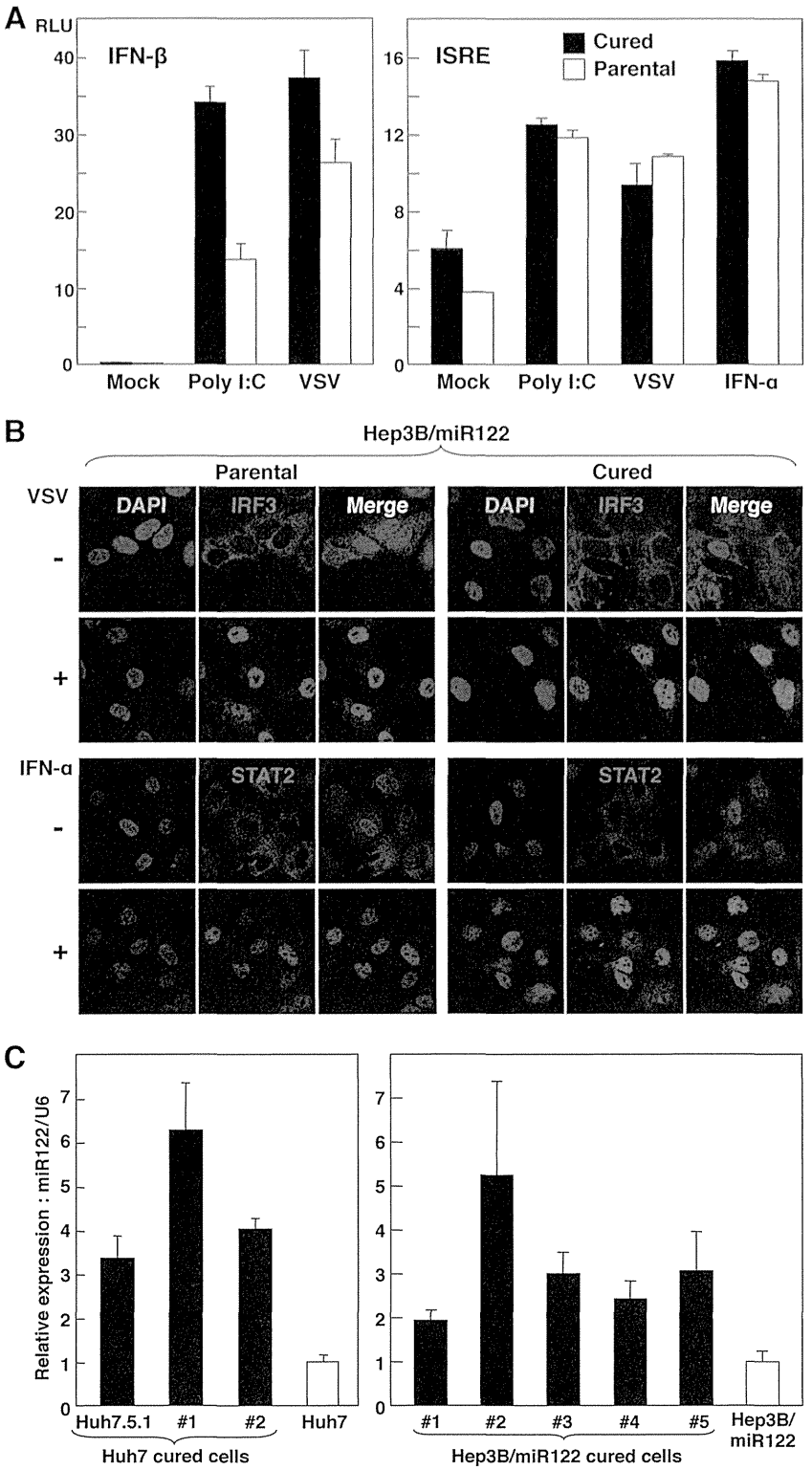

FIG 6 Cured Hep3B/miR122 cells facilitate efficient propagation of HCVcc through enhanced expression of miR122. (A) (Left) Hep3B/miR122 parental cells and cured cells of clone 5 were cotransfected with pIFNβ-Luc and pRL-TK and then infected with the VSV NCP mutant at an MOI of 0.01 or transfected with 1 μg of poly(I·C) at 24 h posttransfection, and luciferase activities were determined at 48 h posttreatment; (right) the cells were cotransfected with pISRE-Luc and pRL-TK and then infected with VSV at an MOI of 0.01 or treated with IFN-α (100 IU/ml) at 24 h posttransfection, and luciferase activities were determined at 48 h posttreatment. (B) (Upper) Hep3B/miR122 parental cells and the cured cells were infected with VSV at an MOI of 0.01, fixed with 4% phosphonoformic acid at 18 h postinfection, and subjected to indirect immunofluorescence assay using rabbit anti-IRF3 antibody, followed by AF488-conjugated anti-rabbit IgG (red); (lower) the cells were treated with IFN-α (100 IU/ml), fixed with 4% paraformaldehyde at 1 h postinfection, and subjected to indirect immunofluorescence assay using rabbit anti-STAT2 antibody, followed by AF488-conjugated anti-rabbit IgG (red). Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (blue). (C) Total RNA was extracted from parental Huh7 and Hep3B/miR122 cells and their cured cells, and the relative expression of miR122 was determined by qRT-PCR by using U6 snRNA as an internal control.

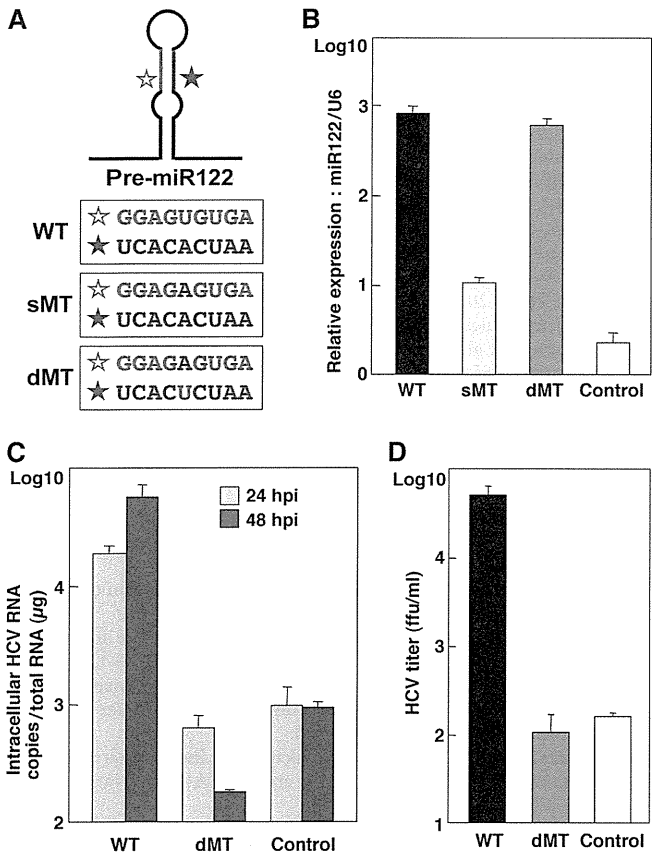

FIG 7 Specific interaction of miR122 with viral RNA is crucial for efficient propagation of HCVcc. (A) Diagram of pre-miR122 and partial nucleotide sequences of wild type (WT) miR122 and mutant miR122 carrying a single mutation (sMT) and double mutations (dMT). (B) Hep3B cells were transduced with lentiviral vectors expressing either WT-, sMT-, or dMT-miR122 or with a control, and the relative expression of miR122 was determined by qRT-PCR by using U6 snRNA as an internal control. (C) Hep3B cells expressing WT- or dMT-miR122 or the control cells were infected with HCVcc at an MOI of 1, and the level of HCV RNA was determined by qRT-PCR at 24 and 48 h postinfection. (D) The culture supernatants were collected at 72 h postinfection, and the viral titers of the supernatants were determined by focus-forming assay using Huh7.5.1 cells.

38, 52). In this study, we assessed the possibility of establishment of human liver cell lines that are susceptible to HCVcc propagation through exogenous expression of miR122 by a lentiviral vector. Although Huh7 cells and their derived cell lines are highly susceptible to propagation of HCVcc, they intrinsically express an abundant amount of miR122. Among the cell lines that we investigated, Hep3B cells exhibit a high sensitivity to HCVcc propagation by expression of miR122 compared to that of Huh7 cells, whereas no sensitivity to HCVcc was observed in the parental Hep3B cells. Therefore, the Hep3B cell line was suggested to be an ideal tool to investigate miR122 function in the life cycle of HCV.

RNA viruses replicate in host cells with high error rates, generating a broad population diversity, which allows rapid adaptation to new environments (33). HCV propagates in the liver of patients with quasispecies heterogeneity and transmits to a new host through contaminated blood or blood products (16). It is known that the complexity of HCV clones significantly decreases during transmission through a genetic bottleneck, resulting in a more homogeneous population. This selection of certain clones is mainly caused by the host factors required for viral replication and immune pressure in a new host and is involved in the early phase of HCV infection in the new environment (18, 25, 32). A sole cell line, Huh7, has been employed in most of the experiments for *in vitro* studies of entry, RNA replication, and particle formation of HCV. Therefore, it has not been possible to assess propagation of HCVcc in human liver cell lines other than Huh7 cells and transmission of HCVcc to liver cell lines of different origins. The establishment of a novel human liver cell line, Hep3B/miR122, for propagation of HCVcc would help to generate new insights into the mutual interaction between HCV and human hepatocytes. Although we are not able to evaluate the effects of the acquired immunity on the induction of the adaptive mutations in cell culture systems, we can assess the host factors involved in the generation of the adaptive mutations by using two different human liver cell lines that support continuous propagation of HCVcc. Further studies are needed to determine the adaptive mutations in the HCV genome by passage in either Hep3B/miR122 or Huh7 cells and in one after the other.

At least seven major HCV genotypes and numerous subtypes have been identified (21), but laboratory strains capable of replicating *in vitro* are limited (36, 64, 68, 70). It is important to establish cell lines that permit the complete propagation of a wide range of HCV genotypes for further understanding of the life cycle of HCV. Although the partial replication of serum-derived HCV in primary hepatocytes in a specialized culture system has been reported (50), development of a simpler and more user-friendly system is required for promotion of research on HCV. It might be feasible to establish new cell culture systems for not only various genotypes of infectious HCV clones but also serum-derived HCV by the expression of miR122 in various human liver cell lines.

While preparing the manuscript, Narbus et al. reported that the expression of miR122 enhances HCV replication in HepG2/CD81 cells (46). Our data also demonstrated that the expression of miR122 increased HCV replication in HepG2/CD81 cells, as shown in Fig. 1D. However, the impact of miR122 expression on the production of infectious particles in HepG2/CD81 cells is significantly lower than that in Huh7 cells (46). Although LH86 (71) and Li23 (30) cell lines derived from human hepatocellular carcinoma have been shown to permit propagation of HCVcc, these cell lines are not well characterized. In contrast, the Hep3B cell line has been utilized in a wide range of research fields for a long time, resulting in the accumulation of many sources of data from genomic and proteomic analyses (1, 47, 55, 63, 67). Moreover, the Hep3B cell line is available from the major cell banks all over the world, which should readily allow reevaluation of the findings in this study. Comparison of the experimental data on HCVcc propagation between Huh7 and Hep3B/miR122 cells might provide a clue to understanding the host factors crucial for the efficient propagation of HCV in human liver cells.

The higher susceptibility to HCVcc propagation of the cured cells derived from Huh7 cells than the parental cells was suggested to be attributable to impairment of the innate immune response (57). However, this is not the only reason for efficient propagation of HCVcc in the Huh7-based cured cell lines (17). It has been shown that cured cell lines, such as Huh7.5.1 and Huh7-Lunet, express a higher level of miR122 than the parental Huh7 cells (13), suggesting that upregulation of miR122 in the cured cells participates in the efficient propagation of HCVcc. However, the level of

miR122 expression in the cured Hep3B cells was not necessarily correlated with the replication efficiency of HCVcc in the present work (Fig. 6C). Most recently, Denard et al. reported that the expression of CREB3L1/OASIS, which specifically prevents division of virus-infected cells, in cured Huh7 cells was reduced compared to that in the parental cells (12), suggesting that CREB3L1/OASIS is also involved in the enhancement of HCVcc propagation in the cured cells.

In this study, we have shown that expression of miR122 confers susceptibility to human liver cell lines for the efficient propagation of HCVcc. Elimination of the HCV genome from the replicon cells of Hep3B/miR122 cells enhanced propagation of HCVcc in accord with the increment of miR122 expression, and propagation of HCVcc in the cured cells was continuously increased in every passage. Furthermore, the interaction between HCV RNA and miR122 was shown to be specific for production of infectious particles in Hep3B/miR122 cells. The establishment of a new permissive cell line for HCVcc allows us not only to investigate the biological function of miR122 on the life cycle of HCV but also to develop novel therapeutics for chronic hepatitis C.

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Hepatitis C Virus NS3/4A Protease Inhibits Complement Activation by Cleaving Complement Component 4

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Abstract

Background: It has been hypothesized that persistent hepatitis C virus (HCV) infection is mediated in part by viral proteins that abrogate the host immune response, including the complement system, but the precise mechanisms are not well understood. We investigated whether HCV proteins are involved in the fragmentation of complement component 4 (C4), composed of subunits C4α, C4β, and C4γ, and the role of HCV proteins in complement activation.

Methods: Human C4 was incubated with HCV nonstructural (NS) 3/4A protease, core, or NS5. Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then subjected to peptide sequencing. The activity of the classical complement pathway was examined using an erythrocyte hemolysis assay. The cleavage pattern of C4 in NS3/4A-expressing and HCV-infected cells, respectively, was also examined.

Results: HCV NS3/4A protease cleaved C4γ in a concentration-dependent manner, but viral core and NS5 did not. A specific inhibitor of NS3/4A protease reduced C4γ cleavage. NS3/4A protease–mediated cleavage of C4 inhibited classical pathway activation, which was abrogated by a NS3/4A protease inhibitor. In addition, co-transfection of cells with C4 and wild-type NS3/4A, but not a catalytic-site mutant of NS3/4A, produced cleaved C4γ fragments. Such C4 processing, with a concomitant reduction in levels of full-length C4γ, was also observed in HCV-infected cells expressing C4.

Conclusions: C4 is a novel cellular substrate of the HCV NS3/4A protease. Understanding disturbances in the complement system mediated by NS3/4A protease may provide new insights into the mechanisms underlying persistent HCV infection.

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Introduction

Hepatitis C virus (HCV) is a single-stranded positive-strand RNA virus of the Flaviviridae family. The viral genome encodes four structural proteins and six non-structural (NS) proteins [1]. NS3/4A, a complex consisting of NS3 with serine protease activity and cofactor NS4A, plays an essential role in processing of HCV proteins. NS3/4A is a target of direct-acting antiviral agents (DAA) [2,3], and use of an NS3/4A protease inhibitor as a DAA markedly increases the therapeutic effect of other anti-HCV agents. Thus, NS3/4A protease may play an important role in interfering with the antiviral response.

HCV has been hypothesized to block the host immune response against persistent infection [4]. Furthermore, the time required for HCV-infected patients to develop hepatic cirrhosis varies among individuals; in particular, the progression of

hepatic fibrosis seems to be slower in HCV carriers with persistent normal alanine aminotransferase (ALT) levels than in chronic hepatitis patients with elevated ALT levels [5]. These differences in clinical features might be caused by variations in the host immune response, but the underling mechanism is unclear.

In the course of proteomic analyses aimed at identifying proteins potentially involved in the pathophysiology of hepatic diseases, we found that a specific peptide fragment of complement component 4 (C4) was significantly more abundant in HCV carriers with persistent normal ALT than in patients with chronic hepatitis [6], as well as more abundant in HCV carriers, regardless of ALT levels, compared to healthy controls. Assuming that C4 expression levels are similar among these groups, this C4 fragment may be generated by post-translational processing in HCV-infected individuals.

The complement system is part of the innate immune system, which can be activated through three pathways: the classical pathway, the mannose-binding lectin pathway, and the alternative pathway. C4, which is involved in the classical- and mannose-binding lectin pathways, can be cleaved by certain cellular protease(s), leading to a cascade of C4 activation [7]. In this study, we provide the first evidence that HCV NS3/4A cleaves C4, and that this cleavage attenuates activation of the classical pathway of complement system.

Materials and Methods

Materials

HCV NS3/4A protease (217 amino acid [aa] fusion protein with NS4A co-factor fused to the N-terminus of NS3 protease domain) with His-tag, HCV core (aa 1–102) with GST-tag, and HCV NS5 (aa 2061–2302) with GST-tag were purchased from AnaSpec (Fremont, CA) or ProSpec (Rehovot, Israel). Isolated human-derived complement components (C1, C2) were obtained from Hycult Biotech (Uden, Netherlands), and C4 and C4-deficient guinea pig serum (C4d-GPS) were purchased from Sigma-Aldrich (St. Louis, MO). VX950, a HCV NS3/4A serine protease inhibitor, was obtained from Selleck Chemicals (Houston, TX). Veronal buffer, sheep erythrocytes, and hemolysin were purchased from Wako (Osaka, Japan), Nippon Biotest Laboratories Inc. (Tokyo, Japan), and Denka Seiken Co. (Tokyo, Japan), respectively.

NS3/4A protease cleavage assay

HCV NS3/4A protease, core, or NS5 (3 µl) and 9 µl of Assay buffer (SensoLyte® 490 HCV Protease Assay Kit, AnaSpec) containing 30 mM dithiothreitol (DTT) were added to C4 (3 µl), and the mixture was incubated at 30°C for 30 min. The solution was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and resolved proteins were stained with Coomassie brilliant blue (CBB). In a separate experiment, VX950 was pre-incubated with NS3/4A protease at 30°C for 30 min, and then incubated with C4 at 30°C for 30 min. Proteins detected by CBB staining were subjected to N-terminal peptide sequence analysis at Nippi Inc. (Tokyo, Japan).

Hemolytic analysis

The method used for hemolytic analysis has been described previously [8,9]. Briefly, intermediates of complement components were sequentially added to sheep erythrocytes sensitized by hemolysin (Ab-sensitized sheep erythrocytes, EA). Dilute erythrocytes and complement components were prepared in Veronal buffer containing 2% gelatin (GVB). To prepare EA, hemolysin was added to 10 ml of erythrocytes (5×10^8 cells/ml) and incubated at 37°C for 30 min. C1 (10 µg) was added to 5 ml of EA, incubated at 30°C for 15 min, and washed twice with GVB (EAC1). NS3/4A protease was prepared in a solution containing 20 mM Tris-HCl (pH 8.0), 20% glycerol, 100 mM KCl, 1 mM DTT, and 0.2 mM EDTA, adjusted to pH 7.5. The reaction solution was adjusted to 2 mM DTT to ensure a uniform effect on C4 activity. C4 was incubated with the NS3/4A protease, and then mixed with 100 µl of EAC1 and incubated at 30°C for 15 min (EAC1-C4). After washing twice with GVB, 1 µl of C2 (0.1 mg/ml) was added and the mixture was incubated at room temperature for 4 min (EAC1-C4-C2). After washing twice again with GVB, 150 µl of 80-fold diluted C4d-GPS was added to 30 µl of EAC1-C4-C2, and the mixture was incubated at 37°C for 30 min. The optical absorbance of the centrifuged supernatant was determined at 415 nm, and the level of hemolysis was calculated using the following formula: Hemolysis (%) = (sample OD_{415} – no C4 OD_{415})/(total hemolysis in distilled water OD_{415} – no C4 OD_{415}) ×100. "No C4" refers to a control sample containing EAC1 not incubated with C4. In a separate experiment, VX950 was first pre-incubated with NS3/4A protease at 30°C for 30 min, and then incubated with C4 at 30°C for 30 min.

Cell culture and transfection

Human hepatoma-derived Huh7.5.1 cells (a kind gift from Dr. F. V. Chisari, The Scripps Research Institute, La Jolla, CA) and human embryonic kidney (HEK) 293T cells were cultured at 37°C under 5% CO_2 in DMEM containing 10% FBS, 100 units/ml penicillin, and 100 g/ml streptomycin. DNA transfections of Huh7.5.1 cells and 293T cells were performed using Lipofectamine LTX/PLUS Reagent (Invitrogen, Carlsbad, CA) and polyethylenimine (Alfa Aesar, Heysham, Lancashire, UK), respectively. The transfection complex was formed at a DNA:reagent ratio of 1:1 (w/w) in OptiMEM (Invitrogen) with incubation for 15 min at room temperature before it was added to the culture.

Preparation of virus stock

The pJ6/JFH1 plasmid was generated by replacing the structural region of the JFH-1 strain with that of the J6CF strain, as described [10]. Cell culture–derived infectious HCV particles (HCVcc) were produced by introducing *in vitro* transcribed RNA from pJ6/JFH1 into Huh-7.5.1 cells by electroporation. The culture supernatant was concentrated using a 100-kDa MWCO Amicon Ultra Centrifugal Filter (Millipore, Bedford, MA). Virus infectivity was measured by indirect immunofluorescence analysis. Virus stocks (1×10^7 focus-forming units/ml) were divided into small aliquots and stored at −80 °C until use.

Plasmids

The C4 expression plasmid pFN21-C4A was purchased from Kazusa DNA Research Institute (Kisarazu, Japan). To create pFN21-C4A delH-Tag, the N-terminal Halo-Tag of pFN21-C4A was removed by digestion with *Hin*dIII and *Pvu*I, followed by blunt-ending with KOD FX neo (Toyobo, Osaka, Japan). pCAG-HA-NS3/4A, which expresses full-length NS3 and NS4 (derived from HCV genotype 1b, Con-1 strain) with an HA tag at the N-terminus of NS3 was generated as described [11]. Point mutation of serine to alanine at position 139 (S139A) in pCAG-HA-NS3/4A was achieved by site-directed mutagenesis using two primers: 5′-TAC TTG AAG GGC TCT GCG GGC GGT CCA CTG C-3′ and 5′-GCA GTG GAC CGC CCG CAG AGC CCT TCA AGT A-3′. The point mutation was confirmed by DNA sequencing.

Immunoprecipitation and immunoblotting

Goat anti-human complement C4 antibody (MP Biomedicals, Santa Ana, CA) was bound to protein G–agarose beads (Thermo Scientific, Rockford, IL) in binding buffer (0.5% Nonidet P-40, 25 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA and protease inhibitor cocktail [Roche, Basel, Switzerland]) for 1 h at room temperature. Culture supernatants were incubated with the beads for 1 h at room temperature, and the immunoprecipitated proteins were eluted by heat treatment for 5 min at 100°C with 2× sample buffer. Culture supernatants were directly mixed with 3× sample buffer at a ratio of 1 volume supernatant to 2 volumes sample buffer (1:2 [v/v]). Cells were solubilized in lysis buffer (1% Triton X-100, 25 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA and protease inhibitor cocktail) on ice. Cell debris was removed by centrifugation, and the resultant supernatants were diluted 1:2 (v/v) with 3× sample buffer. Precipitated proteins, culture supernatants, and cell lysates were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore). After blocking in 4% BlockAce (DS Pharma Biomedical, Osaka, Japan), the blots were incubated with the indicated primary antibodies, followed by the secondary antibody in TBST (25 mM Tris [pH 7.5], 150 mM NaCl, and 0.1% Tween 20). The primary antibodies used were anti-C4γ (clone H-291, Santa Cruz Biotechnology, Dallas, TX), anti-human complement C4, anti-HA (Sigma, St. Louis, MO), anti-HCV core (clone 2H9) and anti-GAPDH (clone 6C5, Santa Cruz Biotechnology). Donkey polyclonal Secondary Antibody to Goat IgG-H&L (HRP) (Abcam, Cambridge, UK), HRP-linked anti-mouse IgG, and HRP-linked anti–rabbit IgG (Cell Signaling Technology, Danvers, MA) were used as secondary antibodies. Finally, proteins were visualized using an enhanced chemiluminescence (ECL) reagent (ECL Select Western Blotting Detection Reagent, GE Healthcare, Little Chalfont, UK).

Statistical analysis

The concentration of proteins detected by Western blots was determined by densitometric analysis using the ImageJ software [12]. Statistical analysis was performed with the SPSS software (SPSS Inc., Chicago, IL) using the Tukey test, with $P < 0.05$ considered to indicate a significant difference.

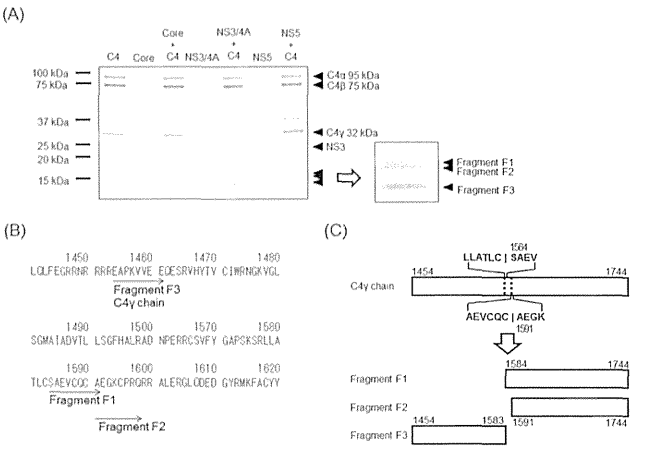

Figure 1. C4 is cleaved by HCV NS3/4A protease at Cys-1583/Ser-1584 or Cys-1590/Ala-1591. (A) HCV NS3/4A protease, core, or NS5 was added to C4, and the products were separated by SDS-PAGE and subjected to CBB staining. Two approximately 17-kDa proteins (Fragment F1 and F2) and a 15-kDa protein (Fragment F3) were detected after incubation of C4 with HCV NS3/4A protease, but not after incubation with core or NS5. (B) Amino acid sequence of aa 1451-1620 region of C4. Protein fragments were analyzed by N-terminal peptide sequencing. The sequences of the N-termini of the 17-kDa proteins (Fragment F1 and F2) were SAEVCQCA and AEGKCPRQ, which are located at aa 1584–1591 and 1591–1598 in C4, respectively. The sequence of the N-terminus of the 15-kDa protein (Fragment F3) was EAPKVVEE, which is located at aa 1454–1461 in C4. (C) Schematic representation of C4γ chain, and Fragment F1, F2 and F3.

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Results

HCV NS3/4A protease cleaves C4 in vitro

To test cleavage of C4 mediated by HCV proteins, C4 (containing subunits C4α, C4β, and C4γ) was mixed with NS3/4A protease, core, or NS5, followed by incubation at 30°C for 30 min. As shown in Figure 1A, doublet bands at 17 kDa (fragments F1 and F2 in the enlarged view) and one band at 15 kDa (fragment F3) were detected in the presence of NS3/4A protease and C4. These bands were not detected after incubation of C4 with core or NS5, or when either core or NS5 were incubated alone.

N-terminal sequence analyses revealed that the bands at approximately 100, 75, and 32 kDa (Figure 1A) represented C4α (N-terminus sequence identified: NVNFQKAI), C4β (KPRLLLFS), and C4γ (EAPKVVEE), respectively. As shown in Figure 1B, the N-terminal sequences of the doublet proteins at 17 kDa were identical to sequences found in C4γ: SAEVCQCA (aa 1584–1591 of C4) and AEGKCPRQ (aa 1591–1598). In addition, the N-terminal sequence of the 15-kDa fragment was EAPKVVEE (aa 1454–1461), indicating that the 15-kDa fragment is the N-terminal region of the C4γ. These results demonstrate that HCV NS3/4A protease cleaves C4 between

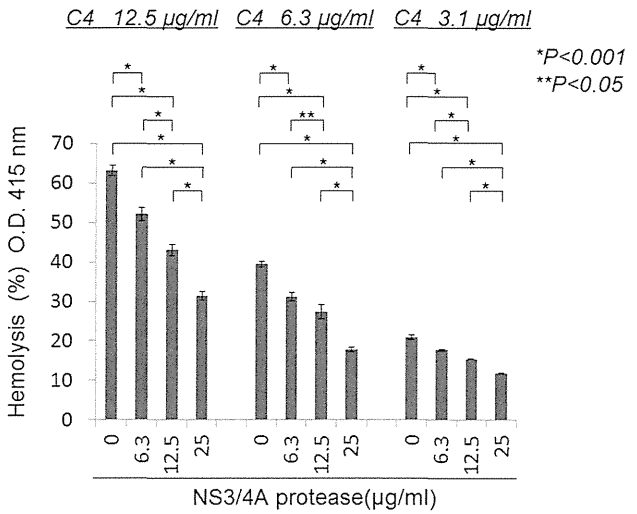

Figure 2. HCV NS3/4A protease inhibits the classical pathway, as assessed by hemolysis. C4 was incubated in the presence or absence of HCV NS3/4A protease, and then C1-sensitized EA (EAC1) was added (EAC1-C4). After washing, C2 was added to form EAC1-C4-C2, and the complex was resuspended in C4d-GPS. The absorbance of the centrifuged supernatant was determined at 415 nm. The grade of hemolysis decreased in the presence of NS3/4A protease in a dose-dependent manner. All measurements were performed in triplicate, and data are expressed as means ± SD.

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either Cys-1583 and Ser-1584 or Cys-1590 and Ala-1591, consistent with the consensus sequence of HCV NS3 proteinase cleavage sites [3,13]. Possible locations for the 15- and 17-kDa fragments of C4γ are shown in Figure 1B and 1C.

HCV NS3/4A protease decreases the activity of the classical pathway of the complement system in a concentration-dependent manner

To examine the functional significance of C4 cleavage by NS3/4A protease, complement components were serially added to EA to reproduce the classical pathway of the complement system. C4, untreated or treated with various concentrations of NS3/4A, was added at various concentrations to the EA-C1 mixture, followed by addition of C2 and C4d-GPS, which were used as sources of C3 and C5-C9. Erythrocyte hemolysis, which is caused by the complement-mediated fusion of erythrocytes, was quantified (Figure 2). NS3/4A treatment significantly decreased hemolysis levels in a concentration-dependent manner. This result, together with those in Figure 1, suggests that the C4 cleavage mediated by NS3/4A protease may contribute to inhibition of complement activation via the classical pathway.

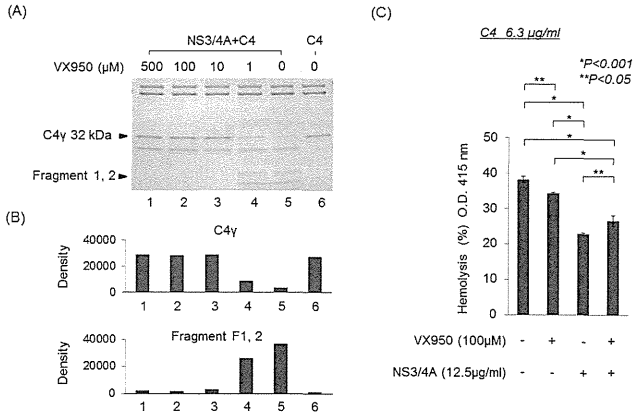

Figure 3. VX950, a HCV NS3/4A protease inhibitor, abrogates cleavage of C4 induced by HCV NS3/4A protease. (A) VX950 was added to HCV NS3/4A protease at the indicated concentrations, and then C4 was added. Proteins were separated by SDS-PAGE for CBB staining. The three C4-derived fragments of 17 kDa and 15 kDa produced by NS3/4A protease action could not be detected after pretreatment with VX950, and this change was accompanied by an increased concentration of the 32-kDa C4γ chain. (B) The C4γ, 17-kDa, and 15-kDa bands were quantified by densitometric analysis using the Image J software. (C) C4 was incubated in the presence or absence of HCV NS3/4A or VX950, and then C1-sensitized EA (EAC1) was added (EAC1–C4). C2 and C4d-GPS were then added, and the absorbance of the supernatant was determined at 415 nm. Hemolysis was inhibited by NS3/4A protease and this inhibition was blocked by VX950. All measurements were made in triplicate, and data are expressed as means ± SD.

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HCV protease inhibitor reduces inactivation of complement by blocking C4 cleavage by NS3/4A protease

We tested the effect of VX950, a specific inhibitor of NS3/4A protease, on C4 cleavage by NS3/4A protease and inhibition of complement activation. As shown in Figure 3A and 3B, under a condition in which more than 80% of 32-kDa C4γ was processed into 17- and 15-kDa fragments in the presence of NS3/4A protease (lanes 5), pretreatment of the protease with 1 μM VX950 moderately inhibited the cleavage of C4γ (lanes 4). The NS3/4A-mediated processing of C4γ into 17- and 15-kDa fragments was almost completely blocked by VX950 at ≥10 μM (lanes 1–3). In the erythrocyte hemolysis assay, the reduction in hemolysis level mediated by NS3/4A significantly recovered in the presence of VX950 (Figure 3C). These results confirmed cleavage of C4γ by NS3/4A and the involvement of the protease in the classical complement pathway.

Cleavage of C4γ in NS3/4A-expressing cells and HCV-infected cells

To determine whether HCV NS3/4A protease cleaves C4 in cells, we analyzed 32-kDa C4γ and its processed fragments in

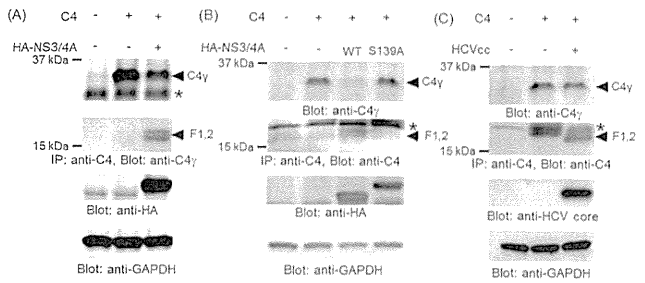

Figure 4. C4 is cleaved by HCV NS3/4 protease in cell cultures. (A) 293T cells were transfected with the indicated plasmids. Anti-C4 immunoprecipitates (IP) of supernatants were separated by SDS-PAGE and analyzed by immunoblotting with anti-C4γ antibody. Detergent-soluble cell lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-HA and anti-GAPDH antibodies. (B) 293T cells were transfected with the indicated plasmids. Culture supernatants were analyzed by immunoblotting with anti-C4γ antibody. Anti-C4 immunoprecipitates (IP) of supernatants were analyzed by immunoblotting with anti-C4 antibody. Detergent-soluble cell lysates were analyzed by immunoblotting with anti-HA and anti-GAPDH antibodies. (C) Huh7.5.1 cells were mock-infected or infected with HCVcc at a multiplicity of infection of 2 for 6 h, followed by mock-transfection or transfection with C4 expression plasmid. Culture supernatants and cell lysates were analyzed as described in (A) and (B). The anti-C4γ antibody was not appropriate for immunoblotting of IP samples derived from Huh7.5.1 cultures because of unavoidable nonspecific cross-reaction. * indicates non-specific reactions in (A) – (C).

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culture medium from 293T cells cotransfected with expression plasmids encoding C4 (pFN21-C4A delH-Tag) and NS3/4A protease (pCAG-HA-NS3/4A). Co-expression of C4 and NS3/4A derived from HCV genotype 1b led to production of the 17-kDa C4γ fragment and reduction in the level of 32-kDa C4γ (Figure 4A). In contrast, the 17-kDa fragment was not detected, and the 32-kDa C4γ level was not changed, when a mutant NS3 with an amino-acid substitution at the catalytic-site (S139A)/4A was co-expressed with C4 (Figure 4B). Next, we investigated C4 cleavage in HCV-infected cultures. In the culture medium of Huh7.5.1 cells infected with HCVcc of strain J6/JFH-1 (genotype 2a) expressing of C4 from pFN21-C4A delH-Tag, the 17-kDa fragment was produced, and the level of 32-kDa C4γ was reduced accordingly (Figure 4C). These data demonstrate that C4γ can be cleaved by HCV NS3/4A, either expressed from a plasmid or in HCV-infected cells, and that proteases of both genotypes 1b and 2a are functional in this cleavage.

Discussion

The results of this study show that C4γ is cleaved by HCV NS3/4A protease *in vitro* and in cell culture. Cleavage of C4 by HCV NS3/4A protease leads to inhibition of activity of the classical complement pathway. C4 cleavage and abrogation of complement activation are blocked by an inhibitor of NS3/4A protease.

HCV NS3/4A protease plays an important role in the replication of non-structural regions [2,3], and might also directly act on the IFN signaling system to inhibit the host immune response and prevent viral clearance, thereby contributing to persistent HCV infection. However, a direct relationship between HCV infection and complement components has not been previously established. Levels of functional C3 or C4 assessed by hemolysis assay are reduced after infection by flaviviruses such as Dengue virus and West Nile virus (WNV) [9,14]. In mice infected with γ-herpesvirus or WNV, genetic deletion of complement C3 or C4 not only enhances mortality but also increases persistent replication of γ-herpesvirus or WNW RNA levels [14,15]. Furthermore, Moulton et al. reported that mousepox virus dissemination was more severe, and viral loads in tissues were higher, in C3-deficient mice; leading to higher mortality than in wild-type mice; those authors concluded that the complement system is critical for slowing viral spread and decreasing tissue titer and damage [16]. Thus, it is likely that the complement system is widely associated with development of viral infection. Further investigation of the role of complement activation mediated by HCV proteins such as HCV NS3/4A protease may provide new insights into development of persistent HCV infection.

Our results indicated that the C4 cleavage site of HCV NS3/4A protease is between either Cys-1583 and Ser-1584 or Cys-1590 and Ala-1591 of C4, both of which are located in the C4γ chain (Figure 1). HCV NS3/4A protease has previously been suggested to cleave at Cys/Thr and Ala/Ser sites [3,13], which is broadly consistent with our results. C4 was also cleaved by HCV NS3/4A protease in HCV-infected cells (Figure 4C), in which unprocessed 32-kDa C4γ and cleaved 17-kDa fragment in the culture medium were observed. In cultures of human hepatoma HepG2 cells, the major fraction of C4α, C4β, and C4γ were present in the culture medium rather than in cells [17,18]. In good agreement with that finding, we detected little C4 in Huh-7–derived cells (data not shown). We speculate that immediately after synthesis, at least a fraction of C4γ can be quickly cleaved by NS3/4A in virally replicating cells, followed by secretion into the culture medium. However, we cannot rule out the possibility that HCV NS3/4A protease is present extracellularly and is functional under some particular conditions, because addition of recombinant antigens derived from the NS3 region to NS4 improves the sensitivity of the anti-HCV test in serum and shortens the window period for seroconversion in patients infected with HCV [19].

Complement components are involved in innate immunity and are responsible for one of the major immunological mechanisms mediated by antibodies [7]. In viral and bacterial infection, these components cause lysis of the outer membrane of virus particles [20] and infected cells [21] by the membrane attack complex C5–C9, ultimately resulting in elimination of the pathogen. Some viruses, such as cytomegalovirus, induce expression of cellular complement inhibitors, for example, decay-accelerating factor and monocyte chemoattractant protein, leading to increased levels of these proteins on the

surfaces of infected cells. Human immunodeficiency virus may incorporate the complement inhibitors into the viral envelope [22,23]. NS1 protein secreted from flaviviruses, such as dengue virus, West Nile virus, and yellow fever virus, not only attenuates activation of the classical and lectin pathways by directly interacting with C4, but also inactivates C4b by interacting with C4-binding protein [9,24]. Thus, NS1 of flaviviruses is considered to play a role in protecting the virus from complement-dependent neutralization. To our knowledge, however, our study provides the first evidence that the viral protease plays a role in protecting the virus from the complement defense system via proteolytic processing of the complement component.

In particular, C4 is involved in the classical and mannose-binding lectin pathways of the complement system, and it is responsible for the major activity of complement components. Upon antibody binding to an antigen, C4 is cleaved into C4a and C4b by the C1q-C1r-C1s complex, and C4b then binds to C2a (C4b2a) on the cell membrane to cleave C3 into C3a and C3b. Subsequently, C3b binds to C4b2a to cleave C5, and finally C5b and C6-C9 form the membrane attack complex to cause lysis of the cell membrane [7]. The erythrocyte hemolysis assay used in this study reproduces this cascade and revealed that HCV NS3/4A protease cleaves C4 and decreases the activity of the classical pathway. The specific assay was constructed to evaluate the function of C4 in the classical pathway by allowing HCV NS3/4A protease to act on C4 alone, without influence from other complement components. Therefore, further work is needed to determine whether HCV NS3/4A protease affects other components.

Several studies have demonstrated that HCV proteins influence complement systems and may be involved in evading antiviral immune responses of the host, as follows. Amet et al. reported that CD59, which may inhibit formation of the membrane attack complex, is incorporated into cultured cells and plasma primary HCV virions and inhibited activation of complement components, whereas administration of a CD59 inhibitor increases the sensitivity of component activation against endogenous HCV viral particles [25]. Banerjee et al. found that the HCV core protein reduces the expression of upstream stimulatory factor (USF)-1, a transcription factor important for basal C4 expression, and that expression of interferon regulatory factor (IRF)-1, which is important for IFN-γ–induced C4 expression, is inhibited by hepatocytes expressing HCV NS5A [26]. Mazumdar et al. showed that NS5A strongly downregulates C3 promoter activity in the presence of IL-1β, acting as an inducer [27]. HCV core inhibits T-cell proliferative responses *in vitro*, and this effect can be reversed by addition of anti–C1q receptor antibody to a T-cell proliferation assay [28]. Here, we identified C4γ as a novel cellular substrate of the HCV NS3/4A protease.

The results of this study suggest that C4γ cleavage by NS3/4A decreased the activity of the classical complement pathway, and might thereby attenuate activation of the complement system. An understanding of the viral protease–mediated inhibition of the complement system should provide new insights into the roles played by immune evasion in persistent HCV infection.

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Author Contributions

Conceived and designed the experiments: SM HU YS MS HT. Performed the experiments: SM KN TS SK KK KO KT TT AM MO. Analyzed the data: SM HU AI KN TS YS. Contributed reagents/materials/analysis tools: HU IS HT. Wrote the manuscript: SM HU TS HT.

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Polymorphisms of the Core, NS3, and NS5a Proteins of Hepatitis C Virus Genotype 1b Associate With Development of Hepatocellular Carcinoma

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Hepatocellular carcinoma (HCC) is one of the common sequelae of hepatitis C virus (HCV) infection. It remains controversial, however, whether HCV itself plays a direct role in the development of HCC. Although HCV core, NS3, and NS5A proteins were reported to display tumorigenic activities in cell culture and experimental animal systems, their clinical impact on HCC development in humans is still unclear. In this study we investigated sequence polymorphisms in the core protein, NS3, and NS5A of HCV genotype 1b (HCV-1b) in 49 patients who later developed HCC during a follow-up of an average of 6.5 years and in 100 patients who did not develop HCC after a 15-year follow-up. Sequence analysis revealed that Gln at position 70 of the core protein (core-Gln^{70}), Tyr at position 1082 plus Gln at 1112 of NS3 (NS3-Tyr^{1082}/Gln^{1112}), and six or more mutations in the interferon/ribavirin resistance-determining region of NS5A (NS5A-IRRDR≥6) were significantly associated with development of HCC. Multivariate analysis identified core-Gln^{70}, NS3-Tyr^{1082}/Gln^{1112}, and α-fetoprotein (AFP) levels (>20 ng/L) as independent factors associated with HCC. Kaplan-Meier analysis revealed a higher cumulative incidence of HCC for patients infected with HCV isolates with core-Gln^{70}, NS3-Tyr^{1082}/Gln^{1112} or both than for those with non-(Gln^{70} plus NS3-Tyr^{1082}/Gln^{1112}). In most cases, neither the residues at position 70 of the core protein nor positions 1082 and 1112 of the NS3 protein changed during the observation period. *Conclusion*: HCV isolates with core-Gln^{70} and/or NS3-Tyr^{1082}/Gln^{1112} are more closely associated with HCC development compared to those with non-(Gln^{70} plus NS3-Tyr^{1082}/Gln^{1112}). (HEPATOLOGY 2013;58:555-563)

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Hepatitis C virus (HCV) is a major etiologic agent of chronic hepatitis worldwide, with the estimated number of infected individuals being more than 180 million. Approximately 15% to 20% of chronically infected individuals undergo liver cirrhosis in a decade or so after infection, with hepatocellular carcinoma (HCC) arising from cirrhosis at an estimated rate of 1% to 4% per year.[1-3] Several host factors such as male gender, older age, elevated α-fetoprotein (AFP) level, advanced liver fibrosis as well as nonresponsiveness to interferon (IFN) therapy have been reported as important predictors of HCC development.[4,5] Recently, a host genetic factor, i.e., the *DEPDC5* locus polymorphism, was reported to be associated with progression to HCC in HCV-infected individuals.[6] On the other hand, it remains controversial as to whether HCV itself plays a direct role in the development of HCC. Experimental data suggest that HCV contributes to HCC by modulating pathways that promote malignant transformation of hepatocytes. HCV core, NS3, and NS5A proteins were shown to be involved in a

Abbreviations: HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN, interferon; IRRDR, interferon/ribavirin resistance-determining region; ISDR, interferon sensitivity-determining region.

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number of potentially oncogenic pathways in cell culture and experimental animal systems.[7] HCV core protein rendered cultured cells more resistant to apoptosis[8,9] and promoted *ras* oncogene-mediated transformation.[10,11] Moreover, transgenic mice expressing the HCV core protein in the liver developed HCC.[12] However, the clinical impact of HCV proteins on HCC development in humans and whether all HCV isolates are equally associated with HCC is yet to be determined. In a clinical setting, HCV core protein mutations at positions 70 (Gln^{70}) and/or 91 (Met^{91}) were closely associated with HCC development.[13-16] Gln^{70} and/or Met^{91} were also linked to resistance to PEG-IFN/ribavirin (RBV) treatment.[17-20] In addition, we and other investigators reported that an N-terminal part of the NS3 protein has the capacity to transform NIH3T3 and rat fibroblast cells[21,22] and to render NIH3T3 cells more resistant to DNA damage-induced apoptosis, which is thought to be a prerequisite for malignant transformation of the cell.[23] Also, the NS5A protein is a pleiotropic protein with key roles in both viral RNA replication and modulation of the host cell functions.[24] In particular, the links between NS5A and the IFN responses have been widely discussed. It was proposed initially that sequence variations within a region in NS5A spanning from amino acids (aa) 2209 to 2248, called the IFN sensitivity-determining region (ISDR), were correlated with IFN responsiveness.[25] Subsequently, in the era of PEG-IFN/RBV combination therapy, we identified a new region near the C-terminus of NS5A spanning from aa 2334 to 2379, which we referred to as the IFN/RBV resistance-determining region (IRRDR).[26,27] The degree of sequence variations within the IRRDR was significantly associated with the clinical outcome of PEG-IFN/RBV therapy. In the context of HCC, several retrospective studies suggested that IFN-based therapy might reduce the risk of HCC development.[4,28-30]

In an attempt to clarify whether viral factors, in particular those within the core, NS3, and NS5A proteins, are involved in HCC development, we carried out a comparative analysis of the aa sequences obtained from HCV patients who developed HCC and those who did not. In addition, we studied the sequence evolution of these genes in the interval between chronic hepatitis C and HCC development over a period of 15 years.

Patients and Methods

Ethics Statement. The study protocol, which conforms to the provisions of the 1975 Declaration of Helsinki, was approved beforehand by the Ethic Committees in Akashi City Hospital and Kobe University Graduate School of Medicine, and written informed consent was obtained from each patient enrolled in this study.

Patients. A total of 49 HCV-infected patients who developed HCC (HCC group) were retrospectively examined. They were followed up (from 1988 to 2003) with an average period until HCC development being 6.5 ± 2.9 years. Paired serum samples at the time of chronic hepatitis C (pre-HCC sample) and HCC development (post-HCC sample) were collected. As a control group, 100 HCV-infected patients who were followed up over a period of 15 years (from 1988 to 2003) without HCC development were retrospectively examined. Serum samples of the control group were available at the time of first visit to the clinic. All patients enrolled in this study were chronically infected with HCV genotype 1b (HCV-1b). HCV subtype was determined as reported previously.[31] Serum HCV RNA titers were quantitated by reverse-transcription polymerase chain reaction (RT-PCR0 with an internal RNA standard derived from the 5′ noncoding region of HCV (Amplicor HCV Monitor test, v. 2.0, Roche Diagnostics, Tokyo, Japan). All patients underwent liver biopsy and were diagnosed as chronic hepatitis. All HCC and 68% (68/100) of non-HCC patients received IFN-monotherapy, either natural IFN alpha (Sumiferon, Dainipponsumitomo Pharmaceutical, Osaka, Japan) at a dose of 6 million units (MU) or recombinant IFN alpha 2b (Intron A; Schering-Plough, Osaka, Japan) at a dose of 10 MU, 3 times a week for 6 months. All HCC patients were nonresponders (NR), who had detectable viremia during the entire course of IFN treatment. On the other hand, 18 (26%) of the 68 non-HCC patients treated with IFN achieved HCV RNA negativity at the end of treatment followed by rebound viremia within 6 months after the treatment and, therefore, they were referred to as relapsers. The other 50 IFN-treated, non-HCC patients were NR. The remaining 32 non-HCC patients did not receive IFN. All patients were

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seen every 2 months and tested for liver function markers during the follow-up period.

Sequence Analysis of HCV Core, NS3, and NS5A Proteins. HCV RNA was extracted from 140 μL of serum using a commercially available kit (QIAmp viral RNA kit; Qiagen, Tokyo, Japan). The core, NS3, and NS5A regions of the HCV genome were amplified as described elsewhere.[26,32-34] The sequences of the amplified fragments were determined by direct sequencing. The aa sequences were deduced and aligned using GENETYX Win software version 7.0 (GENETYX, Tokyo, Japan). The numbering of aa was according to the polyprotein of the prototype of HCV-1b; HCV-J.[35]

Statistical Analysis. Statistical differences in the baseline parameters of HCC and control groups were determined by Student's t test for numerical variables and Fisher's exact probability or chi-square tests for categorical variables. Likewise, statistical differences in viral mutations between HCC and control groups were determined by Fisher's exact probability test. Kaplan-Meier analysis was performed to estimate the cumulative incidence of HCC. The data obtained were evaluated by the log-rank test. Univariate and multivariate logistic analyses were performed to identify variables that independently associated with HCC development. Variables with $P < 0.1$ in univariate analysis were included in a backward stepwise multivariate logistic regression analysis. The odds ratios and 95% confidence intervals (95% CI) were calculated. All statistical analyses were performed using SPSS v. 16 software (Chicago, IL). Unless otherwise stated, $P < 0.05$ 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 AB719460 through AB719842.

Results

Demographic Characteristics of HCC and Control Groups. The clinical characteristics of HCC and control groups are shown in Table 1. The HCC group had significantly higher titers of ALT, AST, and AFP, and higher fibrosis staging score than that of the control group. There was no significant difference in viremia titers between the two groups.

Correlation Between Core Protein Sequence Polymorphism and HCC Development. HCV core protein sequences were obtained from all (49/49) and 94% (94/100) of pre-HCC and control patients' sera, respectively. Comparative sequence analysis revealed that 22 (45%) of 49 HCV isolates in the pre-HCC sera (pre-HCC isolates) and 59 (63%) of 94 HCV isolates from the control group (control isolates) had wild-core (Arg^{70}/Leu^{91}) (Table 2). The difference between HCC and control groups was hovering at a statistically significant level ($P = 0.05$). When the sequence pattern at position 70 alone was examined, a stronger association with HCC was observed. We found that 21 (43%) of 49 pre-HCC isolates had Gln^{70} while only 13 (14%) of 94 control isolates did ($P = 0.0002$). On the other hand, there was no significant correlation between sequence pattern at position 91 and HCC. Thus, a single mutation at position 70 (Gln^{70}) was the only polymorphic factor within core protein that was significantly associated with HCC development. It should be noted that there was no significant correlation between Gln^{70} and the degree of fibrosis progression (data not shown).

Table 1. Demographic Characteristics of HCC and Control Groups

Factor	HCC	Control	P Value
Age	57.3 ± 7.0*	56.4 ± 8.3	0.54
Sex (male/female)	31/18	54/46	0.29
ALT (IU/L)	159.4 ± 79.8	129.7 ± 51.5	0.007
AST (IU/L)	113.0 ± 62.2	91.6 ± 44.1	0.017
AFP (ng/L)	29.1 ± 33.7	18.4 ± 4.4	0.002
Platelets (x $10^4/mm^3$)	16.2 ± 2.8	16.2 ± 2.4	0.88
Inflammation grading score	8.7 ± 0.9	8.4 ± 1.2	0.05
Fibrosis staging score	2.4 ± 0.5	2.2 ± 0.5	0.02
HCV-RNA (KIU/mL)	593.4 ± 112.3	618.1 ± 95.9	0.17

*Mean ± SD. HCC, hepatocellular carcinoma; ALT, alanine aminotransferase; AST, aspartate transaminase; AFP; α-fetoprotein.

Correlation Between NS3 Protein Sequence Polymorphism and HCC Development. Sequences of NS3 serine protease domain (aa 1027 to 1146) were obtained from 94% (46/49) and 93% (93/100) of pre-HCC and control isolates, respectively. We found that 29 (63%) of 46 pre-HCC isolates had Tyr and Gln at positions 1082 and 1112, respectively (Tyr^{1082}/Gln^{1112}), while 39 (42%) of 93 control isolates did (Table 2). The difference in the proportion between pre-HCC and control isolates was statistically significant ($P = 0.029$). On the other hand, there was no significant correlation between Tyr^{1082}/Gln^{1112} and the degree of fibrosis progression (data not shown).

Correlation Between NS5A Protein Sequence Polymorphism and HCC Development. NS5A protein sequences were obtained from 92% (45/49) and 74% (74/100) of pre-HCC and control isolates, respectively. Twenty-four (53%) of 45 pre-HCC isolates had IRRDR of 6 or more mutations (IRRDR≥6)

Table 2. Correlation Between HCC and Sequence Polymorphic Factors of Core, NS3 and NS5A

HCV Protein	Factor	No. of Subjects / No. of Total* HCC	Control	*P* Value
Core	Wild-core (Arg^{70}/Leu^{91})	22/49 (45%)	59/94 (63%)	0.05
	Non-wild-core	27/49 (55%)	35/94 (37%)	
	Gln^{70}	21/49 (43%)	13/94 (14%)	0.0002
	Non-Gln^{70}	28/49 (57%)	81/94 (86%)	
	Leu^{91}	37/49 (76%)	70/94 (74%)	1.0
	Non- Leu^{91}	12/49 (24%)	24/94 (26%)	
NS3	Tyr^{1082} / Gln^{1112}	29/46 (63%)	39/93 (42%)	0.029
	Non-(Tyr^{1082} / Gln^{1112})	17/46 (37%)	54/93 (58%)	
NS5A	IRRDR≥6	24/45 (53%)	15/74 (20%)	0.0003
	IRRDR≤5	21/45 (47%)	59/74 (80%)	
	ISDR≥3	11/45 (24%)	8/74 (11%)	0.07
	ISDR≤2	34/45 (76%)	66/74 (89%)	
	Asn^{2218}	11/45 (24%)	3/74 (4%)	0.002
	Non-Asn^{2218}	34/45 (76%)	71/74 (96%)	

*Number of subjects with a given factor / total number of HCC or control. HCC, hepatocellular carcinoma; Arg^{70}, arginine at position 70 of the core protein; Leu^{91}, leucine at position 91 of the core protein; Gln^{70}, glutamine at position 70 of the core protein; Tyr^{1082}, tyrosine at position 1082 of NS3; Gln^{1112}, glutamine at position 1112 of NS3; IRRDR, interferon/ribavirin resistance-determining region; ISDR, interferon sensitivity-determining region; Asn^{2218}, asparagine at position 2218 of NS5A-ISDR.

while only 15 (20%) of 74 control isolates did (Table 2; $P = 0.0003$). We also found that pre-HCC isolates tended to have a higher degree of sequence heterogeneity in ISDR than control isolates, although not statistically significant due probably to the small number of cases examined; 11 (24%) of 45 pre-HCC isolates and 8 (11%) of 74 of control isolates had ISDR with three or more mutations ($P = 0.07$). Moreover, Asn at position 2218 (Asn^{2218}) within the ISDR was found in 24% (11/45) of pre-HCC isolates and only in 4% (3/74) of the control isolates ($P = 0.002$), suggesting that Asn^{2218} is significantly associated with development of HCC.

Cumulative HCC Incidence on the Basis of Core-Gln^{70}, NS3-Tyr^{1082}/Gln^{1112}, NS5A-IRRDR≥6, and NS5A-Asn^{2218}. Follow-up study revealed that the cumulative HCC incidence in patients infected with HCV-1b isolates with core protein of Gln^{70} and those of non-Gln^{70}, respectively, was 29% and 5% at the end of 5 years, 56% and 23% at the end of 10 years, and 63% and 26% at the end of 15 years (Fig. 1A), with the differences between the two groups being statistically significant ($P < 0.0001$; Log-rank test). Likewise, the cumulative HCC incidence in patients infected with HCV-1b isolates with NS3 of Tyr^{1082}/Gln^{1112} and those of non-(Tyr^{1082}/Gln^{1112}), respectively, was 15% and 7% at the end of 5 years, 37% and 24% at the end of 10 years, and 45% and 24% at the end of 15 years ($P = 0.02$) (Fig. 1B). Also, the cumulative HCC incidence in patients infected with HCV-1b isolates of IRRDR≥6 and those of IRRDR≤5, respectively, was 18% and 10% at the end of 5 years, 59% and 22% at the end of 10 years, and 63% and 27% at the end of 15 years ($P = 0.0002$) (Fig. 1C). Similarly, the cumulative HCC incidence in patients infected with HCV-1b isolates of Asn^{2218} and those of non-Asn^{2218}, respectively, was 31% and 9% at the end of 5 years, 77% and 28% at the end of 10 years, and 77% and 33% at the end of 15 years ($P = 0.0003$) (Fig. 1D).

Identification of Independent Factors Correlated With HCC Development by Univariate and Multivariate Logistic Regression Analyses. In order to identify significant independent factors associated with HCC development, all available data of baseline patients' parameters and core, NS3, and NS5A polymorphic factors were first analyzed by univariate logistic analysis. This analysis yielded eight factors that were significantly associated with HCC development: core-Gln^{70}, NS3-(Tyr^{1082}/Gln^{1112}), NS5A-IRRDR≥6, NS5A-Asn^{2218}, increased levels of ALT (>165 IU/L), AST (>65 IU/L), and AFP (>20 ng/L), and fibrosis staging score (≥3). Subsequently, those eight factors were entered in multivariate logistic regression analysis. This analysis identified two viral factors, core-Gln^{70} and NS3-(Tyr^{1082}/Gln^{1112}), and a host factor, AFP levels (>20 ng/L), as independent factors associated with HCC development (Table 3).

The vast majority of pre-HCC isolates (85%; 39/46) had core-Gln^{70} and/or NS3-Tyr^{1082}/Gln^{1112} and only 15% (7/46) had non-(Gln^{70} plus NS3-Tyr^{1082}/Gln^{1112}). By contrast, about a half of control isolates (52%; 46/89) had non-(Gln^{70} plus NS3-Tyr^{1082}/Gln^{1112}) (Fig. 2A). The difference in the proportion between HCC and control groups was statistically significant ($P < 0.0001$). Furthermore, the cumulative HCC incidence after 15-year follow-up was highest (63%) among patients with core-Gln^{70} plus NS3-(Tyr^{1082}/Gln^{1112}), whereas it was lowest (11%) among patients with non-(Gln^{70} plus NS3-Tyr^{1082}/Gln^{1112}) (Fig. 2B), with the difference being statistically significant ($P < 0.0001$; Log-rank test).

Evolution of the Sequences of the Core, NS3, and NS5A Proteins During the Follow-up Period From Chronic Hepatitis to HCC Development. Finally, we investigated sequence evolution of the core protein, NS3 and NS5A (IRRDR and ISDR) during the follow-up period from chronic hepatitis to HCC development by comparing the sequences between pre-HCC and

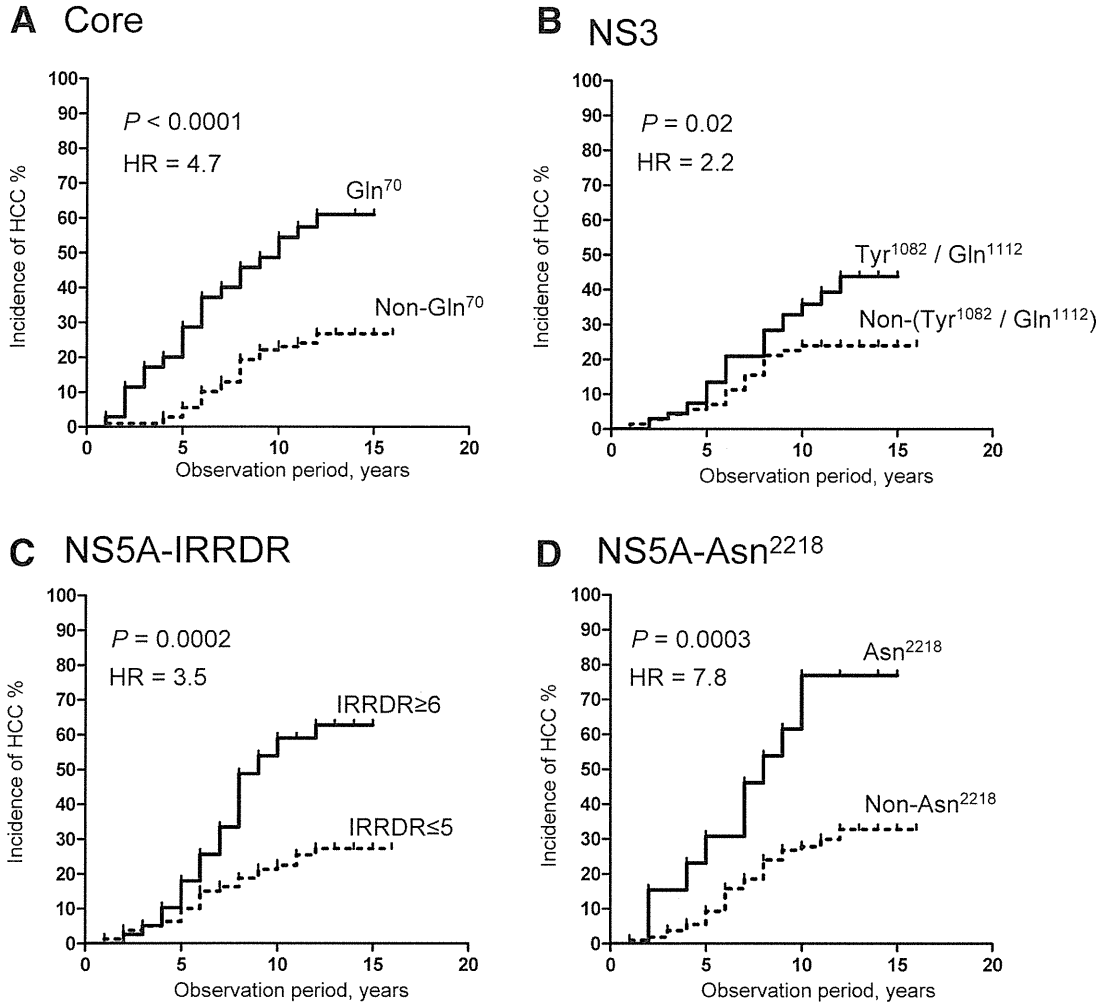

Fig. 1. Cumulative HCC incidence on the basis of HCV-1b sequence patterns. (A) Position 70 of the core protein. The numbers of core-Gln^{70} and non-Gln^{70} analyzed were 34 and 109, respectively. (B) Positions 1082 and 1112 of NS3. The numbers of NS3-(Tyr^{1082}/Gln^{1112}) and non-(Tyr^{1082}/Gln^{1112}) analyzed were 68 and 71, respectively. (C) NS5A-IRRDR. The numbers of NS5A-IRRDR≥6 and IRRDR≤5 analyzed were 39 and 80, respectively. (D) NS5A-Asn^{2218}. The numbers of NS5A-Asn^{2218} and non-Asn^{2218} analyzed were 14 and 105, respectively.

post-HCC isolates. The residue at position 70 of the core protein was conserved in 91% (41/45) of sequence pairs analyzed. The substitutions observed at this position were from Arg^{70} and His^{70} each to Gln^{70} in two cases and from Gln^{70} to Arg^{70} in the other two cases. The residues at positions 1082 and 1112 of NS3 were conserved in 95% (41/43) and 100% (43/43), respectively, of the sequence pairs analyzed.

Table 3. Univariate and Multivariate Regression Analyses to Identify Independent Factors Associated With HCC

	Univariate		Multivariate	
Variable	Odds Ratio (95% CI)	*P* Value	Odds Ratio (95% CI)	*P* Value
Core-Gln^{70}	0.23 (0.10 - 0.52)	0.0004	6.8 (2.1 - 23.0)	0.001
NS3-Tyr^{1082} / Gln^{1112}	2.4 (1.1 - 4.9)	0.029	3.4 (1.1 - 10.0)	0.03
NS5A-IRRDR≥6	4.5 (2.0 - 10.0)	0.0003		
NS5A-Asn^{2218}	7.7 (2.0 - 29.0)	0.002		
AFP (>20 ng/L)	12 (5.1 - 30.0)	0.0001	19.5 (4.7 - 80.0)	0.0001
ALT (>165 IU/L)	4.0 (1.8 - 8.6)	0.0006		
AST (>65 IU/L)	3.9 (1.5 - 10.0)	0.003		
Fibrosis staging score (≥3)	2.4 (1.1 - 4.9)	0.02		

Gln^{70}, glutamine at position 70 of the core protein; Tyr^{1082}, tyrosine at position 1082 of NS3; Gln^{1112}, glutamine at position 1112 of NS3; IRRDR, interferon/ribavirin resistance-determining region; Asn^{2218}, asparagine at position 2218 of NS5A-ISDR, ALT, alanine aminotransferase; AST, aspartate transaminase; AFP; α-fetoprotein; IFN; interferon.

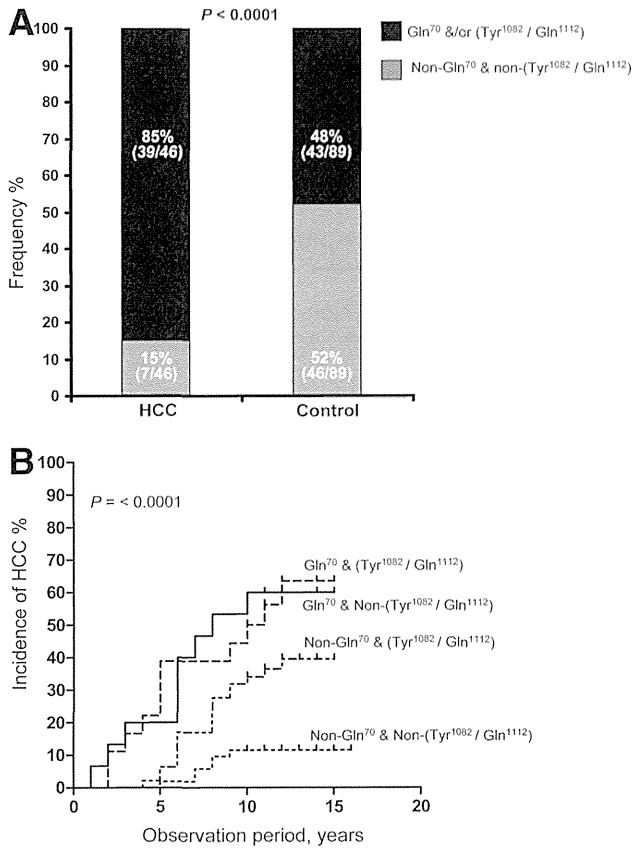

Fig. 2. (A) Proportions of HCV-1b isolates of the HCC high-risk group (core-Gln^{70} and/or NS3-[Tyr^{1082}/Gln^{1112}]) and the low-risk group (non-Gln^{70} and non-[Tyr^{1082}/Gln^{1112}]) among HCC and control groups. (B) Cumulative HCC incidence on the basis of different combined sequence patterns of position 70 of the core protein and positions 1082 and 1112 of NS3. Core-Gln^{70} and NS3-(Tyr^{1082}/Gln^{1112}), n = 18; core-Gln^{70} and non-(Tyr^{1082}/Gln^{1112}), n = 16; non-Gln^{70} and NS3-(Tyr^{1082}/Gln^{1112}), n = 48; non-Gln^{70}/non-(Tyr^{1082}/Gln^{1112}), n = 53.

IRRDR and ISDR showed a high degree of sequence evolution. IRRDR sequences were different between pre-HCC and post-HCC isolates in 66% (25/38) of cases analyzed (Fig. 3). IRRDR sequences tended to be more polymorphic at the time of HCC occurrence. Frequency of HCV isolates with IRRDR≥6 was significantly higher in post-HCC isolates than in pre-HCC isolates; IRRDR≥6 was found in 47% (18/38) of post-HCC isolates compared to 24% (9/38) of pre-HCC isolates ($P = 0.03$). On the other hand, ISDR≥3 was found in 21% (8/38) of post-HCC isolates compared to 11% (4/38) of pre-HCC isolates, with the difference between the two groups being not statistically significant ($P = 0.3$).

Discussion

HCC is one of the common long-term complications of HCV infection. However, whether HCV itself plays a direct role in the development of HCC and whether all HCV isolates are equally associated with HCC development remain to be determined. HCV core, NS3, and NS5A proteins have been reported to affect a wide variety of potentially oncogenic pathways in cell culture and experimental animal systems.[7] In the present study, we demonstrated that HCV isolates with core-Gln^{70}, NS3-Tyr^{1082}/Gln^{1112} or NS5A-IRRDR≥6 were closely associated with HCC development. In addition, a follow-up study revealed that sequence patterns at position 70 of the core protein and positions 1082 and 1112 of NS3 did not significantly alter during the progression from chronic hepatitis to HCC while NS5A-IRRDR showed a significantly higher degree of sequence heterogeneity in post-HCC than in pre-HCC isolates.

Correlation between polymorphisms at positions 70 and 91 of HCV-1b core protein and IFN-based treatment outcome was extensively studied, especially in a Japanese population.[17-20] Interestingly, the same mutations were also associated with progression to HCC in the Japanese population with HCV-1b infection.[13] Results obtained in the present study confirmed and emphasized the significant association between the mutation at position 70 (core-Gln^{70}), but not at position 91, and HCC development (Tables 2, 3; Fig. 1A). Despite the clinical evidence that strongly supports the correlation between core-Gln^{70} and HCC development, the molecular mechanism underlying this correlation is still obscure. Delhem et al.[36] found that tumor-derived HCV core proteins, but not nontumor-derived ones, interact with and activate double-stranded RNA-dependent protein kinase (protein kinase R or PKR), which might modulate viral persistence and carcinogenesis. Gln^{70} was found in two of the three tumor-derived sequences, whereas Arg^{70} was found in two of the three nontumor-derived ones.

As for the NS3 protein of HCV, the possible link between an N-terminal portion of NS3 encoding viral serine protease (aa 1027 to 1146) and hepatocarcinogenesis was reported.[21,22] However, information about the relationship between NS3 sequence diversity and HCC development is still limited. We previously reported a significant correlation between predicted secondary structure of an N-terminal portion of NS3 and HCC development.[34] In the present study, we demonstrated that HCV patients infected with HCV isolates with NS3-(Tyr^{1082}/Gln^{1112}) were at a higher risk to develop HCC than those infected with HCV isolates with non-Tyr^{1082}/Gln^{1112} (Tables 2, 3; Fig. 2B). Computer-assisted secondary structure analysis of NS3 revealed that Tyr^{1082} was associated with the

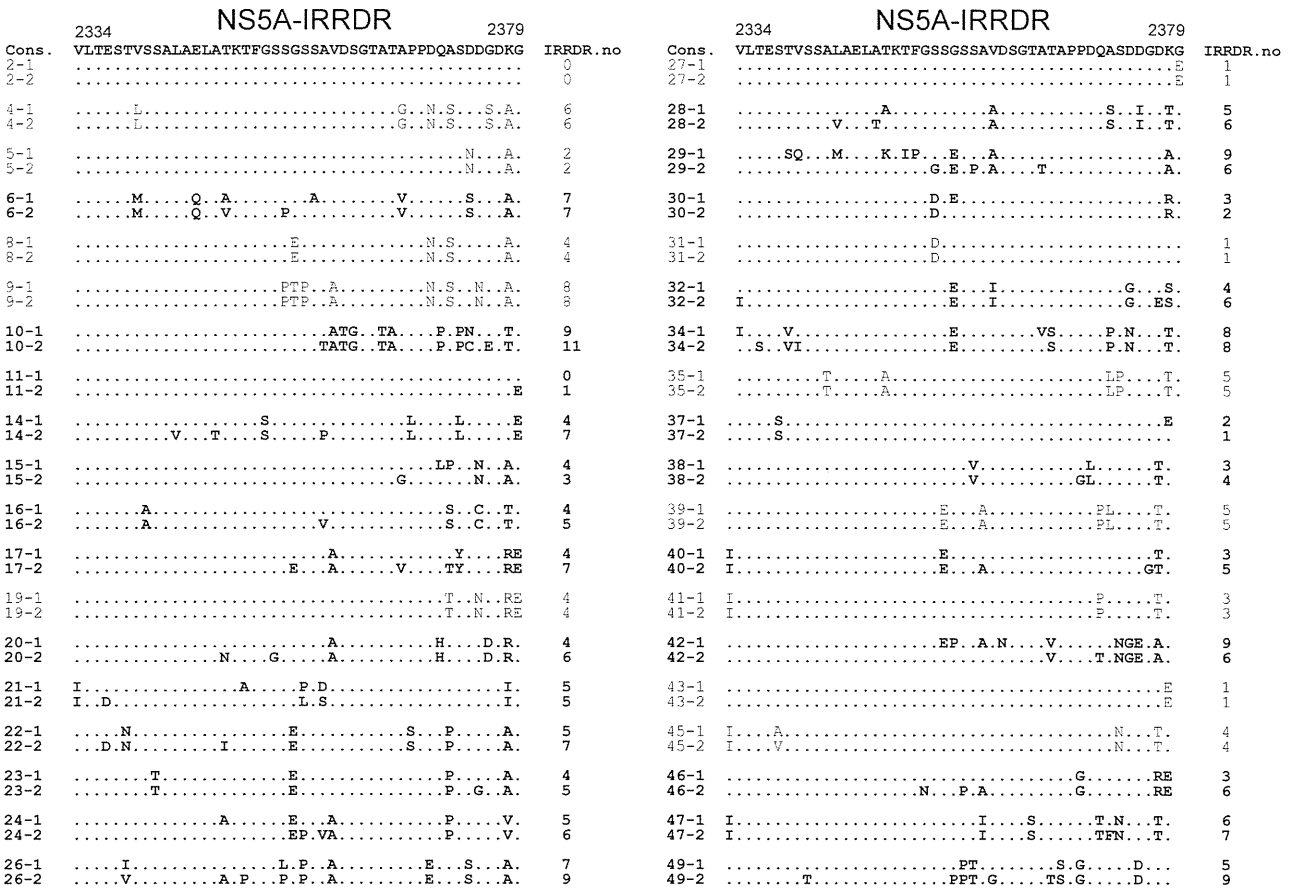

Fig. 3. Pairwise comparison of IRRDR sequences of HCV-1b during the follow-up period between chronic hepatitis and HCC development. Sequence pairs that differ between pre-HCC (numbered with -1) and post-HCC isolates (numbered with -2) are shown. The consensus sequence (Cons.) is shown at the top. The numbers along the sequence indicate the aa positions. Dots indicate residues identical to those of the Cons. sequence. The numbers of IRRDR mutations are shown on the right.

presence of a turn structure at around position 1083 while Phe[1082] was associated with the absence of the turn structure.[34] Notably, the catalytic triad of NS3 serine protease consists of His[1083], Asp[1107], and Ser[1165].[37] Since positions 1082 and 1112 are in close vicinity of the catalytic triad, sequences diversity at these positions might influence the serine protease activity and also pathogenicity of HCV. Large-scale, multicenter clinical studies as well as more detailed experimental studies at the molecular and cellular levels are needed to clarify the importance of sequence diversity at positions 1082 and 1112 of NS3 in HCV-mediated hepatocarcinogenesis.

HCV heterogeneity in NS5A-ISDR and NS5A-IRRDR are correlated with IFN-responsiveness.[17,18,25,26] As IFN-based therapy reduces the risk of HCC development,[4,28-30] we were interested to investigate whether there is a correlation between sequence heterogeneity in NS5A and development of HCC. Our present results revealed that a high degree of sequence heterogeneity in IRRDR (IRRDR≥6) was closely associated with HCC development (Table 2). We previously reported that IRRDR≥6 was significantly associated with good responses to PEG-IFN/RBV combination therapy.[26,27] These results collectively suggest that oncogenic properties and PEG-IFN/RBV responsiveness are independent viral characteristics and that PEG-IFN/RBV therapy helps eliminate oncogenic HCV isolates, thus reducing the risk of HCC development.

Position 2218 of NS5A, located within ISDR, appears to tolerate a wide range of aa substitutions as observed in different HCV-1b isolates.[25,38,39] Interestingly, Asn at position 2218 (Asn[2218]) was detected significantly more frequently in pre-HCC isolates than in the control isolates. Further studies are needed to determine the possible importance of this residue in hepatocarcinogenesis.

Another focus of attention is how the sequences of the core protein, NS3, and NS5A-IRRDR evolve during the interval between chronic hepatitis and HCC development. One of the significant advantages of the

present study was that we could conduct a longitudinal investigation by analyzing the target sequences of pre- and post-HCC isolates. We found that core-Gln^{70} and NS3-(Tyr^{1082}/Gln^{1112}) were well conserved in each paired sample. This indicates that core-Gln^{70} and NS3-(Tyr^{1082}/Gln^{1112}) were already present before the development of HCC. Non-Gln^{70} of the core protein and non-Tyr^{1082} and non-Gln^{1112} of NS3 were also well conserved in each paired sample. These results imply the possibility that these sequence patterns were not a result of HCC but, rather, they were a possible causative factor for the development of HCC. We hypothesize, therefore, that HCV isolates with core-Gln^{70} and/or NS3-(Tyr^{1082}/Gln^{1112}) are highly oncogenic, whereas those with non-(Gln^{70} plus NS3-Tyr^{1082}/Gln^{1112}) are less oncogenic. It is not clear yet as to whether these oncogenic mutations were present from the very beginning of HCV infection or if they emerged at a certain timepoint (before the initiation of follow-up) during the long-term persistence through an adaptive viral evolution in the host. More comprehensive follow-up study is needed to address this issue. In any case, the core-Gln^{70} and NS3-(Tyr^{1082}/Gln^{1112}) would be considered an index for prediction of HCC development. On the other hand, IRRDR in NS5A is more tolerant for sequence evolution. IRRDR in post-HCC isolates showed a significantly higher degree of sequence heterogeneity compared with that in pre-HCC isolates. This observation suggests that IRRDR is under strong selective pressure during the course of HCV infection and that the high degree of IRRDR heterogeneity (IRRDR$\geq$6) in HCV isolates from patients with HCC may not be a causative factor for development of HCC.

In conclusion, the present results suggest the possibility that patients infected with HCV isolates with core-Gln^{70} and/or NS3-(Tyr^{1082}/Gln^{1112}) are at a higher risk to develop HCC compared to those with non-(Gln^{70} plus NS3-Tyr^{1082}/Gln^{1112}).

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