires both N-terminal (aa 1–175) and central (aa 237–302) regions for the downregulation of Syk kinase activity (Table 1).

To address the relevance of the interaction between NS5A and Syk, the possible effect(s) of NS5A on Syk-mediated cellular signalling in Huh-7 cells was examined. Ectopic

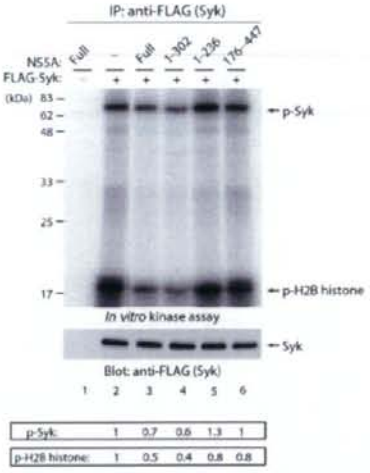

Fig. 5. NS5A downregulates Syk kinase activity. Myc-tagged NS5A and FLAG-tagged Syk were expressed in Huh-7 cells. Cell lysates were immunoprecipitated with anti-FLAG antibody and the immunoprecipitates were subjected to an *in vitro* kinase assay using H2B histone as substrate. Phosphorylation of Syk (p-Syk) and H2B histone (p-H2B histone) was visualized by autoradiography (upper panel). Efficient immunoprecipitation of Syk was verified (lower panel). Arbitrary units of Syk kinase activities, represented by the phosphorylation values of p-Syk and p-H2B histone normalized to the amounts of immunoprecipitated Syk, are shown at the bottom.

expression of Syk alone mediated signal transduction to induce tyrosine phosphorylation of a wide variety of cellular proteins, either directly or indirectly (Fig. 6a, lanes 1 and 3). Hyperosmolarity stress (400 mM sorbitol treatment) enhanced Syk-mediated tyrosine phosphorylation of cellular proteins (Fig. 6a, lanes 3 and 4), with the result being consistent with the previous observation (Miah *et al.*, 2004). Interestingly, co-expression of NS5A decreased Syk-mediated tyrosine phosphorylation of cellular proteins both in the absence and presence of hyperosmolarity stress (Fig. 6a, lanes 7 and 8). The phosphorylation of Syk on Tyr^{352} and/or $Tyr^{525/526}$ is a marker for Syk activation. Using these parameters, we confirmed that co-expression of NS5A inhibited Syk activation both in the absence and presence of hyperosmolarity stress (Fig. 6b).

PLC-γ1 has been reported to be a downstream molecule of Syk-mediated signal transduction (Law *et al.*, 1996). Our results demonstrated that NS5A inhibited PLC-γ1 phosphorylation, probably through downregulation of Syk kinase activity, both in the absence and presence of hyperosmolarity stress (Fig. 6c).

Table 1. Summary of NS5A deletion mutational analysis of the interaction with Syk and inhibition of Syk kinase activity

NS5A mutant	Interaction with Syk	Inhibition of Syk
NS5A(1–447; full)	+	+
NS5A(1–302)	+	+
NS5A(1–236)	+	–
NS5A(176–447)	–	–

NS5A expressed in the context of HCV RNA replication interacts with Syk in Huh-7.5 cells

The interaction of NS5A with Syk was examined further using Huh-7.5 cells harbouring an HCV subgenomic RNA replicon. The results obtained clearly demonstrated that NS5A expressed in the context of HCV RNA replication interacted with Syk (Fig. 7a). It is well known that NS5A takes two forms, p56 and p58, with the former being the basally phosphorylated form and the latter the hyperphosphorylated form (Kaneko *et al.*, 1994; Song *et al.*, 1999). It is noteworthy that Syk interacted with p56 more efficiently than with p58.

We also examined the interaction of NS5A with Syk in Huh-7.5 cells infected with the J6/JFH-1 strain of HCV. The results demonstrated that NS5A interacted with Syk in HCV-infected cells (Fig. 7b). These results collectively suggested that the NS5A–Syk interaction occurs in the context of virus replication, where NS5A is primarily utilized to form the viral replication complex. In this connection, HCV J6/JFH-1 replication was not affected significantly by ectopically expressed Syk in Huh-7.5 cells (data not shown). This observation, however, does not necessarily exclude the possibility that the NS5A interaction with Syk exerts certain biological effect(s) on the host cell's fate.

Syk kinase activity is suppressed in the context of HCV RNA replication

We then examined Syk kinase activity in the HCV subgenomic RNA-harbouring Huh-7.5 cells. An *in vitro* kinase assay demonstrated that Syk kinase activities, represented by autophosphorylation of Syk (p-Syk) and phosphorylation of a substrate (p-H2B histone), were significantly suppressed in HCV RNA-replicating cells compared with the control (Fig. 7c). These results suggested the possibility that Syk kinase activity is downregulated through an NS5A–Syk interaction in HCV-infected hepatocytes as well.

DISCUSSION

The non-receptor protein tyrosine kinase Syk is expressed in a wide variety of haematopoietic cell lineages (Taniguchi *et al.*, 1991). It is also expressed in human mammary

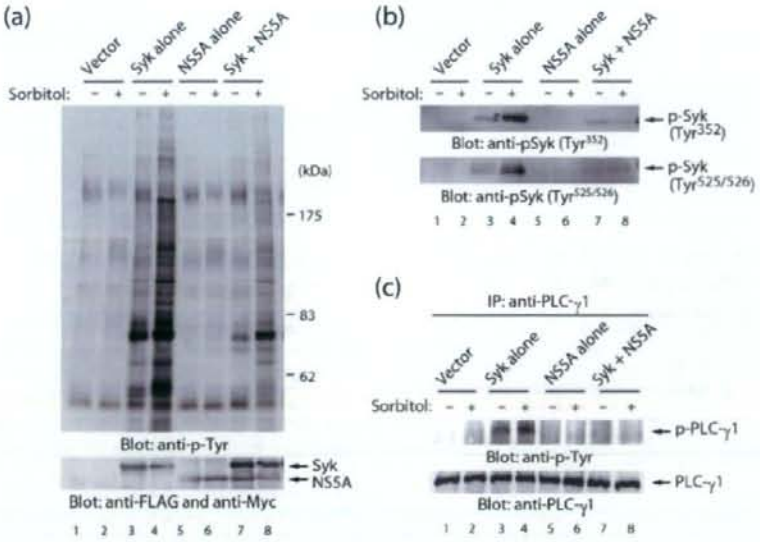

Fig. 6. NS5A suppresses hyperosmolarity stress-induced Syk-mediated tyrosine phosphorylation of cellular proteins. (a) Myc-tagged NS5A was expressed in Huh-7 cells with or without FLAG-tagged Syk. The cells were incubated with or without 400 mM sorbitol for 30 min and then lysed in lysis buffer. Half of the cell lysate was probed with anti-phosphotyrosine (p-Tyr) antibody (upper panel) and the remaining half with anti-FLAG and anti-Myc antibodies (bottom). (b) Cell lysates were probed with anti-p-Syk(Tyr^{352}) (upper panel) or anti-p-Syk(Tyr$^{525/526}$) antibody (lower panel). (c) Cell lysates were immunoprecipitated using anti-PLC-γ1 antibody and probed with anti-p-Tyr antibody (upper panel). Efficient immunoprecipitation of PLC-γ1 was verified (lower panel).

(Coopman *et al.*, 2000) and airway epithelial cells (Ulanova *et al.*, 2005), nasal fibroblasts (Yamada *et al.*, 2001) and hepatocytes (Tsuchida *et al.*, 2000). These results suggest that Syk plays a general physiological role in non-haematopoietic cells as well. The first report of Syk having a role in cancer was a study of mammary epithelial cells (Coopman *et al.*, 2000). Since then, there have been several reports that Syk functions as a tumour suppressor in the process of malignant tumour development, such as gastric cancer (Wang *et al.*, 2004) and leukaemia (Goodman *et al.*, 2001). To look into the possible relevance of Syk in HCV-infected hepatocytes and also the possible involvement of Syk in HCC development, we first examined Syk expression in hepatocytes obtained from HCV-infected and uninfected subjects. We found that Syk was expressed near the plasma membrane of hepatocytes of HCV-infected patients, with a patchy pattern, whereas it was expressed rather diffusely in the cytoplasm of normal, uninfected hepatocytes (Fig. 1).

We also demonstrated that NS5A interacted with Syk and inhibited its kinase activity when expressed ectopically in Huh-7 cells (Figs 2, 5 and 6). The NS5A interaction with Syk was observed even in the context of HCV RNA replication (Fig. 7a, b) and Syk kinase activity was inhibited in HCV RNA replicon-harbouring cells (Fig. 7c). It is likely, therefore, that Syk is a binding partner of NS5A and is functionally inhibited in HCV-infected hepatocytes as well. Whilst an N-terminal portion of NS5A (aa 1–175) was responsible for the binding to Syk, a central portion (aa 237–302) was also required for the inhibition of Syk kinase activity (Figs 3 and 5). It has been reported that NS5A associates with the non-receptor protein tyrosine kinases Lyn and Fyn, members of the Src family kinases, through the proline-rich region of NS5A (aa 343–356) and the SH3 domain of the kinases, thereby inhibiting and activating the kinase activities of Lyn and Fyn, respectively (Macdonald & Harris, 2004; Macdonald *et al.*, 2004). In contrast, Syk does not possess an SH3 domain but has two tandem SH2 domains. These SH2 domains are known to interact with diphosphorylated ITAM of immune receptors, resulting in activation of Syk in an autocrine or paracrine manner (Sada *et al.*, 2001; Yanagi *et al.*, 1995). However, it is unlikely that the NS5A–Syk interaction occurs through its ITAM-related sequence in the same manner as that observed for immune receptors, as NS5A mutants with a mutated ITAM-like sequence still interacted with Syk (Fig. 3). Also, the SH2 domains of Syk are not the only binding sites for NS5A (Fig. 4). These results suggest that the mechanism

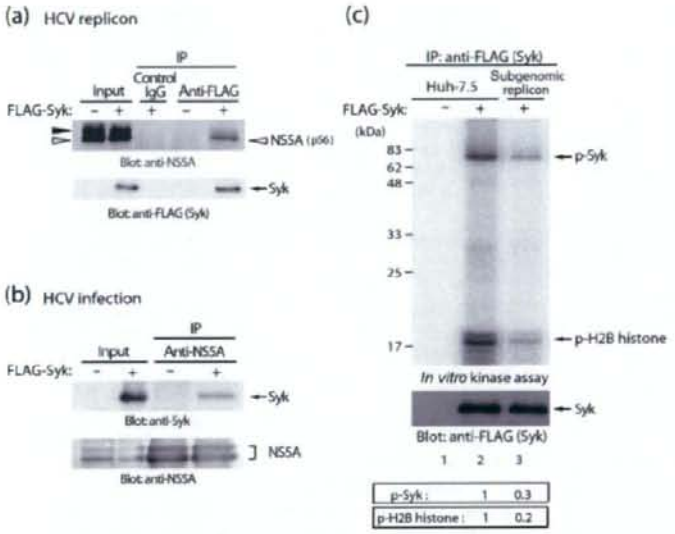

Fig. 7. NS5A expressed in the context of HCV RNA replication interacts with Syk and inhibits its kinase activity. (a) FLAG-tagged Syk was expressed in HCV RNA replicon-harbouring Huh-7.5 cells. Cell lysates were immunoprecipitated with anti-FLAG antibody or control IgG and probed with anti-NS5A (upper panel) or anti-FLAG antibody (lower panel). Filled and open arrowheads indicate the hyperphosphorylated (p58) and hypophosphorylated forms of NS5A (p56), respectively. (b) FLAG-tagged Syk was expressed in HCV J6/JFH-1-infected Huh-7.5 cells. Cell lysates were immunoprecipitated with anti-NS5A polyclonal antibody and probed with anti-Syk monoclonal antibody. (c) FLAG-tagged Syk was expressed in HCV RNA replicon-harbouring Huh-7.5 cells. Cell lysates were immunoprecipitated with anti-FLAG antibody and the immunoprecipitates were subjected to an *in vitro* kinase assay using H2B histone as substrate. Phosphorylation of Syk (p-Syk) and H2B histone (p-H2B histone) was visualized by autoradiography (upper panel). Efficient immunoprecipitation of Syk was verified (lower panel). Arbitrary units of Syk kinase activities, represented by the phosphorylation values of p-Syk and p-H2B histone normalized to the amounts of immunoprecipitated Syk, are shown at the bottom.

underlying the NS5A–Syk interaction differs from what has been observed for Syk and its interacting proteins in immune cells. It is possible that multiple regions of NS5A are involved in the interaction with Syk. Alternatively, NS5A may interact with Syk indirectly through the other host protein(s) that binds directly to Syk.

Syk is activated by cytokine stimulation, hyperosmolarity shock, oxidative stress and engagement with integrin (Corey *et al.*, 1994; Gao *et al.*, 1997; Miah *et al.*, 2004). However, the biological relevance of Syk in hepatocytes has not yet been demonstrated. We have shown in the present study that hyperosmolarity stress-induced activation of Syk resulted in increased tyrosine phosphorylation of endogenous PLC-γ1 (Fig. 6c). This result suggests that activated Syk sends signals to PLC-γ1 in hepatocytes, as observed in immune cells (Law *et al.*, 1996). Our findings that NS5A associates with Syk strongly suggest that NS5A affects the Syk signalosome to alter the signal transduction elicited by the Syk–PLC-γ1 interaction.

Phosphorylation of tyrosine residues in the linker region of Syk is required for immune receptor signalling. Genetic studies have demonstrated that phosphorylation of Tyr^{348} and Tyr^{352} in the linker region of Syk is involved in regulating tyrosine phosphorylation of LAT (linker for activating T cells), SLP-76 and PLC-γ1 and -γ2, and affects Ca^{2+} mobilization triggered by aggregation of the high-affinity IgE receptor (Simon *et al.*, 2005; Zhang *et al.*, 2002). We observed that NS5A downregulated phosphorylation of Tyr^{352} of Syk (Fig. 6b), which correlated with the inhibition of Syk kinase activity. The phosphorylation state of Tyr^{352} also correlated well with the tyrosine phosphorylation state of PLC-γ1. This suggests the possibility that Ca^{2+} mobilization is affected in HCV-infected hepatocytes through the NS5A-mediated downregulation of Tyr^{352} phosphorylation on Syk.

Unlike ectopically expressed Syk, endogenously expressed Syk in B cells under normal conditions is not tyrosine phosphorylated (Wienands *et al.*, 1996). Pervanadate stimulation is known to induce tyrosine phosphorylation of endogenous Syk. We examined the possible interaction of endogenous Syk and NS5A. Our results demonstrated that NS5A interacted with endogenous Syk when the cells were treated with pervanadate, but not when the cells were left untreated (Fig. 2d). These results suggest that NS5A interacts with the tyrosine-phosphorylated, active form of Syk.

Whilst Syk is commonly expressed in normal human breast tissues, benign breast lesions and low-tumorigenic breast

cancer cell lines, only a minimal or even an undetectable level of Syk expression has been demonstrated in invasive breast carcinoma tissues and cell lines (Coopman *et al.*, 2000). DNA methylation of the CpG sites in the *syk* gene promoter has been reported to be responsible for the loss or marked reduction of Syk expression in breast cancer (Yuan *et al.*, 2001). Moreover, Yuan *et al.* (2006) reported that DNA methylation of the *syk* gene in hepatitis B virus-associated HCC cancerous tissue was highly correlated with Syk expression and that the patients with a methylated *syk* gene had a significantly lower overall survival rate after hepatectomy than those with an unmethylated *syk* gene. In contrast, our results revealed that the expression levels of Syk did not differ between normal and HCV-infected hepatocytes (Fig. 1k) or between cancerous and non-cancerous hepatocytes (data not shown). At the functional level, however, NS5A downregulated Syk kinase activity in Huh-7 cells (Fig. 6). Moreover, Syk kinase activity was downregulated in cells harbouring an HCV RNA replicon (Fig. 7c). These results collectively suggest that NS5A is involved, at least partly, in the suppression of Syk kinase activity in HCV-infected cells. It is also interesting to assume that the NS5A-mediated Syk inhibition plays an important role in the development of HCC, although the precise molecular mechanism(s) is yet to be determined. Recently, a possible mechanism by which breast cancer cells become invasive was proposed: human breast cancer cells express and secrete a group of chemokines called growth-related oncogene (GRO)-α, GRO-β and GRO-γ, and their production is regulated by Syk (Li & Sidell, 2005). It would be interesting to examine the possible effects of NS5A and HCV RNA replication on the levels of GRO expression and secretion.

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Sequence Variation in Hepatitis C Virus Nonstructural Protein 5A Predicts Clinical Outcome of Pegylated Interferon/Ribavirin Combination Therapy

Ahmed El-Shamy,[1] Motoko Nagano-Fujii,[1] Noriko Sasase,[2] Susumu Imoto,[2] Soo-Ryang Kim,[2] and Hak Hotta[1,3]

A substantial proportion of hepatitis C virus (HCV)-1b–infected patients still do not respond to interferon-based therapy. This study aims to explore a predictive marker for the ultimate virological response of HCV-1b–infected patients treated with pegylated interferon/ribavirin (PEG-IFN/RBV) combination therapy. Nonstructural protein 5A (NS5A) sequences of HCV in the pretreated sera of 45 patients infected with HCV-1b were analyzed. The mean number of mutations in the variable region 3 (V3) plus its upstream flanking region of NS5A (amino acid 2334-2379), referred to as IFN/RBV resistance-determining region (IRRDR), was significantly higher for HCV isolates obtained from patients who later achieved sustained virological response (SVR) by PEG-IFN/RBV than for those in patients undergoing non-SVR. The receiver operating characteristic curve analysis estimated six mutations in IRRDR as the optimal threshold for SVR prediction. Indeed, 16 (76%) of 21 SVR, but only 2 (8%) of 24 non-SVR, had HCV with six or more mutations in IRRDR (IRRDR ≥ 6) ($P < 0.0001$). All of 18 patients infected with HCV of IRRDR of 6 or greater examined showed a significant (≥1 log) reduction or disappearance of serum HCV core antigen titers within 24 hours after initial dose of PEG-IFN/RBV, whereas 10 (37%) of 27 patients with HCV of IRRDR of 5 or less did ($P < 0.0001$). The positive predictive value of IRRDR of 6 or greater for SVR was 89% (16/18; $P = 0.0007$), with its negative predictive value for non-SVR being 81% (22/27; $P = 0.0008$). *Conclusion:* A high degree (≥6) of sequence variation in IRRDR would be a useful marker for predicting SVR, whereas a less diverse (≤5) IRRDR sequence predicts non-SVR. (HEPATOLOGY 2008;48:38-47.)

Hepatitis C virus (HCV) infection is the major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma in industrialized countries. However, HCV infection is curable, and its complications can be prevented by antiviral therapy.[1,2] Currently, the most effective treatment of chronic HCV infection is based on a combination of pegylated interferon (PEG-IFN) and ribavirin (RBV).[3] Even with this treatment regimen, however, sustained virological response (SVR) rates for those infected with the most resistant genotypes, HCV-1a and HCV-1b, still hover at approximately 50%.[3,4] Considering the high cost and the significant side effects associated with this combination therapy, it is worthy to identify patients most likely to benefit from therapy.[5] Predictors of IFN-based therapy can be classified into two categories, pretreatment and on-treatment factors. Pretreatment factors comprise host factors, such as age, sex, obesity, ethanol consumption, hepatic iron overload, fibrosis, immune responses, and coinfection with other viruses, and viral factors, which mainly include viral genotypes and viral load. On-treatment factors are mainly related to the viral kinetics within

Abbreviations: aa, amino acid; CI, confidence interval; CNR, complete nonresponse; ETR, end-of-treatment response; EVR, early virological response; HCV, hepatitis C virus; IFN, interferon; IRRDR, interferon/ribavirin resistance-determining region; ISDR, interferon sensitivity-determining region; NS5A, nonstructural protein 5A; nt, nucleotide; PEG, pegylated; PKR-BD, double-stranded RNA-activated protein kinase-binding domain; RBV, ribavirin; RT-PCR, reverse transcription polymerase chain reaction; SVR, sustained virological response; V3, variable 3.

From the [1]Division of Microbiology, Kobe University Graduate School of Medicine, Kobe, Japan; [2]Division of Gastroenterology, Kobe Asahi Hospital, Kobe, Japan; and [3]International Center for Medical Research and Treatment, Kobe University Graduate School of Medicine, Kobe, Japan.

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Address reprint requests to: Hak Hotta, M.D., Ph.D., Division of Microbiology, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan. E-mail: hotta@kobe-u.ac.jp; fax: (81)-78-382-5519.

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the first few weeks of treatment.[6] Because the HCV genotype is one of the major factors affecting IFN-based therapy response, IFN resistance is, at least partly, genetically encoded by HCV itself.[7] In this context, nonstructural protein 5A (NS5A), one of the HCV nonstructural proteins, has been widely discussed for its correlation with IFN responsiveness. Enomoto et al.[8,9] proposed that the sequence variations within a region in NS5A, called the IFN sensitivity-determining region (ISDR), is correlated with IFN responsiveness. It was further demonstrated that ISDR and its adjacent sequence was able to bind to double-stranded RNA-activated protein kinase (PKR), one of the important antiviral proteins of the host cell, to inhibit its enzymatic activity and, therefore, the combined region is called PKR-binding domain (PKR-BD).[10,11] A significant correlation between sequence variation in PKR-BD and IFN responsiveness was also reported.[12] In addition, there are some reports that showed a correlation between IFN responsiveness and the sequence diversity of the variable region 3 (V3) [amino acids (aa) 2356 to 2379] or its surrounding regions near the carboxy terminus of NS5A.[12-20]

We have recently reported that a high degree of sequence variations in the V3 and the pre-V3 regions (aa 2334-2355) of NS5A, which we collectively refer to as IFN/RBV resistance-determining region (IRRDR) (aa 2334-2379), was closely correlated with early virological response (EVR) by week 16 in HCV-1b–infected patients treated with PEG-IFN and RBV.[21] In the current study, we aimed to follow up our previous observations to investigate whether the degree of sequence variation in IRRDR could also correlate with SVR on PEG-IFN/RBV combination therapy.

Patients and Methods

Patients. A total of 45 patients chronically infected with HCV-1b, whose diagnoses had been made based on anti-HCV antibody detection, HCV subtype determination according to the method by Okamoto et al.,[22] and clinical follow-up, were treated with PEG-IFN α-2b (1.5 μg/kg body weight, once weekly, subcutaneously) and RBV (600-800 mg daily, per os), according to a standard treatment protocol for Japanese patients established by a hepatitis study group of the Ministry of Health, Labour, and Welfare, Japan, at Kobe Asahi Hospital, Hyogo Prefecture, Japan. All the patients were confirmed negative for hepatitis B surface antigen using chemiluminescent immunoassay (Abbott Japan Co., Ltd., Tokyo, Japan). Serum samples were collected from the patients at intervals of 4 weeks before, during, and after the treatment, and tested for HCV RNA by reverse transcription polymerase chain reaction (RT-PCR), as reported previously.[21] The quantification of serum HCV RNA titers was performed by RT-PCR with an internal RNA standard derived from the 5′ noncoding region of HCV (Amplicor HCV Monitor test, version 2.0, Roche Diagnostics, Tokyo, Japan). The thresholds of the low-range and high-range measurements of this assay were 50 and 600 IU/mL, respectively. HCV core antigen in the sera was also quantitatively measured by chemiluminescent immunoassay (Abbott Japan Co., Ltd., Tokyo, Japan). The threshold of this assay is 20 fmol/L.

The study protocol was approved beforehand by the Ethic Committee in Kobe Asahi Hospital, and written informed consent was obtained from each patient before the treatment.

NS5A Sequence Analysis. HCV RNA was extracted from 140 μL serum using a commercially available kit (QIAmp viral RNA kit; QIAGEN, Tokyo, Japan). For amplification of the NS5A region of the HCV genome, the extracted RNA was reverse transcribed and amplified for full-length NS5A using SuperScript One-step RT-PCR for long templates (Invitrogen, Tokyo, Japan) and a set of primers, NS5A-F1 [5′-TACTCCCTGCCATCCTCTCTCCTG-3′; sense, nucleotides (nt) 5974-5997] and NS5A-F2 (5′-CTCCTTGAGCACGTCCCGGT-3′; antisense, nt 7777-7796). The resultant RT-PCR product was subjected to a second-round PCR by using Platinum Taq DNA polymerase (Invitrogen) and an inner set of primers, NS5A-F3 (5′TCTCCAGCCTTACCATCACYCA-3′; sense, nt 6172-6193) and NS5A-F4 (5′-CGGTARTGRTCGTCCAGGAC-3′; antisense, nt 7761-7780). The samples that were not amplifiable (nos. 3, 23, 47, 61, 65, and 69) using the aforementioned primers were amplified using primer sets reported previously.[23] Reverse transcription was performed at 45°C for 30 minutes and terminated at 94°C for 2 minutes, followed by the first-round PCR over 35 cycles, with each cycle consisting of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 68°C for 90 seconds. The second-round PCR was performed under the same condition. The amplified fragments were purified with QIA quick PCR purification kit (QIAGEN), and visualized by agarose gel electrophoresis and ethidium bromide staining. The sequences of the amplified fragments were determined by direct sequencing without subcloning using Big Dye Deoxy Terminator cycle sequencing kit and ABI 337 DNA sequencer (Applied Biosystems, Inc, Japan). The aa sequences were deduced and aligned using GENETYX Win software version.7.0 (GENETYX Corp., Tokyo, Japan). Numbering of aa throughout the complete manuscript is according to the poly protein of HCV genotype 1b prototype HCV-J.[24]

Statistical Analysis. Statistical difference in the parameters, including all available patients' demographic, biochemical, hematological, and virological data as well as IRRDR sequence variations factors, was determined between different patients' groups by Student *t* test for numerical variables, and Fisher's exact probability test for categorical variables. In the case of multiple comparisons for various regions of NS5A, *P* values were adjusted by the Bonferroni method to reduce the probability of erroneously classifying nonsignificant hypothesis as significant. Although there are five regions of comparison (full-NS5A, N-half, ISDR, PKR-BD and IRRDR), the ISDR is entirely within the PKR-BD, and all the regions fall within the full-NS5A. Therefore, it would be reasonable to adjust the *P* values for three regions of comparison. Accordingly, the *P* value for a test was multiplied by 3. To evaluate the optimal threshold of IRRDR mutations for SVR prediction, the receiver operating characteristic curve was constructed and the area under the curve as well as the sensitivity and specificity were calculated. Subsequently, univariate and multivariate logistic analyses were performed to identify variables that independently predict SVR. The odds ratios and 95% confidence intervals (95% CI) were also calculated. Kaplan-Meier HCV survival curve analysis was performed based on serum HCV-RNA positivity data during treatment period (48 weeks) according to the number of IRRDR mutations (IRRDR $\geq$ 6 and IRRDR $\leq$ 5). The HCV death event was estimated as the first time point of HCV-RNA clearance after initiation of the treatment. The data obtained were evaluated by the log-rank test. Positive and negative predictive values of SVR predictors 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 less than 0.05 was considered statistically significant.

Nucleotide Sequence Accession Numbers. The sequence data reported in this article have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB285035 through AB285081, and AB354116 through AB354118.

Results

Virological Responses of the Patients Treated with PEG-IFN and RBV. Proportions of various virological responses of the patients treated with PEG-IFN/RBV combination therapy are shown in Table 1. Of 45 patients enrolled in this study, 23 (51%), 31 (69%), and 21 (47%) patients, respectively, achieved EVR by week 12 [EVR(12w)], end-of-treatment response (ETR), and sustained virological response (SVR). Among 23 patients with EVR(12w), 22 (96%) and 18 patients (78%) achieved ETR and SVR, respectively. This indicates that EVR(12w) was significantly correlated with ETR and SVR ($P < 0.0001$). A total of 24 patients (53%) failed to achieve SVR, and they were referred to as non-SVR. Non-SVR can be divided into two categories: (i) complete nonresponse (CNR), which is defined by continued presence of serum HCV RNA up to the end of the treatment, and (ii) relapse, which is defined by transient disappearance of HCV RNA at a certain time point followed by reappearance of HCV RNA either before or after the end of the treatment. CNR represented 24% (11/45) of all cases and 46% (11/24) of non-SVR. Thirteen (29%) of 45 patients underwent relapse. Among 13 relapsers, 3 (23%) patients had rebound in HCV viremia before the end of the treatment and, hence, were defined as undergoing viral breakthrough, whereas 10 (77%) patients had rebound in HCV viremia after the end of the treatment, defined as ETR-relapsers.

Table 1. Proportions of Various Virological Responses of Patients Treated With PEG-IFN/RBV

Virological Response	Proportion
EVR(12w)	51% (23/45)*
ETR	69% (31/45)
SVR	47% (21/45)
Non-SVR	53% (24/45)
CNR	24% (11/45)
Relapse	29% (13/45)
ETR-relapse	22% (10/45)
Viral breakthrough	7% (3/45)

*No. of patients/no. of total.

Abbreviations: PEG-IFN/RBV, pegylated-interferon/ribavirin; EVR, early virological response; ETR, end-of-treatment response; SVR, sustained virological response; CNR, complete nonresponse.

Demographic characteristics of patients with SVR, non-SVR, CNR, and relapse are summarized in Table 2. Age, sex, body weight, hemoglobin levels, or gamma guanosine triphosphate titers did not significantly differ between SVR and non-SVR or CNR. However, patients with SVR showed a trend toward having significantly higher platelet counts than those with non-SVR and CNR. Also, the mean initial titers of HCV core antigen for non-SVR and CNR, respectively, were 1.6 times and 2.3 times higher than that for SVR, although the difference was not statistically significant. HCV RNA titers were almost the same among them.

Correlation Between Virological Responses and the Sequence Variation of IRRDR of HCV NS5A Obtained from the Pretreated Sera. The entire NS5A region of the HCV genome was amplified from the pretreated sera and the aa sequences deduced. We compared

Table 2. Demographic Characteristics of Patients With SVR, Non-SVR, CNR, and Relapse

Factor	SVR	Non-SVR	CNR	Relapse	*P* Value		
					SVR versus Non-SVR	SVR versus CNR	SVR versus Relapse
Age	56.5 ± 8.0*	59.9 ± 10.6	59.4 ± 10.0	60.3 ± 11.5	NS†	NS	NS
Sex (male/female)	12/9	13/11	6/5	7/6	NS	NS	NS
Body weight (kg)	58.5 ± 9.4	59 ± 13.2	61.0 ± 10.8	57.8 ± 15.3	NS	NS	NS
Platelets ($\times 10^4/mm^3$)	18.3 ± 4.4	15.0 ± 4.9	12.3 ± 3.9	16.8 ± 4.9	0.02‡	0.001‡	NS
Hemoglobin (g/dL)	14.1 ± 1.3	13.7 ± 1.4	14.4 ± 1.3	14.3 ± 1.5	NS	NS	NS
γ-GTP (IU/L)	43.5 ± 28.7	51.6 ± 35.7	62.8 ± 40.3	43.8 ± 30.5	NS	NS	NS
HCV-RNA (KIU/mL)	1326 ± 1256	1667 ± 1311	1488 ± 1228	1818 ± 1408	NS	NS	NS
HCV core antigen (fmol/L)	6183 ± 6894	9830 ± 1214	14,033 ± 17,089	6481 ± 4023	NS	NS	NS

*Mean ± SD.
†Not significant.
‡Student *t* test.
Abbreviations: SVR, sustained virological response; CNR, complete nonresponse; γ-GTP, gamma guanosine triphosphate.

each NS5A sequence with a consensus sequence inferred from aligning the previously published NS5A-1b sequences.[8] In this connection, the consensus sequence for IRRDR differs from the corresponding sequence of a prototype strain of IFN resistance HCV-1b (HCV-J; DDBJ/EMBL/Genbank accession no. D90208) by a single residue at position 2367 (Ala instead of Gly). Because Ala^{2367} was conserved in 95% of the reported sequences, we used the IRRDR consensus sequence in this study. As shown in Table 3, the mean number of aa substitutions in the entire NS5A obtained from patients with SVR was significantly greater compared with non-SVR and relapse. There was no difference in the number of mutations in an N-terminal half of NS5A (aa 1972-2208), the ISDR (aa 2209-2248) or the PKR-BD (aa 2209-2274) between the different patients' groups. Conversely, we found a more obvious significant difference in the mean numbers of aa mutations within a region consisting of the pre-V3 and V3 regions, which we refer to as IRRDR, between SVR and other patients' groups (Table 3).

To estimate a cutoff number of mutations in IRRDR predicting SVR, the receiver operating characteristics analysis was performed. The result revealed that six mutations were an optimal number of mutations to predict SVR, because it achieved the highest sensitivity (76%) combined with the highest specificity (92%) and yielded an area under the curve of 0.81 (Fig. 1).

Indeed, only 2 (8%) of 24 patients with non-SVR, in contrast to 16 (76%) of 21 patients with SVR, had HCV with IRRDR of 6 or greater, with the difference between the two groups being statistically significant ($P < 0.0001$) (Table 4). Furthermore, none of 11 patients with CNR had HCV of IRRDR of 6 or greater, and the difference between SVR and CNR was statistically significant ($P < 0.0001$). Similarly, only 2 (15%) of 13 relapsers (10 ETR-relapsers + 3 patients with viral breakthrough) had HCV of IRRDR greater than or equal to 6, with the result demonstrating significant difference between SVR and relapse ($P = 0.001$).

Table 3. Average Numbers of aa Mutations Within Different Regions of HCV NS5A Obtained From Pretreated Sera of Patients With SVR, Non-SVR, CNR, and Relapse

NS5A Region	No. of Mutations*				*P* Value†		
	SVR	Non-SVR	CNR	Relapse	SVR versus Non-SVR	SVR versus CNR	SVR versus Relapse
Full-NS5A (aa 1972-2419)	24.9 ± 6.1*	19.7 ± 4.3	20.1 ± 5.2	19.4 ± 3.5	0.012	0.144	0.03
N-half (aa 1972-2208)	9.2 ± 1.9	8.6 ± 1.9	9.0 ± 2.4	8.2 ± 1.2	NS‡	NS	NS
ISDR (aa 2209-2248)	2.1 ± 2.8	1.2 ± 1.1	1.7 ± 1.4	0.8 ± 0.7	NS	NS	NS
PKR-BD (aa 2209-2274)	3.8 ± 3.4	2.5 ± 2.0	2.9 ± 2.4	2.1 ± 1.5	NS	NS	NS
IRRDR (aa 2334-2379)	6.1 ± 2.1	3.9 ± 1.4	3.7 ± 0.9	4.0 ± 1.8	0.0006	0.003	0.018

*Mean ± SD.
†The *P* values obtained with Student *t* test were adjusted using the Bonferroni method (see Materials and Methods).
‡Not significant.
Abbreviations: SVR, sustained virological response; CNR, complete nonresponse; aa, amino acid; ISDR: interferon sensitivity-determining region; PKR-BD, double-stranded RNA-activated protein kinase-binding domain; IRRDR, interferon/ribavirin resistance-determining region.

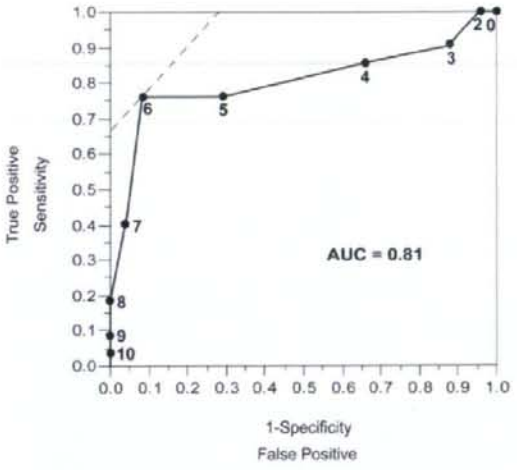

Fig. 1. The receiver operating characteristic curve analysis of IRRDR sequence variation for SVR prediction. The curve depicted with solid line shows an area under the curve of 0.81. Solid circles with numerals plotted on the curve represent different numbers of IRRDR mutations analyzed. The dashed line in the upper left corner indicates the optimal number of IRRDR mutations for SVR prediction, which yields the highest sensitivity (76%) and the highest specificity (92%).

When the IRRDR sequences obtained from all 45 patients were aligned along with the consensus sequence (Fig. 2), we noticed that 10 (48%) of 21 patients with SVR had alanine at position 2360 (Ala^{2360}), whereas only 3 (13%) of 24 patients with non-SVR and none of 11 patients with CNR did ($P = 0.02$ and 0.006, respectively) (Table 4). Similarly, 9 (43%) of 21 patients with SVR had threonine at position 2378 (Thr^{2378}), whereas only 3 (13%) of 24 patients with non-SVR and none of 11 patients with CNR did ($P = 0.04$ and 0.01, respectively).

To identify significant independent SVR predictors, we first entered all available baseline patients' features and IRRDR sequence variations data in univariate logistic analysis. As had been expected, this analysis yielded four factors significantly associated with SVR: IRRDR mutations, either continuous variable ($P < 0.0001$) or dichotomized at 6 ($P < 0.0001$), Ala^{2360} ($P = 0.002$), Thr^{2378} ($P = 0.019$), and platelet count ($P = 0.017$). Subsequently, we analyzed these four factors by multivariate logistic analysis. When the IRRDR mutations were dichotomized at 6, the multivariate analysis identified only the IRRDR of 6 or greater criterion as the independent predictor of SVR (odds ratio = 16.0; CI, 2.4-104.3; $P = 0.004$) (Table 5). However, when the IRRDR mutations were analyzed as a continuous variable, the multivariate analysis yielded IRRDR mutations (odds ratio = 1.8; CI, 1.1-3.1; $P = 0.02$) and Ala^{2360} (odds ratio = 9.3; CI, 1.1-78.8; $P = 0.04$) as independent SVR predictors.

Figure 3A shows the viral clearance rates of patients infected with HCV of IRRDR of 6 or greater and those with IRRDR of 5 or less at 4-week intervals during the whole observation period (72 weeks). All of 18 patients infected with HCV of IRRDR 6 or greater cleared the virus by week 16 and remained free of viremia thereafter until the end of the PEG-IFN/RBV treatment (week 48). Within 4 weeks after the cessation of the combination therapy, however, 2 (11%) of the 18 patients underwent relapse (ETR relapse). Conversely, 16 (59%) of the 27 patients with HCV of IRRDR of 5 or less cleared the virus by week 32. Of the 16 patients who once cleared the virus, 3 (19%) and 8 (50%) underwent relapse to become viral breakthrough and ETR relapsers, respectively.

Kaplan-Meier HCV survival curve analysis confirmed that, after the initiation of the IFN/RBV treatment, HCV clearance was achieved significantly more rapidly in patients infected with HCV isolates with IRRDR of 6 or greater than those with IRRDR of 5 or less, with the difference between the two groups being statistically significant ($P < 0.0001$) (Fig. 3B).

Sequence Analysis of ISDR and PKR-BD of HCV NS5A Obtained from Pretreated Sera. As described, there was no difference in the mean number of mutations in ISDR or PKR-BD between SVR and non-SVR or CNR (Table 3). Only four patients had HCV with four or

Table 4. Correlation Between NS5A Sequence Variation and Virological Responses of the Patients

	No. of Subjects / no. of Total*				P Value†		
Criteria	SVR	Non-SVR	CNR	Relapse	SVR versus Non-SVR	SVR versus CNR	SVR versus Relapse
IRRDR ≥ 6	16/21 (76%)	2/24 (8%)	0/11 (0%)	2/13 (15%)	< 0.0001	< 0.0001	0.001
Ala^{2360}	10/21 (48%)	3/24 (13%)	0/11 (0%)	3/13 (23%)	0.02	0.006	NS‡
Thr^{2378}	9/21 (43%)	3/24 (13%)	0/11 (0%)	3/13 (23%)	0.04	0.01	NS

*Total no. of SVR, Non-SVR, CNR, or relapse.

†Fisher's exact test.

‡Not significant.

Abbreviations: SVR, sustained virological response; CNR, complete nonresponse; IRRDR, interferon/ribavirin resistance-determining region; Ala^{2360}, alanine at position 2360; Thr^{2378}, threonine at position 2378.

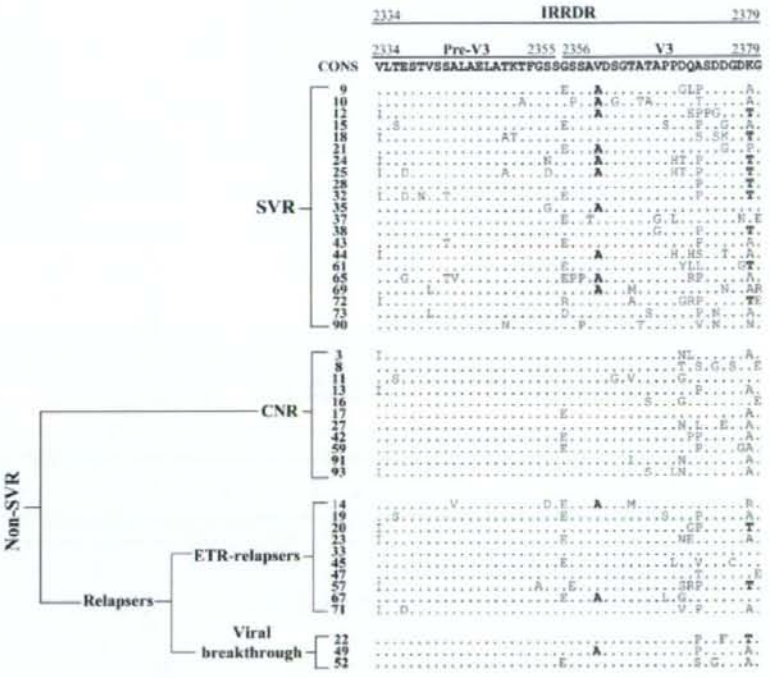

Fig. 2. Sequence alignment of IRRDR (Pre-V3 plus V3 regions) of NS5A of HCV-1b obtained from the pretreatment sera. The consensus sequence (Cons) is shown on the top. The Cons sequence differs from a prototype sequence of IFN-resistant HCV-1b (HCV-J; DDBJ/EMBL/GenBank accession no. D90208) by a single residue at position 2367 (alanine instead of glycine). The numbers along the sequence indicate aa positions. Dots indicate residues identical to those of the Cons sequence. Ala^{2360} and Thr^{2378} are written in boldface.

more mutations in ISDR (data not shown), the criterion for IFN-sensitive HCV strains according to Enomoto et al.[8,9] Although there appeared to be a trend for patients with HCV having four or more mutations in ISDR toward SVR (3 of 4), the difference was not statistically significant. Also, the prevalence of HCV with four or more mutations in ISDR was not significantly different between SVR (3 of 21; 14.3%) and non-SVR (1 of 24; 4.2%). It would be interesting to note, however, that all three HCV strains with four or more mutations in ISDR obtained from SVR (nos. 10, 65, and 72) had HCV of IRRDR of 6 or greater, whereas the only one strain with four or more mutations in ISDR from non-SVR (no. 13) had three mutations in IRRDR (data not shown). It is thus possible that the IRRDR sequence variation is associated with PEG-IFN/RBV responsiveness more closely than is the ISDR variation.

Table 5. Multivariate Logistic Regression Analysis to Identify Independent SVR Predictors

Factor	Odds (95% CI)	*P* value
Multivariate analysis 1		
IRRDR ≥ 6	16.0 (2.4-104.3)	0.004
Ala^{2360}	7.1 (0.8-66.8)	0.09
Thr^{2378}	4.1 (0.5-30.9)	0.17
Platelets	1.1 (0.9-1.4)	0.27
Multivariate analysis 2		
IRRDR mutations as a continuous variable	1.8 (1.1-3.1)	0.02
Ala^{2360}	9.3 (1.1-78.8)	0.04
Thr^{2378}	4.9 (0.7-33.3)	0.1
Platelets	1.2 (1.0-1.5)	0.08

Only factors that were significantly associated with SVR in univariate analysis were included in multivariate logistic regression analysis.

Abbreviations: IRRDR, interferon/ribavirin resistance-determining region; Ala^{2360}, alanine at position 2360; Thr^{2378}, threonine at position 2378; CI, confidence interval.

Correlation Between Rapid Reduction of HCV Core Antigen Titers and the Sequence Variation in IRRDR of HCV NS5A Obtained from the Pretreated Sera. As stated before, there was no significant difference in the mean values of initial HCV core antigen titers between patients with SVR and those with non-SVR (Table 2). However, we observed a strong association of SVR with rapid reduction of HCV core antigen titers during the very early stages of treatment, that is, 24 hours and 1, 2, and 4 weeks after the initiation of treatment (data not shown). Therefore, we analyzed whether the degree of sequence variation in IRRDR correlated with the very rapid reduction (24 hours after the first dose of PEG-IFN/RBV) of HCV core antigen titers. The result obtained clearly demonstrated a significant correlation between IRRDR of 6 or greater and the very rapid reduction of HCV core antigen titers 24 hours and 1, 2, and 4 weeks

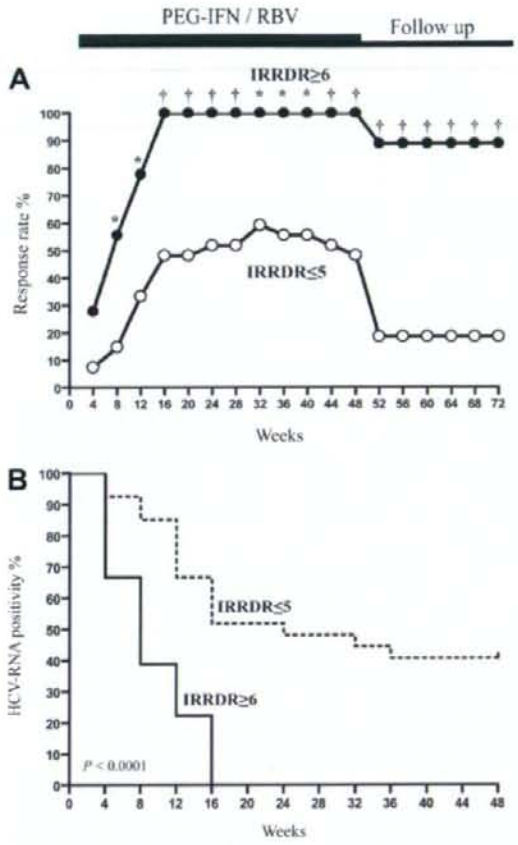

Fig. 3. Time course of HCV clearance during IFN/RBV treatment and follow-up period. (A) The viral clearance rates of patients infected with HCV isolates with six or more mutations in IRRDR (IRRDR ≥ 6) or five or fewer mutations (IRRDR ≤ 5) at 4-week intervals during the whole observation period. *$P < 0.01$; †, $P < 0.001$ (Fisher's exact probability test). (B) Kaplan-Meier HCV survival curve analysis based on serum HCV-RNA positivity during IFN/RBV treatment course (48 weeks) for HCV isolates with IRRDR of 6 or greater and IRRDR of 5 or fewer. $P < 0.0001$ (log-rank test).

after the initiation of treatment (Table 6). Most notably, all 18 patients infected with HCV isolates of IRRDR of 6 or greater achieved significant (≥1 log) reduction or disappearance of serum HCV core antigen titers 24 hours after the first dose of PEG-IFN/RBV.

Proposed Markers for Prediction of Various Virological Responses During PEG-IFN/RBV Combination Therapy. As described, IRRDR of 6 or greater and Ala[2360] were statistically selected as independent SVR predictors. Therefore, we aimed to assess their predictability, in terms of positive and negative predictive values, for various virological responses to PEG-IFN/RBV combination therapy (Table 7). IRRDR greater than or equal to 6 could predict EVR(12w), ETR, and SVR with the positive predictive values of 78% ($P = 0.01$), 100% ($P = 0.000007$), and 89% ($P = 0.0007$), respectively. Moreover, the negative predictive value of IRRDR of 6 or greater for non-SVR was 81% ($P = 0.0008$). Thus, IRRDR of 6 or greater would be useful to predict not only SVR but also non-SVR. Similarly, Ala[2360] could also predict ETR and SVR with positive predictive values of 92% ($P = 0.002$) and 77% ($P = 0.046$), respectively.

Discussion

A substantial proportion of HCV-1b–infected patients do not respond to IFN/RBV combination therapy. Given the significant side effects and high cost associated with this combination therapy, it would be of great utility if clinicians could predict, either before or during the treatment, which patients would, or would not, achieve SVR. Useful predictors of SVR must have a high positive predictive value; conversely, useful predictors of non-SVR must have high negative predictive value.[5] Most recent studies have focused on the possible correlation between the likelihood of achieving SVR and viral clearance kinetics during the first few months of the treatment.[25,26] Conversely, some studies dealt with the possible correlation between SVR and sequence variation within a part of NS5A, especially the V3 region.[12-20]

Table 6. Significant Correlation Between the Rapid Reduction of HCV Core Antigen Titers and IRRDR Sequence Variations

	No. of Patients With Significant Reduction of HCV Core Antigen Titers / No. of Total							
Criteria	24 Hours* (≥ 1 log)†	P Value‡	1 Week* (≥ 1 log)†	P Value‡	2 Weeks* (≥ 1.5 log)†	P Value‡	4 Weeks* (≥ 2 log)†	P Value‡
IRRDR ≥ 6	18/18	<0.0001	15/18	0.002	13/18	0.016	16/18	0.0007
IRRDR ≤ 5	10/27		9/27		9/27		10/27	

*Period after initiation of IFN/RBV therapy.

†Criteria of the significant reduction of HCV core antigen titers. Two (both at 24 hours and 1 week) and three patients (both at 2 and 4 weeks) who achieved disappearance of serum HCV core antigen were also considered to meet these criteria.

‡Fisher's exact test.

Abbreviations: IRRDR, interferon/ribavirin resistance-determining region; IFN/RBV, interferon/ribavirin.

Table 7. Positive Predictive Value, Negative Predictive Value, Sensitivity and Specificity of IRRDR ≥ 6 and Ala^{2360} on the Likelihood of Achieving Various Virological Responses

Virological Response	IRRDR ≥ 6				Ala^{2360}			
	PPV	NPV	Sensitivity	Specificity	PPV	NPV	Sensitivity	Specificity
EVR (12W)	78% (14/18)	67% (18/27)	61% (14/23)	82% (18/22)	69% (9/13)	56% (18/32)	39% (9/23)	82% (18/22)
ETR	100% (18/18)	56% (15/27)	58% (18/31)	100% (14/14)	92% (12/13)	41% (13/32)	39% (12/31)	93% (13/14)
SVR	89% (16/18)	81% (22/27)	76% (16/21)	92% (22/24)	77% (10/13)	66% (21/32)	48% (10/21)	88% (21/24)

Abbreviations: IRRDR, interferon/ribavirin resistance-determining region; Ala^{2360}, alanine at position 2360; EVR, early virological response; ETR, end-of-treatment response; SVR, sustained virological response; PPV, positive predictive value; NPV, negative predictive value.

We previously reported that a high degree of sequence variation (≥6 mutations) in IRRDR was significantly correlated with the EVR by week 16 in HCV-1b–infected patients treated with PEG-IFN/RBV combination therapy.[21] In the current follow-up study, we aimed to investigate whether the IRRDR sequence variation is correlated also with SVR. By using different statistical approaches, the results obtained clearly demonstrated that the high degree of sequence variation in IRRDR (IRRDR ≥6) significantly correlated with SVR, whereas the low degree of sequence variation in this region (IRRDR ≤5) correlated with non-SVR. Nearly two-thirds of patients with SVR had HCV of IRRDR of 6 or greater, whereas only 2 (8%) of 24 patients with non-SVR did ($P < 0.0001$) (Table 4). Notably, 16 of the 18 patients infected with HCV of IRRDR of 6 or greater achieved SVR. Accordingly, the positive predictive value and negative predictive value of IRRDR greater than or equal to 6 for SVR and non-SVR were 89% ($P = 0.0007$) and 81% ($P = 0.0008$), respectively (Table 7). Our current results thus strongly suggest that IRRDR greater than or equal to 6 would be a useful marker for prediction of SVR.

It was reported that the determination of HCV core antigen levels in the serum was an accurate and reliable alternative to monitor HCV RNA titers and that rapid reduction of HCV core antigen levels within a few weeks after the initiation of the therapy could predict treatment outcome in patients receiving PEG-IFN/RBV combination therapy.[27-29] Indeed, we found a strong association between the likelihood of achieving SVR and rapid reduction of HCV core antigen during the first 4 weeks of PEG-IFN/RBV combination therapy. More importantly, we found a significant correlation between the rapid reduction of HCV core antigen titers and the degree of sequence variation in IRRDR. Notably, all the patients infected with HCV of IRRDR greater than or equal to 6 showed a significant (≥1 log) reduction or disappearance of serum HCV core antigen titers 24 hours after the first dose of PEG-IFN/RBV (Table 6). This, in particular, suggests a possible influence of IRRDR of 6 or greater on HCV replication kinetics during IFN-based therapy because the direct effect of IFN begins a few hours after the first dose. Moreover, IRRDR greater than or equal to 6 was significantly associated with rapid clearance of serum HCV RNA as early as week 8 during PEG-IFN/RBV combination therapy (Fig. 3). These results collectively reinforce the possible correlation between the sequence variation in IRRDR and HCV clearance by the IFN-based therapy.

We also examined whether the criterion of IRRDR of 6 or greater was applicable to previously reported studies, for which information on both treatment outcome (responder versus nonresponder) and IRRDR sequences are available.[8,13] As shown in Table 8, the average numbers of amino acid variations from the same consensus sequence used in the current study were significantly larger for SVR than for non-SVR in a study with Japanese patients ($P = 0.003$)[8] and hovered at nearly a significant level in a study with European patients ($P = 0.06$).[13] More importantly, the criterion of IRRDR greater than or equal to 6 could significantly differentiate between responders and nonresponders in the Japanese study ($P = 0.003$) and also hovered at nearly a significant level in the European study ($P = 0.058$). It should be noted that, in the latter study, there were only three patients who had HCV with IRRDR of 6 or greater, all of whom became SVR. Taken together, these results suggest the useful application of IRRDR of 6 or greater as an SVR marker even in different geographical regions, although the prevalence of HCV with IRRDR of 6 or greater may vary with different regions of the world.

Although we observed significant correlation between the overall number of mutations in IRRDR and PEG-IFN/RBV responsiveness, we also found particular amino acid mutations, Ala^{2360} and Thr^{2378}, that were significantly associated with SVR (Table 4 and Fig. 2). In particular, Ala^{2360} was identified as an independent SVR marker. In this connection, it should be noted that four of five HCV isolates of IRRDR of 5 or less obtained from patients with SVR had either Ala^{2360} or Thr^{2378}. Furthermore, 20 (95%) of 21 HCV isolates obtained from pa-

Table 8. Comparative Analysis of the Mean Numbers of aa Mutations in IRRDR in Previously Reported Japanese and European Studies

Study	Factor	SVR	Non-SVR	*P* Value
Enomoto et al.[8]	No. of IRRDR mutations	7.6 ± 2.6*	3.7 ± 2.0	0.003†
	No. of patients with IRRDR ≥ 6	8	1	0.003‡
	IRRDR ≤ 5	1	8	
Duverlie et al.[13]	No. of IRRDR mutations	5.6 ± 2.3	4.2 ± 0.8	0.06†
	No. of patients with IRRDR ≥ 6	3	0	0.056‡
	IRRDR ≤ 5	5	11	
This study	No. of IRRDR mutations	6.1 ± 2.1	3.9 ± 1.4	0.0006†
	No. of patients with IRRDR ≥ 6	16	2	< 0.0001‡
	IRRDR ≤ 5	5	22	

NOTE. Same consensus sequence was used in this comparative analysis.
*Mean ± SD.
†Student *t* test.
‡Fisher's exact test.
Abbreviations: aa, amino acid; IRRDR, interferon/ribavirin resistance-determining region; SVR, sustained virological response.

tients with SVR had either one of the three factors (IRRDR ≥6, Ala^{2360}, or Thr^{2378}). To our knowledge, there is no known CD4 or CD8 epitope(s) in IRRDR so far reported. Interestingly, however, Neumann-Haefelin et al.[30] recently identified an HLA-A26 $CD8^+$ T-cell epitope located at position 2416, 37 aa distant from IRRDR. This epitope was shown to be targeted in all patients with acute resolving HCV infection examined. Further studies are needed to elucidate the role(s) for the distal carboxy terminal region of NS5A, including IRRDR, in both IFN/RBV responsiveness and T cell–mediated virus clearance.

In conclusion, our results suggest that a high degree of sequence variation in IRRDR (IRRDR ≥6), and a particular aa mutation (Ala^{2360}) to a lesser extent, would be a useful marker to predict SVR.

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