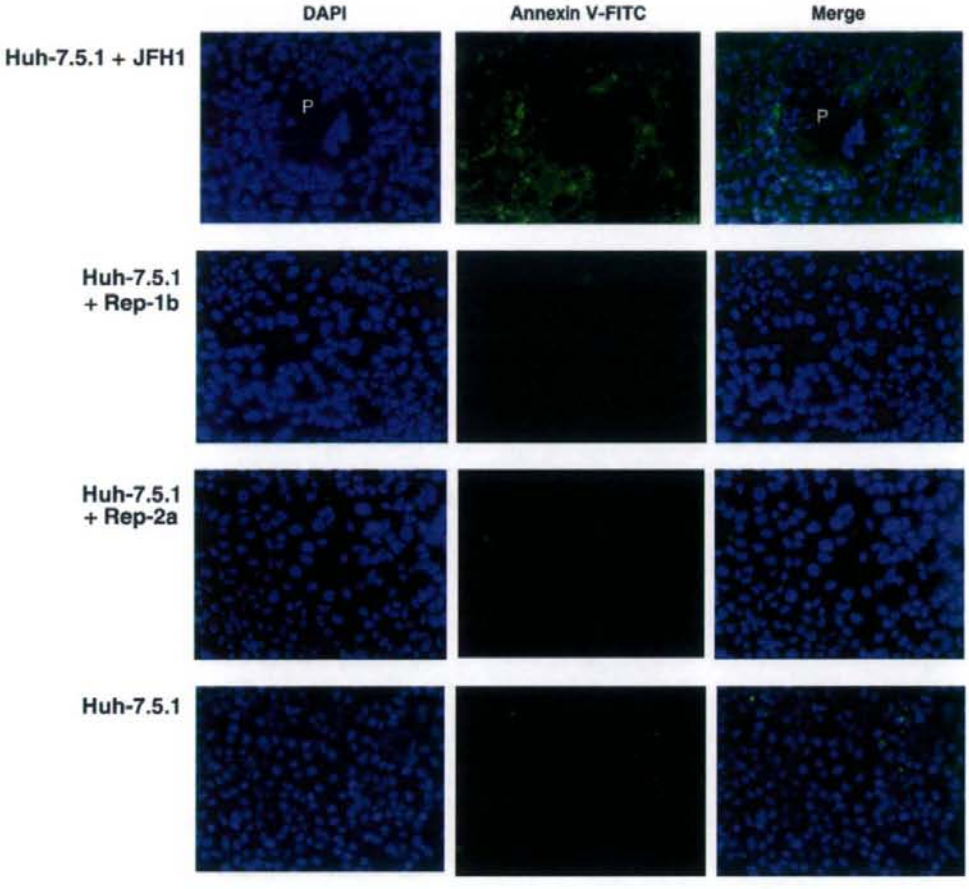

Fig. 4. HCV-JFH1 infection induces apoptosis and leads to plaque formation. The HCV-JFH1 culture supernatant was transferred onto uninfected Huh-7.5.1 cells plated on 22-mm round micro cover glasses in 60-mm-diameter plates at density of 2×10^5 cells per plate. After ~5 h incubation, the supernatant was replaced with medium containing 0.8% methylcellulose. Thirteen days after infection, cover glasses were incubated with 100 μl of staining solution containing Annexin V-FITC at room temperature for 10 to 15 min. The cells that express HCV subgenomic replicons were also incubated and stained with Annexin V-FITC. Rep 1b, Rep-Feo; Rep 2a, SGR-JFH1 (see Materials and methods).

JFH1 replication activates expression of ER stress-related proteins

Cellular stresses such as virus infections prevent protein folding and maturation in the endoplasmic reticulum (ER) and result in the accumulation of misfolded proteins (ER stress) (Kaufman, 1999; Pahl, 1999), triggering the unfolded protein response (UPR). The UPR leads to global shut-off of protein translation and to apoptotic cell death (Ferri and Kroemer, 2001; Mori, 2000; Munro and Pelham, 1986). We and other groups have previously reported that subgenomic or genomic HCV replication induces ER stress and triggers UPR (Nakagawa et al., 2005; Tardif et al., 2002). Therefore, we next studied expression of the ER stress-related proteins, GRP78 and phosphorylated eIF2-alpha, in JFH1-infected cells (Fig. 5). GRP78 is one of the ER chaperones whose expression is induced by ER stress through cleavage and nuclear translocation of ATF6. The eIF2-alpha is phosphorylated by PER-like ER kinase (PERK) on ER stress, causing direct global inhibition of initiation of protein translation (Harding et al., 1999). Huh-7.5.1 cells were infected with HCV-JFH1 supernatant and harvested on the fourth and seventh days post-infection (Fig. 5). As the expression of HCV core protein increased, expression levels of GRP78 and phosphorylated eIF2-alpha also increased substantially. Suppression of virus replication by interferon-alpha treatment led to a decrease of cellular GRP78 and phosphorylated eIF2-alpha. Interferon-alpha treatment did not eliminate the expression of HCV completely, though the levels of core and phosphorylated eIF2-alpha expression apparently decreased compared with the JFH-1 infected Huh-7.5.1 cells at seventh days post-infection. These findings demonstrated that HCV-JFH1 infection induced ER stress.

Persistence of ER stress activates apoptosis signaling pathways, including the induction of C/EBP homologous protein (CHOP) and activation of JNK kinase and caspase12, leading to cell death (Ferri and Kroemer, 2001). As shown in Fig. 5, the

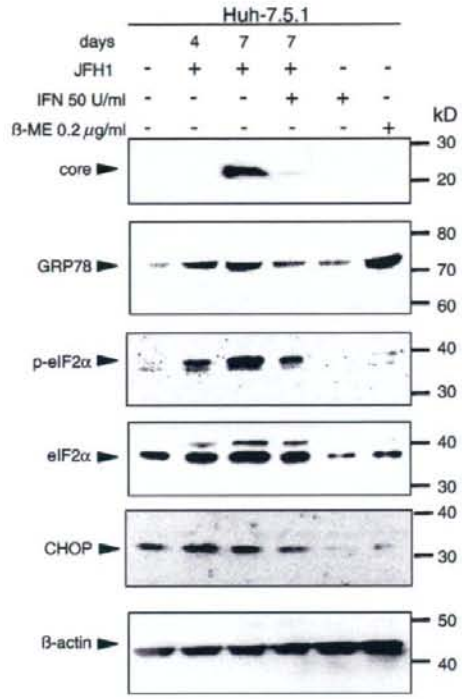

Fig. 5. Expression of ER stress-related proteins in HCV-JFH1 infected cells. The supernatant of JFH1-transfected Huh-7.5.1 cells was transferred onto uninfected Huh-7.5.1 cells. The cells were harvested at 4 and 7 days after infection. The JFH1-infected cells were also cultured with interferon (50 U/ml) or 2-mercaptoethanol (0.2 μg/ml) and harvested after 48 h after treatment. 2-Mercaptoethanol was used as a positive control to induce ER stress (Nakagawa et al., 2005). Western blotting was performed using anti-core, anti-GRP78, anti-phospho-eIF2-alpha (p-eIF2α), anti-eIF2-alpha, anti-GADD153/CHOP, and anti-beta-actin antibodies. β-ME, 2-mercaptoethanol.

level of CHOP expression was apparently increased in JFH1-infected Huh-7.5.1 cells.

To determine whether ER stress contributes to the formation of cytopathic plaques, JFH1-infected cells were incubated in methylcellulose-containing medium and double immunofluorescence staining of the plaques was performed. As shown in Fig. 6, overexpression of GRP78 was colocalized with HCV-core-positive cells with and without CPE. Together with the result shown in Fig. 4, these findings suggest that ER stress is induced in the HCV-JFH1-infected cells and these responses may be involved in development of apoptosis and the formation of cytopathic plaques.

A cytopathic clone could be isolated and this had acquired a high infection efficiency and increased cytopathogenicity

The plaque assay enabled differential quantification of viral infectivity and cytopathogenicity by the immunofluorescence detection of HCV core protein in JFH1-infected, plaque-developed cultures. The number of plaques, as well as infectious foci, was linearly proportional to the dilution of an inoculum (Fig. 7B). It was revealed that only a few populations of HCV-positive foci developed cytopathic plaques (Fig. 3B and Table 1). The infectious focus-forming units and plaque-forming units were 5.6×10^3 FFU/ml and 9.7×10^2 PFU/ml, respectively (Table 1).

To determine whether the difference between the cytopathic and noncytopathic HCV-JFH1 replication might be attributable to viral factors, we isolated clones from each cytopathic plaque. JFH1-infected Huh-7.5.1 cells were incubated in DMEM containing methylcellulose. Cytopathic plaques became visible at ~1 week after inoculation. We isolated cells from each plaque using a cloning cylinder, subcultured, and transferred supernatant onto uninfected Huh-7.5.1 cells. To our surprise, infection of naive cells with plaque-derived supernatants led to massive cell death at 10 days post-infection (Fig. 8A). The supernatant of these cells was transferred again onto uninfected Huh-7.5.1 cells again. Immunofluorescence assay revealed that almost 100% of the cells were positive for HCV core protein (Fig. 8B). The infectivity and cytopathogenicity of this isolated plaque (Pl #1) were 4.9×10^3 FFU/ml and 3.0×10^3 PFU/ml respectively (Table 1), much higher than the parental JFH1 clone. Moreover, the ratio of PFU to FFU in a plaque-isolated clone (Pl #1) was significantly higher than that of parental JFH1 clone (0.58 and 0.17 respectively) (Table 1 and Figs. 7B and C). We next performed an infection experiment of the parental JFH1 and a plaque-derived clone by adjusting infectious titers of the inocula by HCV core antigen levels. As shown in Fig. 8C, virus from cytopathic plaque (Pl #1, #2, #3) showed significantly higher elevation of core antigen levels in supernatants than the parental JFH1 in every time point. The second round isolation of plaques from the Pl #1 subclone (Pl #1-1, #1-2 and #1-3 in the Table 3) showed consistently higher replication efficiency and cytopathogenicity. These results indicated that JFH1 subclones isolated from cytopathic plaques showed significantly higher infection efficiency and greater cytopathic effects than the original JFH1.

The isolated plaque had amino acid substitutions clustered in the NS5B region

To determine whether there are viral mutations in the cytopathic JFH1 subclone (Pl #1), we performed sequence analyses. As shown in Table 2, 11 nucleotide changes were found in the cytopathic plaque, and 9 of these were non-synonymous mutations (81.8%). In particular, 6 of the 11 mutations (9153, 9232, 9293, 9295, 9353, and 9355) were clustered in the C terminal half of the NS5B region. We also performed sequence analyses of the Pl #1-isolated subclones, Pl #1-1, #1-2, and #1-3, and other clones that had been independently isolated from different plaques, Pl #2, #3, and #4 (Table 3). Those subclones showed similar mutations within NS5B region. The C2438S, P2934S, and S3001N substitutions were redundantly appeared in the 4 plaque-isolated clones and in all three Pl #1-derived subclones. In contrast, no mutations were found in the virus from infectious foci without plaque formation. These results showed an evidence that certain amino acid mutations were directly associated with the viral replication efficiency and cytopathogenicity.

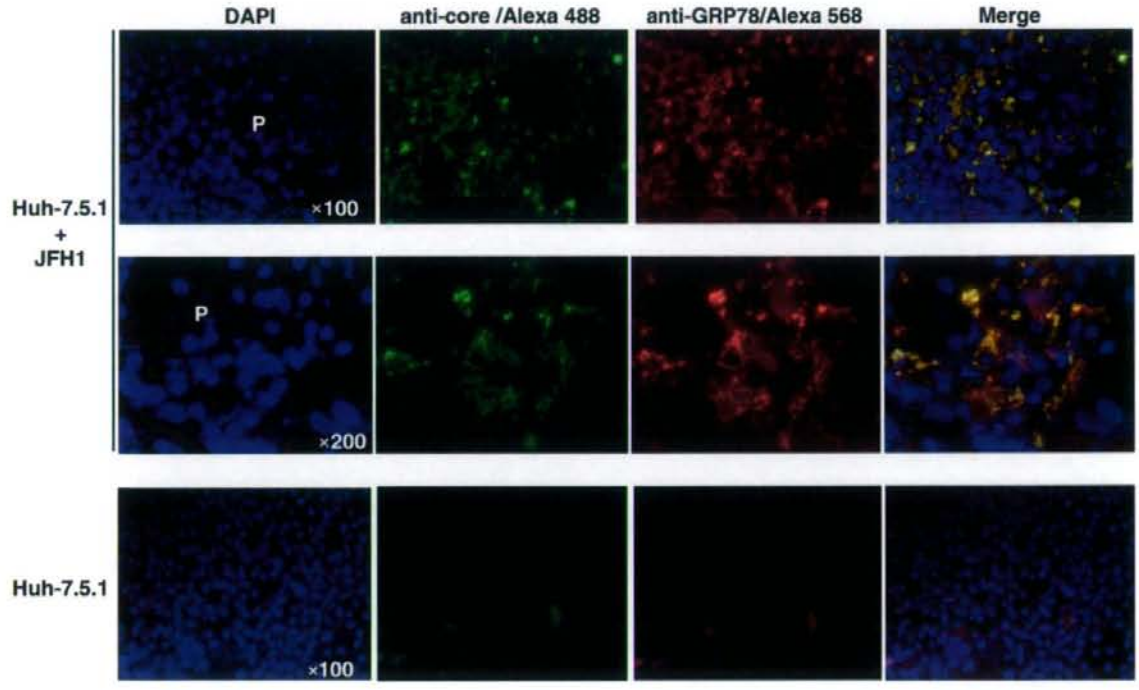

Fig. 6. Co-expression of HCV core and GRP78 in the cytopathic plaque. The HCV-JFH1 culture supernatant was transferred onto uninfected Huh-7.5.1 cells plated on 22-mm round micro cover glasses in 60-mm-diameter plates at density of 4×10^5 cells per plate. After ~5 h incubation, the supernatant was replaced with medium containing 0.8% methylcellulose. Double immunofluorescence was performed 10 days after infection using mouse anti-core antibody and goat-anti-GRP78 antibody.

Introduction of NS5B mutations in JFH1 clone showed higher replication efficiency and cytopathogenicity

We finally investigated on phenotypes of the amino acid mutations identified in the isolated cytopathic subclones. We constructed mutant clones from the wild type JFH1 plasmid, in which three amino acid mutations in NS5B region were individually introduced; T7662A, C9153T, and G9295C (see Tables 2 and 3). Transfection of the mutant HCV-RNAs showed that all mutants developed massive cell death on 10 days after transfection and that their extents of the CPE were apparently greater than the wild type JFH1 clone (Fig. 9A). The levels of core antigen in the culture medium were significantly higher in the mutant clones than in the wild type (Fig. 9B). Furthermore, the expression levels of cellular HCV core protein were significantly higher in the mutant clones than in the wild type with the order of T7662>C9153≫G9295C>JFH1 (Fig. 9C).

Discussions

Our results show that replication of HCV-JFH1 resulted in morphologic changes to the host cells, which are characterized by massive cell death (Figs. 1–3). These observations suggested that HCV infection and replication could cause CPE on the host cells. The development of the CPE involved virus protein-induced ER stress and subsequent apoptotic cell death (Figs. 4–6). The JFH1/ΔE1-E2 with deletion of the HCV envelope proteins-infected Huh-7.5.1 cells did not induce the CPE (Fig. 2A), which indicates that the key factors of plaque formation are not only viral replication but also the propagation of virus particles and re-infection. We took advantage of the HCV-induced CPE and developed a plaque assay using highly permissive Huh-7.5.1 cells. The assay revealed that the HCV-induced cytopathogenicity varied between infectious foci with cytopathic and noncytopathic infection (Fig. 3B). Interestingly, isolated JFH1 subclones from the plaques showed significantly increased infectivity and cytopathogenicity (Table 1 and Fig. 8). Viral genetic analyses showed nine amino acid substitutions; among them five were clustered in the C terminal half of the NS5B region, which might contribute to virus replication efficiency and cytopathogenicity (Table 2).

Cytopathic effects are key characteristics of the *Flaviviridae* that include Japanese encephalitis virus (JEV) (Vaughn and Hoke, 1992), West Nile Virus (Borisevich et al., 2006), yellow fever virus (Quaresma et al., 2006), dengue virus (DEN) (Despres et al., 1993), and bovine viral diarrhea virus (BVDV) (Mendez et al., 1998) and also of viruses such as adenovirus (Shinoura et al., 1999), Epstein–Barr virus (Sato et al., 1989), poliovirus (Yanagiya et al., 2005), and influenza virus (Hinshaw et al., 1994). The *Flaviviridae* utilizes the ER as the primary site for genomic replication and protein synthesis (Jordan et al., 2002; Su et al., 2002; Tardif et al., 2004). It has been reported that apoptotic cell death mediated by virus-induced ER stress contributes to the cytotoxicity of JEV, BVDV, and DEN-2

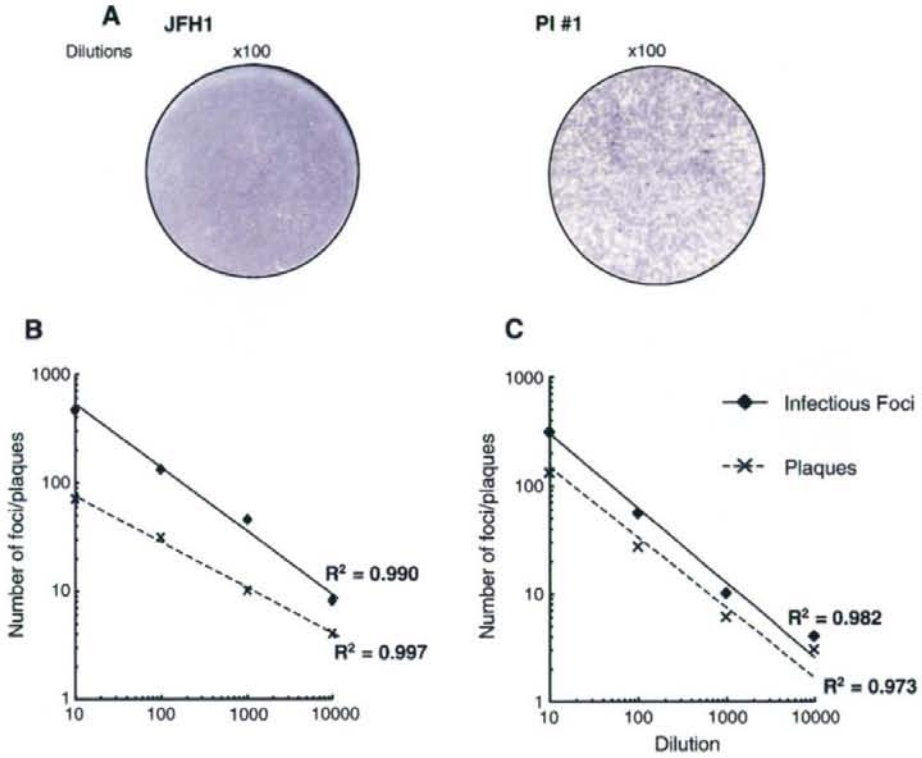

Fig. 7. Correlation of infectious foci or plaques with dilution of an inoculum. (A) Plaque assay. Huh-7.5.1 cells were seeded in collagen-coated 60-mm-diameter plates at density of 4×10^5 cells per plates and were incubated at 37 °C under 5.0% CO_2. After overnight incubation, HCV-JFH1 (left panel) or plaque-purified clone (PI #1) (right panel) infected culture supernatants were serially diluted in a final volume of 2 ml per plates and transferred onto the cell monolayers. After ~5 h of incubation, the inocula were removed, and the cell monolayers were overlaid with 8 ml of culture medium containing 0.8% methylcellulose. After 7 days of culture under normal conditions, formation of cytopathic plaque was visualized by staining with 0.08% crystal violet. (B and C) The PFU-adjusted culture supernatant of parental HCV-JFH1 (B) or plaque-purified clone (PI #1) (C) was transferred at various dilutions onto uninfected Huh-7.5.1 cells, and the plaque assay and immunocytochemistry were performed (described above). The infectivity and cytotoxicity were quantified by counting HCV-positive foci and cytopathic plaque respectively. The horizontal axis showed dilutions of the viral supernatant and the vertical axis showed the number of infectious foci or plaques.

(Jordan et al., 2002; Su et al., 2002; Yu et al., 2006). In DEN-2-infected cells, the NS2B-3 protein causes XBP1 splicing and induces ER stress (Yu et al., 2006). These findings are consistent with our results for HCV in that the JFH1 infection induced ER stress and unfolded protein responses and led to apoptotic cell death and formation of plaques.

The ER is closely associated with viral replication and assembly. Most of the HCV structural and nonstructural proteins accumulate in the ER membrane and form a membranous web that is characterized by a convoluted ER structure (Gosert et al., 2003). Moreover, the folding and assembly of HCV proteins require interaction with ER chaperone proteins such as calreticulin, BiP/GRP78, and heat shock protein-90 (HSP90) (Choukhi et al., 1998; Waxman et al., 2001). The ER stress, which is induced by virus replication, involves three different mechanisms (Tardif et al., 2002): transcriptional induction, translational attenuation, and protein degradation. In our study, both GRP78 and phosphorylated eIF2-alpha proteins were induced as viral proteins increased in concentration in HCV-JFH1 infected cells, and the GRP78 or annexin V and HCV core proteins co-localize in cytopathic plaques, showing that HCV infection and replication induce UPR and that ER stress-mediated apoptosis causes the viral cytopathic effects on host cells.

Several HCV structural and nonstructural proteins are involved in the ER stress. The structural glycoproteins, E1 and E2, interact with ER chaperones (Choukhi et al., 1998; Liberman et al., 1999), HCV NS4B induces UPR through ATF6 or the IRE1-XBP1 pathway (Zheng et al., 2005), and HCV core triggers apoptosis by inducing ER stress and ER calcium depletion both *in vitro* and *in vivo* (Benali-Furet et al., 2005).

Table 1
Cytopathogenicity and infectivity of JFH1 clones

	PFU/ml [a]	FFU/ml [b]	PFU/FFU
JFH1	$9.7 \pm 3.8 \times 10^2$ [c]	$5.6 \pm 0.9 \times 10^3$	0.17±0.05
PI #1	$3.0 \pm 1.9 \times 10^3$	$4.9 \pm 1.6 \times 10^3$	0.58±0.21

[a] PFU, plaque-forming unit.
[b] FFU, focus-forming unit.
[c] Values are displayed as mean ± S.D.

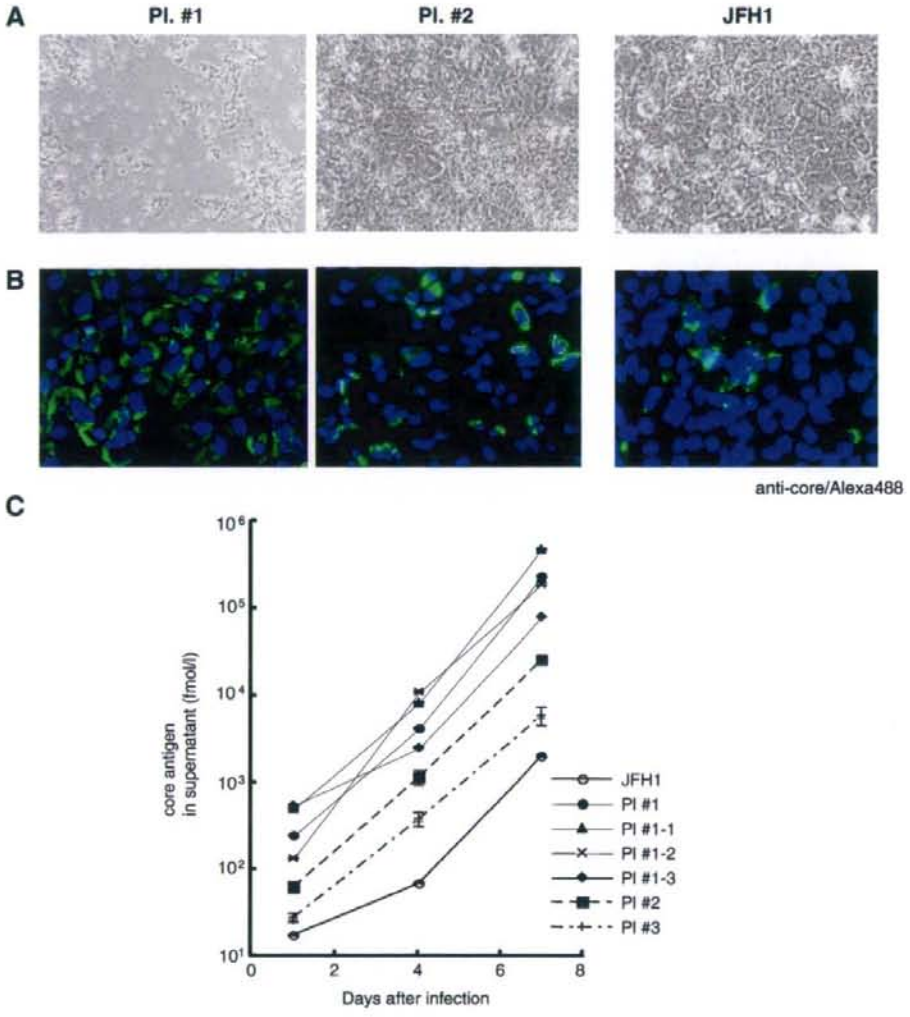

Fig. 8. The isolation of cytopathic plaques. The HCV-JFH1 culture supernatant was transferred at various dilutions onto uninfected Huh-7.5.1 cells. After ~5 h incubation, the supernatant was removed then infected cells were cultured in 0.8% methylcellulose-containing medium in 60-mm-diameter plates. Cytopathic plaques were detectable at 8 days after infection. Cells from each plaque were isolated using a cloning cylinder, subcultured, and transferred onto uninfected Huh-7.5.1 cells. (A) Observation by phase-contrast microscopy at 10 days of culture. (B) After 15 days of culture, the supernatant was transferred onto uninfected Huh-7.5.1 cells and an immunofluorescence assay was performed 5 days after infection using anti-core antibody. (C) Supernatants from parental JFH1, plaque-derived viruses (Pl #1, #2, and #3) and the second round isolation of plaques from the Pl #1 subclones (Pl #1-1, #1-2, and #1-3) were inoculated onto Huh-7.5.1 cells with PFU-adjusted doses, respectively. HCV core antigen levels in culture medium were measured on the days indicated. Inoculation and the assays were done in triplicate. The S.D.s were within 4% in each plot.

HCV E2 induces ER stress at lower levels but binds to PERK and inhibits phosphorylation of eIF2-alpha at high levels of expression (Pavio et al., 2003). These reports have shown that HCV may induce ER stress and regulate subsequent intracellular responses to promote its survival in hepatocytes. Consistently with these reports, our findings that HCV-JFH1 induces the expression of an ER chaperon protein and phosphorylation of eIF2-alpha indicates that robust replication of HCV-JFH1 produces unfolded proteins in the ER, leading to activation of ATF6 and stimulation of the transcription of ER chaperon proteins to promote protein folding. HCV-JFH1-induced unfolded proteins also activate PERK, which phosphorylates eIF2-alpha to inhibit the protein translation. Furthermore, the severe ER stress finally activates apoptosis signaling pathways at the early stage of viral infection. Although which HCV-JFH1 gene product is involved in ER stress-mediated apoptosis is not identified in our study, such proteins may contribute to the regulation of ER stress signaling in the host cell that leads to viral survival or cell death.

The plaque assay is often used to quantify virus infectious titers by visualizing the viral-induced CPE. However, due to the noncytopathic nature of HCV and the lack of highly permissive

Table 2
Nucleotide changes and amino acid substitutions in the cytopathic JFH1 subclone

Nucleotide*	Amino acid*
A1353G	M334V
C2842A	T843K
G3402A	G1017S
A5819G	Synonymous
T7662A	C2438S
C9153T	P2934S
G9232A	G2960D
G9293C	Synonymous
G9295C	R2985P
C9353A	H3000Q
G9355A	S3001N

* Nucleotide and amino acid numbers were derived from pJFH1full (Wakita et al., 2005).

host cell lines, detection of HCV-infected cells commonly relied on visualization of the infected focus by immunostaining HCV proteins (Zhong et al., 2005). Disadvantages include the costs of the antibodies and substrate, additional steps for assay and detection, and microscopic examination to count the foci. By using a highly permissive host cell line and optimizing several conditions, we have developed a plaque assay for HCV. Because the HCV-JFH1 strain is not absolutely cytopathic and does not kill all infected cells, the calculated plaque-forming units do not directly reflect HCV infectious titer but rather reflect cytopathogenicity or the percentage of cytopathic clones in the total infectious foci.

The HCV plaque assay revealed that JFH1 infection and replication developed cytopathic and noncytopathic infectious cell foci (Fig. 3B). One would suspect that the different outcomes of HCV replication might be attributable to the clonal heterogeneity of the host cells. However, there are several pieces of evidence that the Huh-7.5.1 cell line, which we used as host, might be a homogenous cell line. Huh-7.5.1 is derived from parental Huh7 cells through two rounds of clonal selection for neomycin resistance that were dependent on permissiveness for the HCV subgenomic replicon (Blight et al., 2002; Zhong et al., 2005). Sumpter et al. have reported that the HCV-permissive feature is due to mutational inactivation of RIG-I, a cytoplasmic double-stranded RNA sensor that induces type-I IFN production (Sumpter et al., 2005). This evidence suggests that the cytopathic HCV replication is attributable to virus factors, in particular, virus genomic alteration and not by clonal variation or evolution of the host cells.

Indeed, the isolation of the plaque-forming HCV subclones and inoculation onto naive cells showed significantly higher replication yields (Fig. 8) and more frequent development of cytopathic plaques (Table 1). These findings indicate that HCV-JFH1 has evolved into cytopathic and noncytopathic subclones. Our results are similar to BVDV infection. BVDV is divided into two biotypes, cytopathic (*cp*) and noncytopathic (*ncp*) strains. Most *cp* strains, which induce strong apoptotic cell death upon infection, develop from the *ncp* strains by RNA recombination such as insertion of cellular sequences, duplications and rearrangements, and deletions and lead to expression of the NS3 protein (Meyers and Thiel, 1996). Kummerer et al. have reported that other *cp* strain had point mutations in NS2 that enhanced cleavage of NS2/3 junction and NS3 production (Kummerer and Meyers, 2000). As for HCV, considering a rapid HCV replication cycle and the poor fidelity of the viral NS5B RNA-dependent RNA polymerase (RdRp) (Bartenschlager and Lohmann, 2000; Kato et al., 2005), evolution of sequence variants may well develop even after a transfection of cloned HCV-RNA. Very recently, *in vitro* permissive subclones of HCV genotype 1a, H77S stain, have been reported, which have five cell culture-adaptive mutations in the NS3, 4A, and 5A regions (Yi et al., 2007). In these clones, introduction of amino acid substitutions in the p7 and NS2 region enhanced production of the virion particles.

Interestingly, sequence analyses of a cytopathic HCV-JFH1 subclone (Pl #1) identified six amino acid substitutions in the NS5B RdRp (Table 2). Three of the six mutations were redundantly appeared in other clones that were independently isolated from the plaques (Table 3). These findings make us speculate that these amino acid substitutions may affect the enzymatic activity of RdRp by altering tertiary structure of the

Table 3
Nucleotide changes and amino acid substitutions in the NS5B regions of the cytopathic JFH1 subclones

Pl #1	#1-1	#1-2	#1-3	Pl #2	Pl #3	Pl #4
						T7623A (S2428T)
T7662A (C2438S)	T7662A (C2438S)	T7662A (C2438S)	T7662A (C2438S)	T7662A (C2438S)	T7662A (C2438S)	
					A7550C	
					C7551A (N2470T)	
						G8259C
						C8260G (A2640R)
C9153T (P2934S)	C9153T (P2934S)	C9153T (P2934S)	C9153T (P2934S)		C9153T (P2934S)	
				G9162T (V2941L)		
					A9201T (I2954F)	
G9232A (G2960D)						
				G9235A (R2965Q)		
G9295C (R2985P)	G9295C (R2985P)		G9295C (R2985P)			
C9353A (H3000Q)	C9353A (H3000Q)					
G9355A (S3001N)	G9355A (S3001N)		G9355A (S3001N)			G9355A (S3001N)

Nucleotide and amino acid numbers were derived from pJFH1full (Wakita et al., 2005).

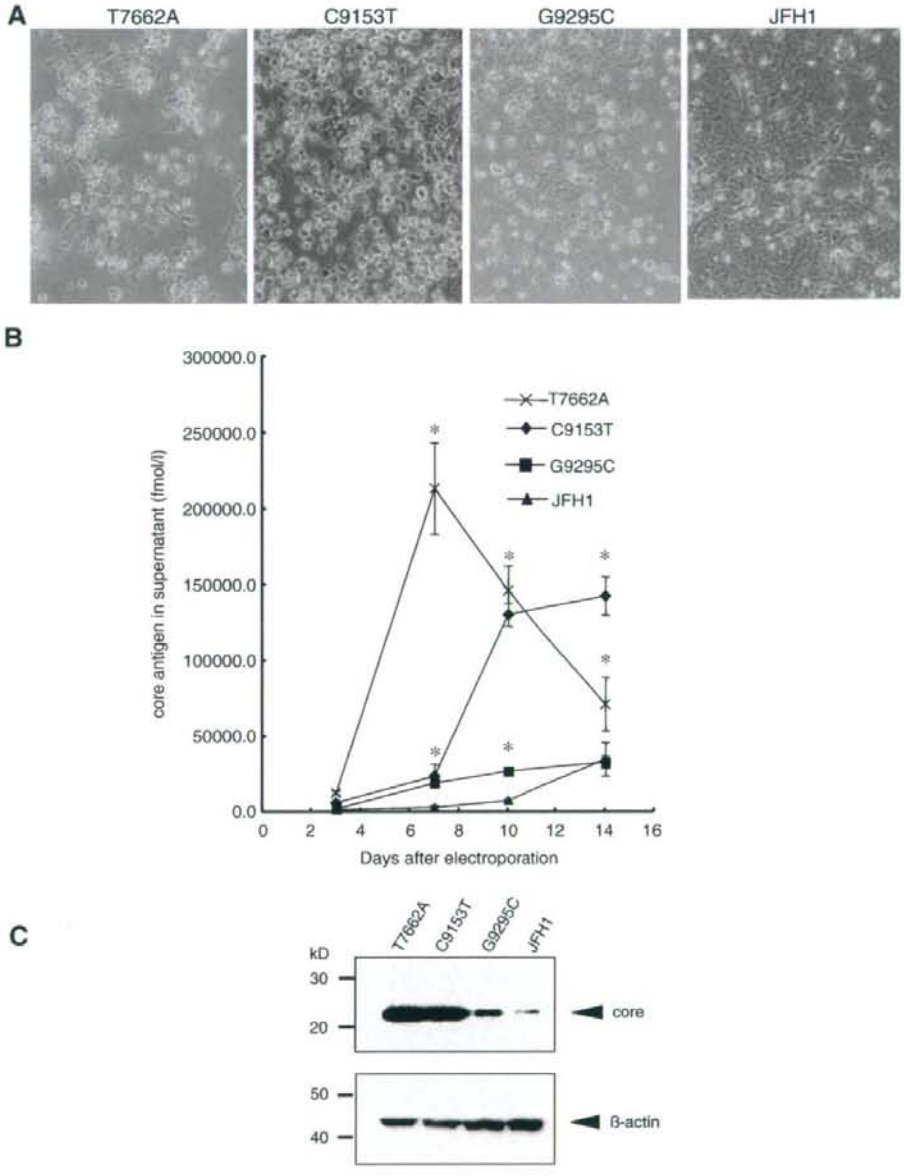

Fig. 9. Introduction of various mutations into the NS5B region of JFH1. The mutations identified in the cytopathic plaque Pl #1; T7662A, C9153T, and G9295C were introduced individually into the parental JFH1. Each JFH1 mutant, T7662A, C9153T, and G9295C, RNA was transfected into Huh-7.5.1 cells by electroporation. The transfected cells were split every 3 to 5 days (see Materials and methods). (A) JFH1 mutants transfected Huh-7.5.1 cells were observed by phase-contrast microscopy at day 7 after transfection. (B) Levels of core antigen in the culture supernatants. The culture supernatants of transfected cells were collected on the days indicated, and the levels of core antigen were measured. Asterisks indicate p-values of less than 0.05. (C) The supernatants of JFH1 mutants transfected Huh-7.5.1 cells were transferred onto uninfected Huh-7.5.1 cells. The cells were harvested at 7 days after infection. Western blotting was performed using anti-core and anti-beta-actin.

thumb domain or affect the quaternary structure of the whole HCV replicase complex by altering surface affinity to other nonstructural proteins. Mapping of the amino acid substitutions in the RdRp tertiary structure has shown that the amino acid 2438 was located on the finger domain, and three amino acids, 2934, 2960, and 2985, were located on the outer surface of the thumb domain, which corresponds to the opposite side of the nucleotide tunnel. The other substitutions, 3000 and 3001, were within the domain of the polypeptide linking the polymerase to the membrane anchor (Lesburg et al., 1999). Very

recently, Zhong et al. have reported that long-term culture of HCV-JFH1 of more than 60 days leads to the evolution of certain mutations in the viral genome (Zhong et al., 2006). They identified amino acid changes in Core, E2, NS3, and NS5A regions, and especially E2 mutation increased infectivity and density changes of viruses. In our present study, however, we could not find those mutations in the virus subclones that we have isolated in the plaque assay technique. The discrepancy might be attributable to the presence or absence of HCV-CPE-induced cell clonal alteration of the host Huh-7.5.1 that occurs concomitantly with viral genetic evolution during long-term cell culture. Further analyses may be necessary to determine the most critical regions that regulate the viral replication efficiency and cytopathogenicity.

Interestingly, the mutant virus clones, T7662A (C2438S), C9153T (P2934S), and G9295C (R2985P), showed considerably higher replication efficiency and cytopathogenicity than the wild type JFH1 clone (Fig. 9). These results strongly suggest that certain NS5B mutations in the plaque-purified strains display more replication-efficient and cytopathic phenotypes. The present data are still preliminary. Further studies may be necessary to fully characterize these mutations and their functions, which include introduction of mutations of the HCV region and of the other plaque-purified viruses and combination of the mutations, and to study their effects on virus protein functions. We are at present analyzing derivative JFH1 clones in which other amino acid mutations were introduced.

Several clinical findings have suggested that HCV is not cytopathic and that antiviral immune responses such as cytotoxic T lymphocytes play important roles in HCV pathogenesis (Cerny and Chisari, 1999). On the other hand, apoptotic cell death is the first cellular response to many hepatotoxic events and has been implicated in the pathogenesis of liver diseases, such as viral hepatitis, autoimmune diseases, alcohol-induced injury, cholestasis, hepatocellular carcinoma, and fulminant hepatic failure (Canbay et al., 2004; Ghavami et al., 2005; Patel and Gores, 1995; Rodrigues et al., 2000; Rust and Gores, 2000; Thompson, 1995). Several clinical studies have shown that fulminant hepatic failure (FHF), from which HCV-JFH1 strain was isolated, showed far more hepatocyte apoptosis, as characterized by caspase activation and Fas-FasL expression, than chronic hepatitis and normal populations (Leifeld et al., 2006; Mita et al., 2005; Ryo et al., 2000). The ER stress markers GRP78 and ATF6 are upregulated in the HCV liver tissue as the histological grade advanced. In addition, GRP78 and ATF6 are upregulated as the histological grade increased in hepatocellular carcinoma (HCC) (Shuda et al., 2003) and proteomic analysis of HCC tissue samples has shown significant upregulation of HSP70 and GRP78 (Chuma et al., 2003; Takashima et al., 2003), indicating that these proteins may play important roles in HCV-induced hepatocarcinogenesis.

In conclusion, the cytopathic mutants of HCV-JFH1 strain were isolated by using plaque assay techniques. A mechanism of the cytopathic effects involved ER stress-mediated apoptosis that was triggered by virus infection. That process of cytopathic effects might explain one aspect of HCV-induced liver injury during acute infection. Further analyses of cellular effects on HCV replication may elucidate the pathogenesis of HCV infection and may define novel host factors as targets of antiviral chemotherapeutics.

Materials and methods

Reagents

Recombinant human interferon alpha-2b was from Schering-Plough (Kenilworth, NJ). Beta-mercaptoethanol was from Wako (Osaka, Japan). Anti-CD81 antibody (JS-81) was from BD Biosciences (Franklin Lakes, NJ) (Morikawa et al., 2007).

Cells and cell culture

Huh-7.5.1 cells (Zhong et al., 2005) (kindly provided by Dr Francis V. Chisari) were maintained in Dulbecco's modified minimal essential medium (DMEM, Sigma) supplemented with 2 mmol/l L-glutamine and 10% fetal bovine serum at 37 °C under 5.0% CO_2.

In vitro RNA synthesis and transfection

A plasmid, pJFH1-full (Wakita et al., 2005), which encodes the full-length HCV-JFH1 sequence, and two control plasmids for pJFH1-full were used; pJFH1/GND that is a replication incompetent mutant with a mutation in the NS5B GDD motif and pJFH1/ΔE1-E2 in which a coding region of the HCV envelope proteins was deleted. The HCV RNA was synthesized using the RiboMax Large Scale RNA Production System (Promega, Madison, WI), with the linearized pJFH1 plasmid as template. After DNaseI (RQ-1 RNase-free DNase, Promega) treatment, the transcribed HCV-RNA was purified using ISOGEN (Nippon Gene, Tokyo, Japan). For the RNA transfection, Huh-7.5.1 cells were washed twice, and 5×10^6 cells were resuspended in Opti-MEM I (Invitrogen, Carlsbad, CA) containing 10 μg of HCV RNA, transferred into a 4-mm electroporation cuvette, and subjected to an electric pulse (1050 μF and 270 V) using the Easy Ject system (EquiBio, Mieddlesex, UK). After electroporation, the cell suspension was left for 5 min at room temperature and then incubated under normal culture conditions in a 10-cm diameter cell culture dish. The transfected cells were split every 3 to 5 days. The culture supernatants were subsequently transferred onto uninfected Huh-7.5.1 cells. The levels of HCV replication and viral protein expression were detected by real-time PCR, western blotting, and immunocytochemistry.

HCV subgenomic replicon constructs

HCV subgenomic replicon plasmid pRep-Feo was derived from the HCV-N strain pHCV1bneo-delS (Tanabe et al., 2004) and pSGR-JFH1 was from the HCV-JFH1 strain (Kato et al., 2003). The replicon RNA was synthesized from pRep-Feo or pSGR-JFH1 and transfected into Huh-7.5.1 cells. After culture in the presence of G418 (Wako), cell lines stably expressing the replicon were established.

Real-time RT-PCR analysis

Total cellular RNA was isolated using ISOGEN (Nippon Gene). Two micrograms of total cellular RNA was used to generate cDNA from each sample using SuperScript II (Invitrogen) reverse transcriptase. Expression of mRNA was quantified using Quanti Tect SYBR Green PCR Master Mix (QIAGEN, Valencia, CA) and the ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). The primers used were as follows: HCV-JFH1 sense (positions 7090 to 7109; 5′-TCA GAC AGA GCC TGA GTC CA-3′), HCV-JFH1 antisense (positions 7404 to 7423; 5′-AGT TGC TGG AGG GCT TCT GA-3′), beta-actin sense (5′-ACA ATG AAG ATC AAG ATC ATT GCT CCT CCT-3′), and beta-actin antisense (5′-TTT GCG GTG GAC GAT GGA GGG GCC GGA CTC-3′).

Quantification of HCV core antigen in the culture supernatant

The culture supernatants of JFH1-RNA transfected Huh-7.5.1 cells were collected on the days indicated, passed through a 0.45 μm filter (MILLEX-HA, Millipore, Bedford, MA), and stored at −80 °C. The levels of core antigen in the culture supernatants were measured using a chemiluminescence enzyme immunoassay (CLEIA) according to the manufacturer's protocol (Lumipulse Ortho HCV Antigen, Ortho-Clinical Diagnostics, Tokyo, Japan).

Western blotting

Western blotting was carried out as described previously (Tanabe et al., 2004; Yokota et al., 2003). Briefly, 10 μg of total cell lysate was separated by SDS–PAGE and blotted onto a polyvinylidene fluoride (PVDF) western blotting membrane. The membrane was incubated with the primary antibodies followed by a peroxidase-labeled anti-IgG antibody and visualized by chemiluminescence using the ECL western blotting Analysis System (Amersham Biosciences, Buckinghamshire, UK). The antibodies used were anti-core mouse monoclonal antibody 2H9 (provided by Dr. Wakita), anti-GRP78 goat monoclonal antibody, anti-GADD153/CHOP rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-eIF2-alpha, anti-phospho-eIF2-alpha rabbit polyclonal antibody (Cell Signaling, Danvers, CA), and anti-beta-actin antibody (Sigma).

Immunocytochemistry

HCV-JFH1-transfected or infected Huh-7.5.1 cells were cultured in Lab-Tek® Chamber Slide™ (Nalge Nunc International, Rochester, NY) or on 22-mm-round micro cover glasses (Matsunami, Tokyo, Japan). For detection of HCV-core and GRP78, cells were fixed with cold acetone for 15 min. The cells were incubated with the primary antibodies for 1 h at 37 °C and with Alexa Fluor 488 goat anti-mouse IgG antibody or Alexa Fluor 568 donkey anti-goat IgG antibody (Molecular Probes, Eugene, OR) for 1 h at room temperature. To analyze apoptosis of HCV-JFH1 infected cells, double staining for annexin V-FITC binding and for cellular DNA using propidium iodide (PI) was performed using an annexin V-Fluorescein Staining Kit (Wako, Osaka, Japan). Cells were visualized by a fluorescence microscopy (BZ-8000, KEYENCE, Osaka, Japan).

Plaque assay

Huh-7.5.1 cells were seeded in collagen-coated 60-mm-diameter plates at a density of $2–4 \times 10^5$ cells per plates and were incubated at 37 °C under 5.0% CO_2 (as described above). After overnight incubation, HCV-infected culture supernatants were serially diluted in a final volume of 2 ml per plates and transferred onto the cell monolayers. After ~5 h of incubation, the inocula were removed, and the cell monolayers were overlaid with 8 ml of culture medium (DMEM, 2 mmol/l L-glutamine and 10% fetal bovine serum) that contained 0.8% methylcellulose. After 7 to 12 days of incubation under normal culture conditions, formation of cytopathic plaque was visualized by staining the cell monolayers with 0.08% crystal violet solution (Sigma). The levels of cytotoxicity were evaluated by counting the plaques and calculating the titer (PFU/ml). Similarly, the titers of infectivity were evaluated by performing immunocytochemistry to detect foci of HCV-core-positive cells and calculating the infectious focus-forming units (FFU/ml).

Sequence analyses

The cDNA from the isolated JFH1 plaque was amplified from cytopathic virus-infected Huh-7.5.1 cells by RT-PCR and subjected to direct sequence determination. Nucleotide sequences were read from both strands using Big Dye Terminator Cycle Sequencing Ready Reaction kits (Applied Biosystems) and an automated DNA sequencer (ABI PRISM® 310 Genetic Analyzer; Applied Biosystems).

Establishment of mutant JFH1 clones

In order to introduce various mutations into the NS5B region of JFH1, plasmid pJFH1 was digested with *Hin*dIII and the DNA fragment encompassing nt. 8231 to 9731 was subcloned into the pBluescriptII SK+ phagemid vector (Stratagene, La Jolla, CA). The following mutations were introduced into the DNA fragment in the subcloning vector by site-directed mutagenesis (Quick-ChangeII Site-Directed Mutagenesis Kit; Stratagene): C9153T and G9295C, respectively. Finally, these *Hin*dIII–*Hin*dIII fragments were subcloned back into the parental plasmid pJFH1. The mutation T7662A-introduced PCR fragment (nt. 7421–7839) was subcloned into the T-Vector (pGEM-T Easy Vector Systems; Promega) and digested with *Rsr*II and *Bsr*GI. Finally, these *Rsr*II–*Bsr*GI fragments were subcloned back into the parental plasmid.

Statistical analyses

Statistical analyses were performed using the Student's *t*-test, and *p*-values of less than 0.05 were considered as statistically significant.

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特集II 高齢者C型慢性肝炎に対する治療のあり方

ISDRからみた高齢者のC型慢性肝炎に対する治療法*

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Key Words：interferon, ISDR, amino acid mutations in HCV core region

はじめに

C型慢性肝炎に対するインターフェロン(interferon：IFN)治療は，PEG-IFNとRibavirin併用療法により格段に進歩した．しかし，いまだ難治例が存在し，1型高ウイルス量症例ではウイルス排除率(Sustained virologic response：SVR)率は50%に満たない[1][2]．とくに高齢者では，治療完遂しても治療効果が劣るとともに，副作用の出現率が高いことから十分な治療が行えないことも，大きな要因と考えられている．一方，治療効果を規定する因子としては，年齢・性別などの宿主因子のみならず，ウイルス側の因子も重要視されている．とくに1b型C型肝炎ウイルス(Hepatitis C virus：HCV)のIFN感受性領域(Interferon sensitivity determining region：ISDR)は，治療効果を規定する因子として重要であることを，われわれは報告してきた[3][4]．そこで，本稿ではISDRからみた高齢者に対するIFN療法について述べる．

1b高ウイルス量症例に対するPEG-IFN/Ribavirin併用療法の治療成績

当科および関連施設で構成する共同研究Y-PERS(Yamanashi-PEG-Interferon α2b- Ribavirin Study)で集積されたPEG-IFN/Ribavirin併用療法を施行した465症例を検討した．初回治療の場合は高ウイルス量症例に限られるが，1b型に対する48週治療，2a型および2b型の24週治療のSVR率はそれぞれ45%(63/140)，77%(17/22)，68%(13/19)であった(図1)．とくに，難治の1b型かつ高ウイルス量症例に関して，60歳未満/以上，男性/女性に分けて検討すると，60歳未満では男女差がないものの，60歳以上では，男性44%に対し女性22%のSVR率で，高齢女性のSVR率がきわめて悪いことが明らかになった(図2)．この理由としては，高齢者，とくに女性では，治療中止・減量率が高いことが考えられた．そこで，治療完遂率と完遂者におけるSVRの関連について検討した．この結果は，60歳以上の女性では治療完遂率は61%と，他と比べて若干劣るものの，有意な差はみられなかった．しかし，SVR率は36%であり，60歳以上の高齢女性は，たとえ治療完遂してもSVR率が悪いことが判明した．したがって，高齢者の治療成績が劣ることは，単に副作用による治療中止・減量によるものではなく，他の原因があると考えられた．これまで，この理由として，HCVの持続感染期間の差による線維化進展の可能性(すなわち高齢者では線維化進展例が多い)や，女性特有のホルモン環境の相違，

* Mutations in ISDR on efficacy of interferon therapy for elderly chronic hepatitis C patients.

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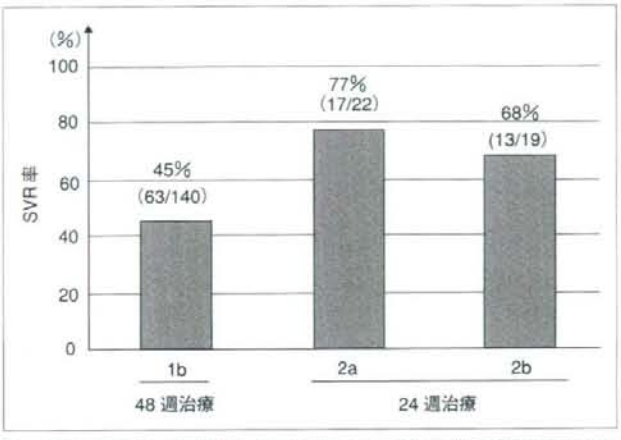

図1 PEG-IFN/Ribavirin併用療法におけるgenotypeとウイルス排除率(SVR)率

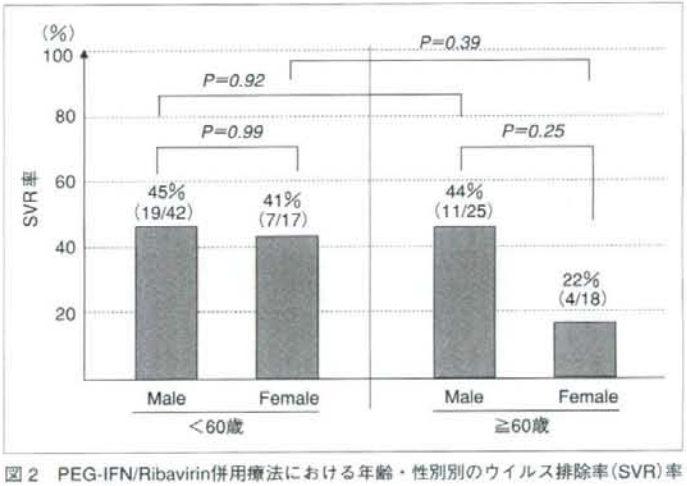

図2 PEG-IFN/Ribavirin併用療法における年齢・性別別のウイルス排除率(SVR)率
(n=102)

宿主の免疫反応によるHCV陰性化時期の遅延などが論じられてきたが，いまだ，明らかな結論は得られていない．

ISDRからみたPEG-IFN/Ribavirin併用療法の治療成績

1b型のHCVにおいては，IFN単独療法の治療成績を規定する因子として，ISDRの関与が明らかになっている．これはHCVのNS5A領域内の40アミノ酸領域で，この領域にアミノ酸変異のまったくない野生型(Wild type)では IFN単独療法のSVR率はきわめて低く，4個以上の変異がある変異型(Mutant type)では，きわめて高いSVR率を示し，1～3個の変異がある中間型(Intermediate type)では，この中間のSVR率を示す．PEG-IFN/Ribavirin併用療法においても，ISDR別にSVR率を検討すると，野生型では37%(29/78)，中間型では44%(19/43)であるのに対し，変異型では80%(12/15)であり，変異型は中間型や野生型に比べ有意にSVR率が高かった(図3)[5]．この理由は，

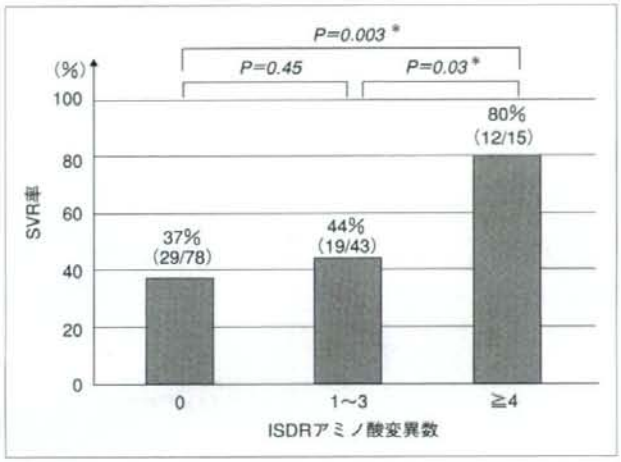

図3　PEG-IFN/Ribavirin併用療法におけるIFN感受性領域ウイルス排除率(SVR)率(1b症例 n=140)

* Significant difference

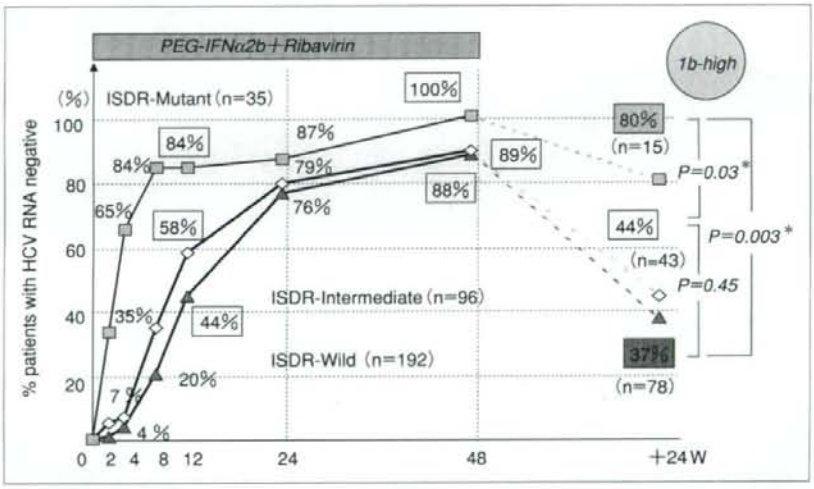

図4　PEG-IFN/Ribavirin併用療法におけるIFN感受性領域(ISDR)と経時的ウイルス陰性化率(1b症例 n=323)

* Significant difference

ISDRが治療早期のウイルス量の減少と関係しているためと考えられている．すなわち，ISDR変異型では野生型・中間型に比較して投与開始2週間ないしは4週間のウイルス量の減少率が高く，12週以内のウイルス消失(Early virologic response：EVR)に影響していた(図4)．EVRは治療効果(SVR)予測する重要な因子とされ[6]，1b型高ウイルス量症例ではEVRが得られた症例では48週間の治療により高率にSVRとなる．したがって，HCVのIFN反応性はウイルスダイナミクスにより判定することが可能であるが，これは，ISDRとも強く関連しているということである．さらに

表1 PEG-IFN/Ribavirin併用療法(1b)のウイルス排除率(SVR)に寄与する因子(多変量解析)(n=140)

	odds ratio (95% CI)	*P* value
年齢(<60歳/≧60歳)	0.453(0.191-1.073)	0.0719
肝線維化(F1/F2~4)	0.356(0.152-0.833)	0.0173
HCV コア蛋白量	0.454(0.196-1.055)	0.0663
ISDR(変異数<2/≧2)	5.125(1.590-16.520)	0.0062

多変量ロジステック回帰分析

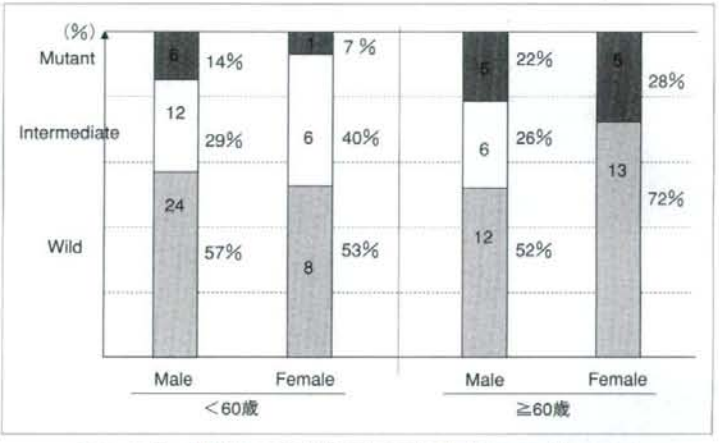

図5 年齢・性別とIFN感受性領域(ISDR)の分布(1b-high)(n=101)

SVRに寄与する因子について宿主側の因子を含めて，多変量ロジステック回帰分析を行なうと，抽出された因子は年齢，肝線維化(F2以上とそれ以外)とISDR変異数(2未満と2以上のみ)のみであり，この中でもISDRがもっとも強い因子であった(表1)．また，ISDRは治療開始前のHCV RNA量と相関しているが，多変量解析ではRNA量はISDRに勝る因子ではなく，投与前のウイルス量を1,000 KIU以上と未満とに分けて検討すると，同等のウイルス量群の中でも，変異型の効果が高いことが判明し，上記を裏づけているものと思われた[7]．

しかし，ISDR変異数が同じであっても治療反応性が異なる症例が存在し，ISDR変異数0ないしは1の難治症例でも，SVR例と非SVR例が存在する．そこで，われわれは，ISDR変異数0ないしは1の症例で，他のウイルス学的条件と臨床的条件がそろった症例で，IFN反応性が良好な症例とそうでない症例について，HCV全ゲノムの相違を検討した．その結果，両者に相違がみられたのは，コア領域とNS2領域のアミノ酸であり，統計学に有意な相違がみられたのはコア領域の70番目のアミノ酸のみであった．さらにこの領域に注目してretrospectiveに，IFN治療効果とこのアミノ酸変異との関連を検討すると，ISDRが0ないしは1の難治が予測される症例であっても，このアミノ酸がHCVのプロトタイプのHCV-Jにみられるアルギニン(R)であれば高率にSVRが期待できるものの，グルタミン(Q)に変異しているとIFN治療反応性がきわめて悪く，主治医の判断で治療を中断した症例や，治療完遂してもSVRにならない症例が多数を占めていた．同様の報告はAkutaらにより，コア領域の70番目と91番目のアミノ酸に変異がみられるとインターフェロン治療効果が劣り，とくに50歳以上の女性ではその傾向が顕著であることが報告されている[8]．

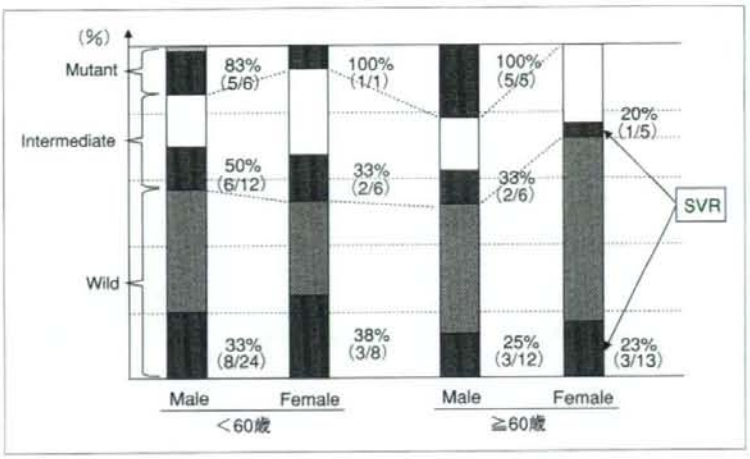

図6　年齢・性別とIFN感受性領域(ISDR)の分布とウイルス排除率(SVR)の関係(n=101)

ISDRと年齢・性別との関連

上記のようにISDRは治療効果と密接に関連しているが，これと年齢・性別との関連についても検討した．本検討では，ISDRの分布は野生型が多くを占め，中間型や変異型は少なかったが，60歳以上の女性には変異型の比率が低いことが判明した(図5)．各年代，男性/女性におけるISDR別の治療成績には差がほとんどみられないことから，ISDRの分布が各年代の治療性成績に関連し，60歳以上の女性のSVR率が低いことの一つの理由にISDRの分布の差が関与していることが推測された(図6)．しかし，この分布の理由については，変異型のHCVが，過去の治療や自然経過で駆逐されており，現在のPEG-IFN/Ribavirin併用療法の治療対象でないことが推測された．そこで，約10年ほど前のIFN単独療法時のISDRについても検討した．しかし，10年ほど前の50歳以上の女性でもISDRのmutant typeは少なく，もともとある年代以上の，女性にはこのtypeが少ない可能性が考えられた．すなわちこの集団の感染経路が異なりISDRの分布が異なる可能性や，女性ではmutant typeは持続感染しにくく，ある年代以降で自然治癒している可能性などが考えられるが，一定の結論が得られるような証拠は存在しない．しかし，かつてのIFN単独療法の時代でも，wild typeやintermediate typeの比率が高い高齢女性の治療成績はきわめて悪く，ISDRが関与している可能性も否定できなかった．しかし，この傾向は他の地域でも普遍的にみられるのかどうかなど，今後の検討課題も存在する．

ISDRからみた高齢者のC型慢性肝炎に対する治療法

これまでの検討で，ISDRはPEG-IFNを含むIFN単独治療ないしはPEG-IFN/Ribavirin併用療法において，治療効果を規定する重要な因子であることが明らかとなった．高齢女性にはISDR mutant typeが少ないことから，治療効果が低いことの一つの説明であることが推測された．しかし，この傾向が，他の地域や集団で普遍的なものであるかどうかなどは，今後の検討に委ねられるものと考えられる．しかしながら，ISDRは治療効果予測因子として非常に重要であり，とくに，副作用の出現率が高い高齢者では，治療法や治療期間を選択するうえで重要な情報となりえる可能性がある．われわれは，年齢・性別などの宿主要因とウイルス型，ウイルス量などのウイルス要因にISDRやコア領域変異を参考に治療方針を個々に検討している．すなわち，1bかつ高ウイルス量で高齢者のきわめて難治が予測される症例であってもISDRがmutant typeであれば積

極的に治療を行うが，wild typeかつコア70番のアミノ酸がQであれば，仮に治療開始してもNull responseであれば，副作用を鑑み早期に治療を中断ないしは，肝癌抑止のための治療に切り替えるなどの検討を行っている．今後は，さらなる症例の蓄積と解析により，ISDRの解析が高齢者を含めた個々の症例に応じたテーラーメード治療を可能にするものと考えられる．

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Interferon sensitivity determining region : ISDR

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索引用語：C型慢性肝炎，インターフェロン，ISDR，個別化医療

1 はじめに

C型慢性肝炎に対する従来のインターフェロン単独療法の，ウイルス排除(SVR: sustained virological response)率は30％程度に過ぎなかったが，ペグインターフェロンとリバビリン併用療法が行われるようになり，約70％でウイルス排除が可能となった．しかし，難治とされるgenotype 1bかつ高ウイルス量の慢性肝炎では，併用療法を48週間行っても，約半数でのみウイルス排除可能であるにすぎない．このインターフェロン治療反応性の違いについては，さまざまな検討がなされてきたが，ウイルス側の要因として，C型肝炎ウイルス(HCV)の遺伝子型(genotype)をはじめとする，さまざまな遺伝子変異の存在が明らかになってきた．特に，HCVの非翻訳領域(Nonstructural region: NS) 5Aに存在するインターフェロン感受性領域(Interferon sensitivity determining region: ISDR)のアミノ酸変異は，治療効果の予測因子として臨床応用可能であることがみいだされている．

2 C型肝炎ウイルス（HCV）の遺伝子構造とインターフェロン感受性領域（Interferon sensitivity determining region: ISDR）

C型肝炎ウイルス(Hepatitis C virus: HCV)は＋1本鎖のRNAウイルスであり，ゲノムの両端に非翻訳領域が存在し，中央部には約3,010個のアミノ酸からなる1本のポリ蛋白前駆体をコードするopen reading frameが存在する．この領域にはHCVの構造蛋白(コア，エンベロープ蛋白)とウイルス増殖に必要な種々の酵素をコードする非構造領域(Nonstructural region: NS)が存在する．このうち，NS5A領域のC末端よりの40アミノ酸(NS5A a.a. 2209-2248)領域は，かつて，1b型のHCVに対するインターフェロン単独療法の著効症例と無効症例の全塩基配列の比較検討から，治療効果に関連した遺伝子領域とし

Minoru SAKAMOTO et al : Interferon sensitivity determining region: ISDR
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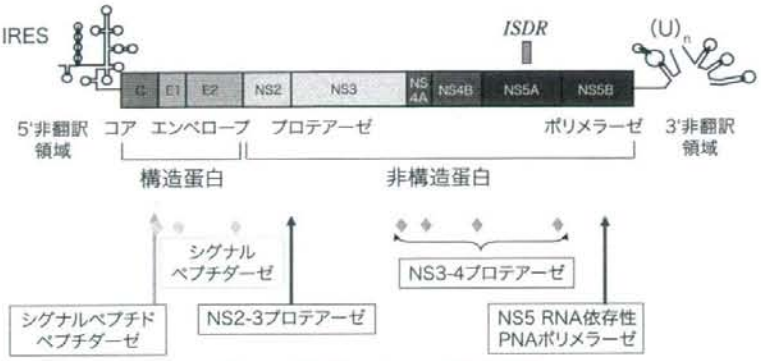

図1　C型肝炎ウイルスの遺伝子構造

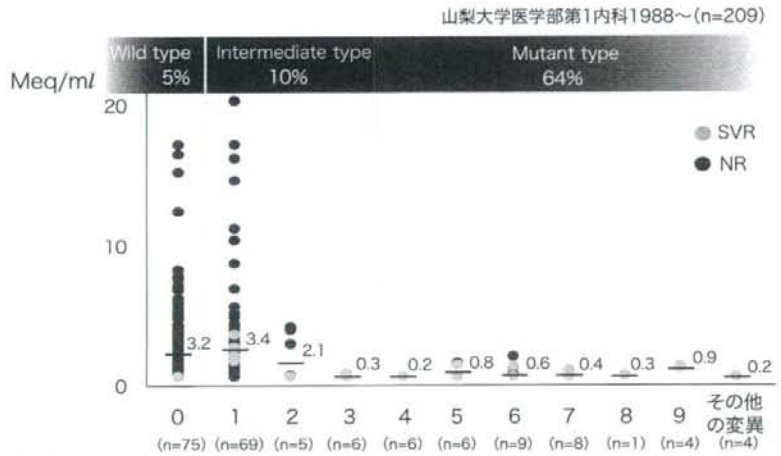

図2　IFN単独療法(6カ月)のISDR変異数と治療効果とウイルス量の関係

てみいだされ，インターフェロン感受性領域(Interferon sensitivity determining region: ISDR)と命名された(図1)[1,2]．すなわち，1b型のHCVの標準株であるHCV-Jと比較して，変異のない野生型(wild type)ではインターフェロン単独療法ではSVRとなる可能性は極めて低いのに対し，4個以上の変異がある変異型(mutant type)では高いSVR率を示し，1～3個の変異がある中間型(intermediate type)ではこの中間のSVR率を示す．この領域はPKR Binding domainのN末端側に位置し，ウイルス増殖と密接に関連している部位と考えられ，レプリコンを用いた細胞培養モデルでも適応変異(adaptive mutations)の集積部位である[3]．さらにISDRを含むNS5A領域から翻訳される蛋白は，種々のtyrosine kinas活性を調節していることも推測されており，この領域のアミノ酸変異が

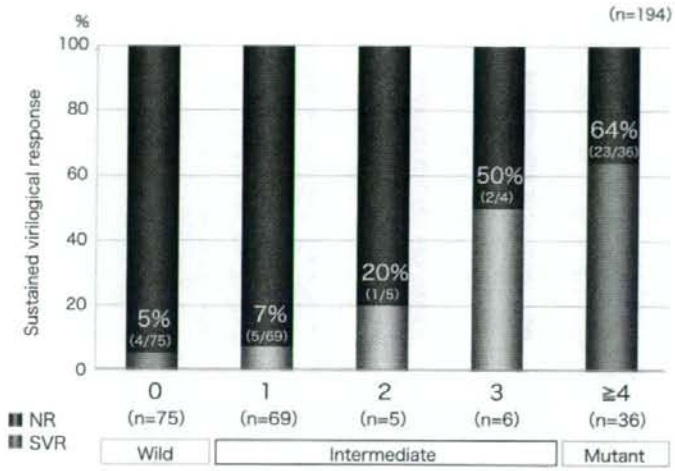

図3　IFN単独療法(6カ月)のISDR別SVR率

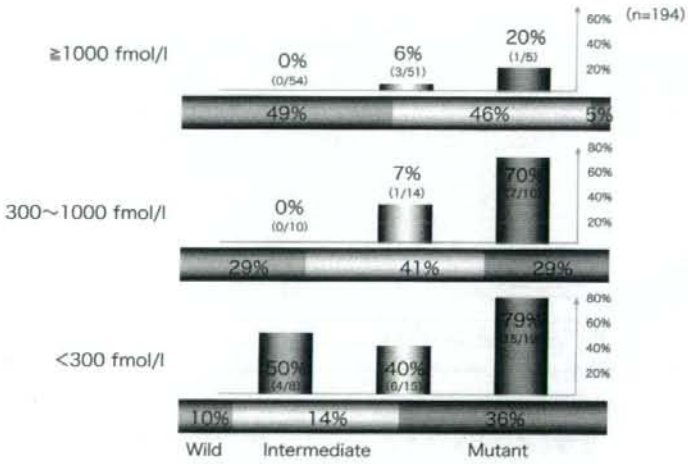

図4　IFN単独療法(6カ月)のHCV量とISDR別SVR率

HCVの増殖やインターフェロン感受性と関連することは，ウイルス学的にも容易に推測される．また，臨床的には，野生型ではウイルス量が多く，変異型ではウイルス量は低く，ISDRの変異数が増すほどウイルス量は減少することが明らかになっている(図2)．

3　インターフェロン単独療法とISDR変異

もともと，ISDRはインターフェロン単独療法の時代にみいだされたもので，1b型のC型慢性肝炎に対するインターフェロン単独6カ月間の治療成績をISDRのアミノ酸変異数