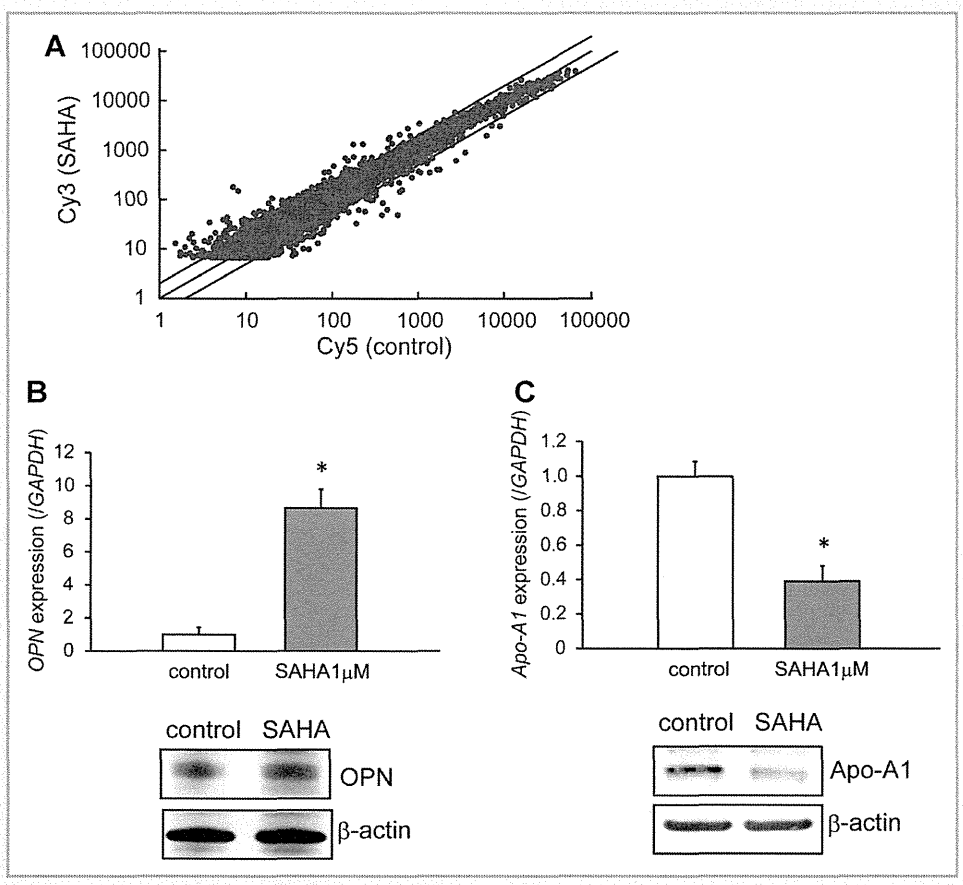

Fig. 4. A: Microarray analysis of OR6 cells after SAHA treatment Standardized data of Cy3 (SAHA)- and Cy5 (control)-stained RNAs are plotted. Global normalization was performed by adjusting to a median Cy3/Cy5 to 1. Lines outside the median line represent twofold difference. Values were normalized according to a median Cy3/Cy5 ratio of 1. B,C: Expression levels of *OPN* and *Apo-A1* in OR6 cells after SAHA treatment Expression levels of *OPN* and *Apo-A1* were examined by quantitative RT-PCR and Western blotting. Means and SDs of gene expression normalized with GAPDH for three experiments are shown. *OPN* expression was significantly up-regulated after treatment with 1 μM SAHA for 72 h compared to control (B: $^*P < 0.001$). *Apo-A1* expression was significantly down-regulated after treatment with 1 μM SAHA for 72 h compared to control (C: $^*P < 0.001$).

DISCUSSION

In the present study, we investigated whether the HDAC inhibitor SAHA affects HCV replication via epigenetic alterations in host cells. To this objective, we used OR6 cells, in which full-length HCV RNA replicates, were used as the replicon system.

The replicon cell culture system was first established by Lohmann et al. [1999] and this system finally developed to the infectious HCV particle producing cell system [Wakita et al., 2005]. In the replicon system, modified HCV genomes can replicate to high levels in human hepatocellular carcinoma cells (HuH-7) and many stably transfected replicon cell lines are established after continuous drug selection with

TABLE I. Up-Regulated Genes in SAHA-Treated OR6 Cells

Name	Description	SAHA/control	$\log_2$ (SAHA/control)	Up
ANXA1	Annexin A1	25.23	4.66	[a]
SPP1	Osteopontin	7.43	2.89	[b]
UBE2L6	Ubiquitin/ISG15-conjugating enzyme E2L6	5.80	2.54	[b]
IFIT1	Interferon-induced rotein with tetratricopeptide repeats 1	5.30	2.40	[b]
TUBA1A	Tubulin alpha-1A chain	4.86	2.28	[b]
KCNJ8	ATP-sensitive inward rectifier potassium channel 8	4.55	2.19	[b]
EFEMP1	EGF-containing fibulin-like extracellular matrix protein 1	4.43	2.12	[b]
KRT23	Keratin, type I cytoskeletal 23	4.07	2.03	[b]

OR6 cells were treated with 1 μM SAHA for 72 h and total RNA was analyzed by microarray (Human Oligo chip 25K). Genes with expression levels of more than 100 and more than a fourfold difference relative to control were selected.

[a]>eightfold.

[b]>fourfold.

TABLE II. Down-Regulated Genes in SAHA-Treated OR6 Cells

Name	Description	SAHA/control	$\log_2$ (SAHA/control)	Down
SERPINC1	Antithrombin-III	0.08	3.58	[a]
GJB1	Gap junction beta-1 protein	0.14	2.80	[b]
F12	Coagulation factor XII	0.17	2.54	[b]
SLCO183	Solute carrier organic anion transporter family member 183	0.22	2.19	[b]
C3	Complement C3	0.22	2.18	[b]
AKR1810	Aldo-keto reductase family 1 member 810	0.23	2.11	[b]
GAL	Galanin	0.24	2.07	[b]
FABP1	Fatty acid-binding protein, liver	0.24	2.06	[b]
APOA1	Apolipoprotein A1	0.25	2.00	[b]

OR6 cells were treated with 1 μM SAHA for 72 h and total RNA was analyzed by microarray (Human Oligo chip 25K). Genes with expression levels of more than 100 and less than 1/4 of control were selected.

[a]<1/16.

[b]<1/4.

neomycin (G418). This cell culture system has been widely used as a tool in the study of HCV virology and drug development [Horscroft et al., 2005]. Development of OR6 cell was originally reported by Ikeda et al. [2005]. The *Renilla* luciferase reporter gene and neomycin resistant gene were introduced in the 5′ untranslated region of genome-length HCV RNA (genotype 1b). This construct containing full-length HCV RNA robustly replicated in the HuH-7 cells after the electroporation and one of the colonies designated OR6 was selected by G418.

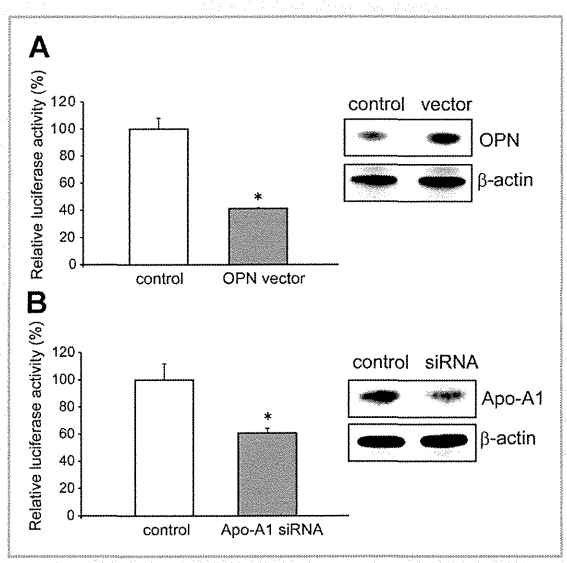

Fig. 5. Reduced HCV replication in OR6 cells after gene transfer. A: HCV replication in OR6 cells after overexpression of the *OPN* gene HCV replication was evaluated by luciferase activity in OR6 cells after transfection with the *OPN* expression vector. Means and SDs of luciferase activities for three experiments are shown. The luciferase activity in OR6 cells after overexpression of the *OPN* gene was significantly reduced. *$P < 0.01$. B: HCV replication in OR6 cells after knockdown of the *Apo-A1* gene HCV replication was evaluated by luciferase activity in OR6 cells after transfection with siRNA for the *Apo-A1* gene. Means and SDs of luciferase activities for three experiments are shown. The luciferase activity in OR6 cells after knockdown of the *Apo-A1* gene was significantly reduced. *$P < 0.05$.

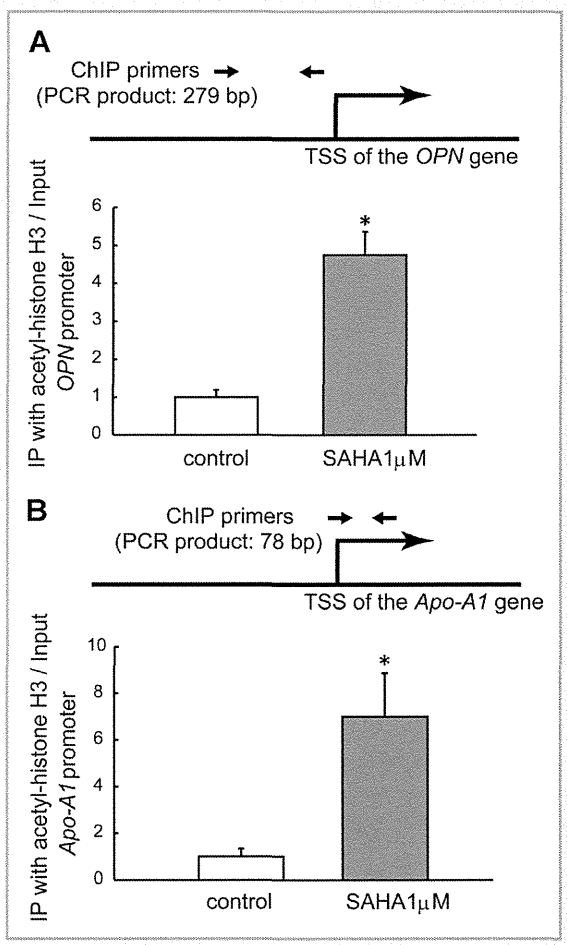

Fig. 6. ChIP assay at the promoter regions of *OPN* and *Apo-A1* in OR6 cells after SAHA treatment levels of acetylated histone H3 around the promoter regions of *OPN* (A) and *Apo-A1* (B) after SAHA treatment were analyzed by ChIP assay. The ChIP primers are located near the TSSs of these genes and should be suitable for histone acetylation assay. The ChIP-PCR products for the *OPN* gene and the *Apo-A1* gene are 279 and 78 bp, respectively. The fraction of immunoprecipitated DNA was calculated as: immunoprecipitated DNA (IP) with anti-acetyl-histone H3 antibody/input DNA. Means and SDs of acetylated histone H3 levels for three experiments are shown. The acetylated histone H3 levels around the promoter regions of *OPN* and *Apo-A1* were significantly increased after SAHA treatment. *$P < 0.001$.

The OR6 cell system facilitates monitoring of HCV RNA replication. However, if cell viability is decreased as a result of drug toxicity, luciferase activity cannot be evaluated accurately. Therefore, we first examined the cellular toxicity of SAHA in OR6 cells and confirmed that SAHA treatment was non-toxic at concentrations below 1 μM as determined by cell counting, viability assay, and MTT assay. Lu et al. have reported the inhibitory effect of HDAC inhibitors including SAHA on hepatocellular carcinoma (HCC). They treated several HCC cell lines with SAHA at various concentrations and show similar results to our results [Lu et al., 2007]. In contrast, the HCV replication rate evaluated by luciferase activity was significantly reduced by treatment with SAHA at concentration below 1 μM in a dose-dependent manner. We also confirmed that another HDAC inhibitor TSA suppresses HCV replication (Fig. 2B). These results indicate that the HDAC inhibitors SAHA and TSA reduced HCV replication without cellular toxicity in OR6 cells.

We next investigated the molecular mechanisms underlying the suppressive effect of SAHA on the HCV replication rate. *miR-122* is a liver-specific miRNA and plays an important role in fatty-acid metabolism in the liver [Esau et al., 2006]. Recent studies have reported that *miR-122* is down-regulated in HCC and has multiple functions as a tumor suppressor during hepatocarcinogenesis [Coulouarn et al., 2009; Fornari et al., 2009]. Moreover, *miR-122* directly binds to the 5′ noncoding region of the HCV genome and modulates HCV RNA replication [Jopling et al., 2005]. It is noteworthy that *miR-122* facilitates HCV replication via binding to the 5′ noncoding region of the viral genome, whereas miRNAs generally function to repress expression of their target genes by binding to 3′ noncoding regions. Epigenetic alterations such as DNA methylation and histone modification are important mechanisms for the regulation of miRNA expression [Saito et al., 2006, 2011]. In this study, we investigated the effect of treatment with HDAC inhibitors on *miR-122* expression in OR6 cells and found that there was no significant difference in *miR-122* expression after treatment with 1 μM SAHA or 0.1 μM TSA, suggesting that *miR-122* is not involved in the suppressive effect of HDAC inhibitors on HCV replication. These findings led us to hypothesize that histone acetylation induced by SAHA treatment does not modulate *miR-122* expression and that HCV replication was inhibited through other pathways.

Microarray analysis showed differential expression of various genes in OR6 cells induced by SAHA treatment for 72 h. Notably, several cytoskeletal genes such as *tubulin alpha-1A chain*, *extracellular matrix protein 1*, and *keratin* were significantly up-regulated by SAHA treatment. We have reported that a close correlation might exist between malignant transformation of HCC and cytoskeletal changes [Kanamori et al., 2011]. The results of this study are similar to those of our previous studies showing that sodium butyrate, another HDAC inhibitor, changed the mobility, fluidity, and adhesive properties of cancer cells into less a malignant phenotype [Saito et al., 1998; Masuda et al., 2000; Wakabayashi et al., 2000; Nakamura et al., 2001; Kaneko et al., 2004]. Another interesting result of the microarray was the up-regulation of interferon-induced protein by SAHA treatment. This finding is also consistent with our previous study, in which sodium butyrate induced interferon-related gene expression [Wakabayashi et al., 2005], suggesting that there may be crosstalk between the interferon-inducing pathway and epigenetic changes by HDAC inhibitors. Up-regulation of interferon-induced protein by HDAC inhibitors may result in suppression of HCV replication.

Besides cytoskeletal genes and interferon-induced proteins, we focused our present study on *OPN* and *Apo-A1*. The *OPN* gene is a key regulator of the Th1-type immune system and Th1-type immunity plays an important role in viral elimination [Patarca et al., 1993; Ashkar et al., 2000]. Recent studies showed that the promoter activity of *OPN* was correlated with the efficacy of interferon-based therapy [Mochida et al., 2004; Naito et al., 2005]. These findings suggest that increased expression of *OPN* can eliminate HCV by activating the Th1-type immune system. Among the genes that were down-regulated by SAHA treatment, we focused on the *Apo-A1* gene because this protein is essential for HCV replication [Mancone et al., 2011]. The *Apo-A1* gene is a protein component of high-density lipoprotein particles and is thought to be necessary for HCV particle formation and maintenance of infectivity. To confirm the results of the microarray analysis, we examined expression of *OPN* and *Apo-A1* in OR6 cells after SAHA treatment by quantitative RT-PCR. We found a significant increase of *OPN* expression and significant reduction of *Apo-A1* expression, which may lead to suppression of HCV replication. Moreover, we overexpressed *OPN* and knocked down *Apo-A1* in OR6 cells using an expression vector and siRNA procedure, respectively. As shown in Figure 5A,B, the luciferase activities were significantly reduced after overexpression of *OPN* and knockdown of *Apo-A1* in OR6 cells. These results indicate that overexpression of *OPN* and reduced expression of *Apo-A1* play critical roles in SAHA-induced suppression of HCV replication.

Since histone acetylation generally activates gene expression, we presumed that up-regulation of *OPN* is mediated by histone H3 acetylation around its promoter region. The ChIP assay results demonstrated that histone H3 acetylation levels of the promoter regions of *OPN* and *Apo-A1* were remarkably increased in SAHA-treated OR6 cells compared to control. Acetylation of lysine residues on histones H3 is correlated with active or open chromatin, which allows various transcription factors access to the promoters of target genes [Yoo and Jones, 2006]. This suggests that up-regulation of *OPN* is induced by histone H3 acetylation through SAHA-mediated epigenetic mechanisms. On the other hand, *Apo-A1* transcription was significantly down-regulated by SAHA treatment in OR6 cells despite acetylation of its promoter region. Although up-regulation of *OPN* expression by SAHA treatment is reasonable, we could not prove the molecular mechanism underlying down-regulation of *Apo-A1* by SAHA treatment at this time. Studies have demonstrated that HDAC inhibitors suppress expression of specific genes such as *adiponectin* and *bcl-2* [Duan et al., 2005; Qiao et al., 2006]. Further studies regarding *Apo-A1* inactivation induced by HDAC inhibitors are necessary.

To our knowledge, this study is the first report showing that the HDAC inhibitor SAHA significantly inhibits HCV replication. We presume that this inhibitory effect on HCV replication may be mediated by up-regulation of *OPN* via SAHA-mediated histone modification in host cells, which is expected to inhibit HCV replication. Another significant benefit of SAHA treatment on HCV replication may be down-regulation of *Apo-A1*, which is essential for the HCV life cycle. These findings suggest that epigenetic therapy

with HDAC inhibitors represents a novel potential treatment strategy for diseases associated with HCV infection such as chronic hepatitis, liver cirrhosis, and HCC.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid for Young Scientists A (23680090 to Y.S.) and Grant-in-Aid for Scientific Research C (#24590993 to H.S.) from the Japan Society for the Promotion of Science (JSPS), the Keio Gijuku Academic Development Fund (to Y.S.), Takeda Science Foundation (to Y.S.), and Inaida Foundation (H.S.).

REFERENCES

Ashkar S, Weber GF, Panoutsakopoulou V, Sanchirico ME, Jansson M, Zawaideh S, Rittling SR, Denhardt DT, Glimcher MJ, Cantor H. 2000. Eta-1 (osteopontin): An early component of type-1 (cell-mediated) immunity. Science 287:860–864.

Butler LM, Zhou X, Xu WS, Scher HI, Rifkind RA, Marks PA, Richon VM. 2002. The histone deacetylase inhibitor SAHA arrests cancer cell growth, up-regulates thioredoxin-binding protein-2, and down-regulates thioredoxin. Proc Natl Acad Sci USA 99:11700–11705.

Chen YC, Su WC, Huang JY, Chao TC, Jeng KS, Machida K, Lai MM. 2010. Polo-like kinase 1 is involved in hepatitis C virus replication by hyper-phosphorylating NS5A. J Virol 84:7983–7993.

Coulouarn C, Factor VM, Andersen JB, Durkin ME, Thorgeirsson SS. 2009. Loss of miR-122 expression in liver cancer correlates with suppression of the hepatic phenotype and gain of metastatic properties. Oncogene 28:3526–3536.

Duan H, Heckman CA, Boxer LM. 2005. Histone deacetylase inhibitors down-regulate bcl-2 expression and induce apoptosis in t(14;18) lymphomas. Mol Cell Biol 25:1608–1619.

Ebinuma H, Saito H, Tada S, Nakamoto N, Ohishi T, Tsunematsu S, Kumagai N, Tsuchimoto K, Tsukada N, Inagaki Y, Horie Y, Takahashi M, Atsukawa K, Okamura Y, Kanai T, Hibi T. 2012. Disadvantages of peginterferon and ribavirin treatment in older patients with chronic hepatitis C: An analysis using the propensity score. Hepatol Int 6:744–752.

Esau C, Davis S, Murray SF, Yu XX, Pandey SK, Pear M, Watts L, Booten SL, Graham M, McKay R, Subramaniam A, Propp S, Lollo BA, Freier S, Bennett CF, Bhanot S, Monia BP. 2006. miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. Cell Metab 3:87–98.

Fornari F, Gramantieri L, Giovannini C, Veronese A, Ferracin M, Sabbioni S, Calin GA, Grazi GL, Croce CM, Tavolari S, Chieco P, Negrini M, Bolondi L. 2009. MiR-122/cyclin G1 interaction modulates p53 activity and affects doxorubicin sensitivity of human hepatocarcinoma cells. Cancer Res 69:5761–5767.

Gal-Yam EN, Saito Y, Egger G, Jones PA. 2008. Cancer epigenetics: Modifications, screening, and therapy. Annu Rev Med 59:267–280.

Gao L, Aizaki H, He JW, Lai MM. 2004. Interactions between viral nonstructural proteins and host protein hVAP-33 mediate the formation of hepatitis C virus RNA replication complex on lipid raft. J Virol 78:3480–3488.

Haas MJ, Alamir AR, Sultan S, Chehade JM, Wong NC, Mooradian AD. 2011. Nicotinic acid induces apolipoprotein A-I gene expression in HepG2 and Caco-2 cell lines. Metabolism 60:1790–1796.

Hahnel A, Wichmann H, Kappler M, Kotzsch M, Vordermark D, Taubert H, Bache M. 2010. Effects of osteopontin inhibition on radiosensitivity of MDA-MB-231 breast cancer cells. Radiat Oncol 5:82.

Hamamoto I, Nishimura Y, Okamoto T, Aizaki H, Liu M, Mori Y, Abe T, Suzuki T, Lai MM, Miyamura T, Moriishi K, Matsuura Y. 2005. Human VAP-B is involved in hepatitis C virus replication through interaction with NS5A and NS5B. J Virol 79:13473–13482.

Horscroft N, Lai VC, Cheney W, Yao N, Wu JZ, Hong Z, Zhong W. 2005. Replicon cell culture system as a valuable tool in antiviral drug discovery against hepatitis C virus. Antivir Chem Chemother 16:1–12.

Ikeda M, Abe K, Dansako H, Nakamura T, Naka K, Kato N. 2005. Efficient replication of a full-length hepatitis C virus genome, strain O, in cell culture, and development of a luciferase reporter system. Biochem Biophys Res Commun 329:1350–1359.

Ikeda M, Abe K, Yamada M, Dansako H, Naka K, Kato N. 2006. Different anti-HCV profiles of statins and their potential for combination therapy with interferon. Hepatology 44:117–125.

Jopling CL, Yi M, Lancaster AM, Lemon SM, Sarnow P. 2005. Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. Science 309:1577–1581.

Kanamori H, Kawakami T, Effendi K, Yamazaki K, Mori T, Ebinuma H, Masugi Y, Du W, Nagasaka K, Ogiwara A, Kyono Y, Tanabe M, Saito H, Hibi T, Sakamoto M. 2011. Identification by differential tissue proteome analysis of talin-1 as a novel molecular marker of progression of hepatocellular carcinoma. Oncology 80:406–415.

Kaneko F, Saito H, Saito Y, Wakabayashi K, Nakamoto N, Tada S, Suzuki H, Tsunematsu S, Kumagai N, Ishii H. 2004. Down-regulation of matrix-invasive potential of human liver cancer cells by type I interferon and a histone deacetylase inhibitor sodium butyrate. Int J Oncol 24:837–845.

Kaul A, Stauffer S, Berger C, Pertel T, Schmitt J, Kallis S, Zayas M, Lohmann V, Luban J, Bartenschlager R. 2009. Essential role of cyclophilin A for hepatitis C virus replication and virus production and possible link to polyprotein cleavage kinetics. PLoS Pathog 5:e1000546.

Kurita S, Higuchi H, Saito Y, Nakamoto N, Takaishi H, Tada S, Saito H, Gores GJ, Hibi T. 2010. DNMT1 and DNMT3b silencing sensitizes human hepatoma cells to TRAIL-mediated apoptosis via up-regulation of TRAIL-R2/DR5 and caspase-8. Cancer Sci 101:1431–1439.

Lauer GM, Walker BD. 2001. Hepatitis C virus infection. N Engl J Med 345: 41–52.

Liang G, Lin JC, Wei V, Yoo C, Cheng JC, Nguyen CT, Weisenberger DJ, Egger G, Takai D, Gonzales FA, Jones PA. 2004. Distinct localization of histone H3 acetylation and H3-K4 methylation to the transcription start sites in the human genome. Proc Natl Acad Sci USA 101:7357–7362.

Lohmann V, Korner F, Koch J, Herian U, Theilmann L, Bartenschlager R. 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 285:110–113.

Lu YS, Kashida Y, Kulp SK, Wang YC, Wang D, Hung JH, Tang M, Lin ZZ, Chen TJ, Cheng AL, Chen CS. 2007. Efficacy of a novel histone deacetylase inhibitor in murine models of hepatocellular carcinoma. Hepatology 46:1119–1130.

Mancone C, Steindler C, Santangelo L, Simonte G, Vlassi C, Longo MA, D'Offizi G, Di Giacomo C, Pucillo LP, Amicone L, Tripodi M, Alonzi T. 2011. Hepatitis C virus production requires apolipoprotein A-I and affects its association with nascent low-density lipoproteins. Gut 60:378–386.

Masuda T, Saito H, Kaneko F, Atsukawa K, Morita M, Inagaki H, Kumagai N, Tsuchimoto K, Ishii AH. 2000. Up-regulation of E-cadherin and I-catenin in human hepatocellular carcinoma cell lines by sodium butyrate and interferon-alpha. In Vitro Cell Dev Biol Anim 36:387–394.

Mishiro T, Ishihara K, Hino S, Tsutsumi S, Aburatani H, Shirahige K, Kinoshita Y, Nakao M. 2009. Architectural roles of multiple chromatin insulators at the human apolipoprotein gene cluster. EMBO J 28:1234–1245.

Mochida S, Hashimoto M, Matsui A, Naito M, Inao M, Nagoshi S, Nagano M, Egashira T, Mishiro S, Fujiwara K. 2004. Genetic polymorphims in promoter region of osteopontin gene may be a marker reflecting hepatitis activity in chronic hepatitis C patients. Biochem Biophys Res Commun 313:1079–1085.

Naito M, Matsui A, Inao M, Nagoshi S, Nagano M, Ito N, Egashira T, Hashimoto M, Mishiro S, Mochida S, Fujiwara K. 2005. SNPs in the promoter region of the osteopontin gene as a marker predicting the efficacy of interferon-based therapies in patients with chronic hepatitis C. J Gastroenterol 40:381–388.

Nakamura M, Saito H, Ebinuma H, Wakabayashi K, Saito Y, Takagi T, Nakamoto N, Ishii H. 2001. Reduction of telomerase activity in human liver cancer cells by a histone deacetylase inhibitor. J Cell Physiol 187:392–401.

Nakamura M, Saito H, Ikeda M, Tada S, Kumagai N, Kato N, Shimotohno K, Hibi T. 2008. Possible molecular mechanism of the relationship between NS5B polymorphisms and early clearance of hepatitis C virus during interferon plus ribavirin treatment. J Med Virol 80:632–639.

Okamoto T, Nishimura Y, Ichimura T, Suzuki K, Miyamura T, Suzuki T, Moriishi K, Matsuura Y. 2006. Hepatitis C virus RNA replication is regulated by FKBP8 and Hsp90. EMBO J 25:5015–5025.

Patarca R, Saavedra RA, Cantor H. 1993. Molecular and cellular basis of genetic resistance to bacterial infection: the role of the early T-lymphocyte activation-1/osteopontin gene. Crit Rev Immunol 13:225–246.

Qiao L, Schaack J, Shao J. 2006. Suppression of adiponectin gene expression by histone deacetylase inhibitor valproic acid. Endocrinology 147:865–874.

Saito H, Ebinuma H, Takahashi M, Kaneko F, Wakabayashi K, Nakamura M, Ishii H. 1998. Loss of butyrate-induced apoptosis in human hepatoma cell lines HCC-M and HCC-T having substantial Bcl-2 expression. Hepatology 27:1233–1240.

Saito Y, Liang G, Egger G, Friedman JM, Chuang JC, Coetzee GA, Jones PA. 2006. Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells. Cancer Cell 9:435–443.

Saito Y, Suzuki H, Matsuura M, Sato A, Kasai Y, Yamada K, Saito H, Hibi T. 2011. MicroRNAs in hepatobiliary and pancreatic cancers. Front Genet 2:66.

Sarasin-Filipowicz M, Krol J, Markiewicz I, Heim MH, Filipowicz W. 2009. Decreased levels of microRNA miR-122 in individuals with hepatitis C responding poorly to interferon therapy. Nat Med 15:31–33.

Schickel R, Boyerinas B, Park SM, Peter ME. 2008. MicroRNAs: Key players in the immune system, differentiation, tumorigenesis and cell death. Oncogene 27:5959–5974.

Takayama T, Ebinuma H, Tada S, Yamagishi Y, Wakabayashi K, Ojiro K, Kanai T, Saito H, Hibi T. 2011. Prediction of effect of pegylated interferon alpha-2b plus ribavirin combination therapy in patients with chronic hepatitis C infection. PLoS ONE 6:e27223.

Tu H, Gao L, Shi ST, Taylor DR, Yang T, Mircheff AK, Wen Y, Gorbalenya AE, Hwang SB, Lai MM. 1999. Hepatitis C virus RNA polymerase and NS5A complex with a SNARE-like protein. Virology 263:30–41.

Vaillancourt FH, Brault M, Pilote L, Uyttersprot N, Gaillard ET, Stoltz JH, Knight BL, Pantages L, McFarland M, Breitfelder S, Chiu TT, Mahrouche L, Faucher AM, Cartier M, Cordingley MG, Bethell RC, Jiang H, White PW, Kukolj G. 2012. Evaluation of phosphatidylinositol-4-kinase IIIalpha as a hepatitis C virus drug target. J Virol 86:11595–11607.

Wakabayashi K, Saito H, Ebinuma H, Saito Y, Takagi T, Nakamura M, Umezawa A, Hata J, Ishii H. 2000. Bcl-2 related proteins are dramatically induced at the early stage of differentiation in human liver cancer cells by a histone deacetylase inhibitor projecting an anti-apoptotic role during this period. Oncol Rep 7:285–288.

Wakabayashi K, Saito H, Kaneko F, Nakamoto N, Tada S, Hibi T. 2005. Gene expression associated with the decrease in malignant phenotype of human liver cancer cells following stimulation with a histone deacetylase inhibitor. Int J Oncol 26:233–239.

Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, Murthy K, Habermann A, Krausslich HG, Mizokami M, Bartenschlager R, Liang TJ. 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 11:791–796.

Watanabe T, Kato H, Kobayashi Y, Yamasaki S, Morita-Hoshi Y, Yokoyama H, Morishima Y, Ricker JL, Otsuki T, Miyagi-Maesima A, Matsuno Y, Tobinai K. 2010. Potential efficacy of the oral histone deacetylase inhibitor vorinostat in a phase I trial in follicular and mantle cell lymphoma. Cancer Sci 101:196–200.

Yoo CB, Jones PA. 2006. Epigenetic therapy of cancer: Past, present and future. Nat Rev Drug Discov 5:37–50.

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

Ahmed El-Shamy,[1,2]* Michiko Shindo,[3]** Ikuo Shoji,[1] Lin Deng,[1] Tadao Okuno,[3] and Hak Hotta[1]

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;00:000–000)

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 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]

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

From the [1]Division of Microbiology, Kobe University Graduate School of Medicine, Kobe, Japan; [2]Department of Virology, Suez Canal University Faculty of Veterinary Medicine, Ismalia, Egypt; and [3]Department of Gastroenterology, Akashi City Hospital, Akashi, Japan.

Received September 3, 2012; accepted December 9, 2012.

Supported in part by Health and Labour Sciences Research Grants from the Ministry of Health, Labour and Welfare, Japan, and a SATREPS Grant from Japan Science and Technology Agency (JST) and Japan International Cooperation Agency (JICA). This study was also carried out as part of Japan Initiative for Global Research Network on Infectious Diseases (J-GRID), Ministry of Education, Culture, Sports, Science and Technology, Japan, and the Global Center of Excellence (G-COE) Program at Kobe University Graduate School of Medicine.

**These authors contributed equally to this work.*

Current address for Ahmed El-Shamy: Division of Liver Diseases, Mount Sinai School of Medicine, New York, New York, USA.

Current address for Michiko Shindo and Tadao Okuno: Okuno Gastroenterology Clinic, Akashi, Hyogo, Japan.

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 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

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

View this article online at wileyonlinelibrary.com.
DOI 10.1002/hep.26205
Potential conflict of interest: Nothing to report.

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) 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

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

HCV Protein	Factor	No. of Subjects / No. of Total*		P Value
		HCC	Control	
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.

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 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

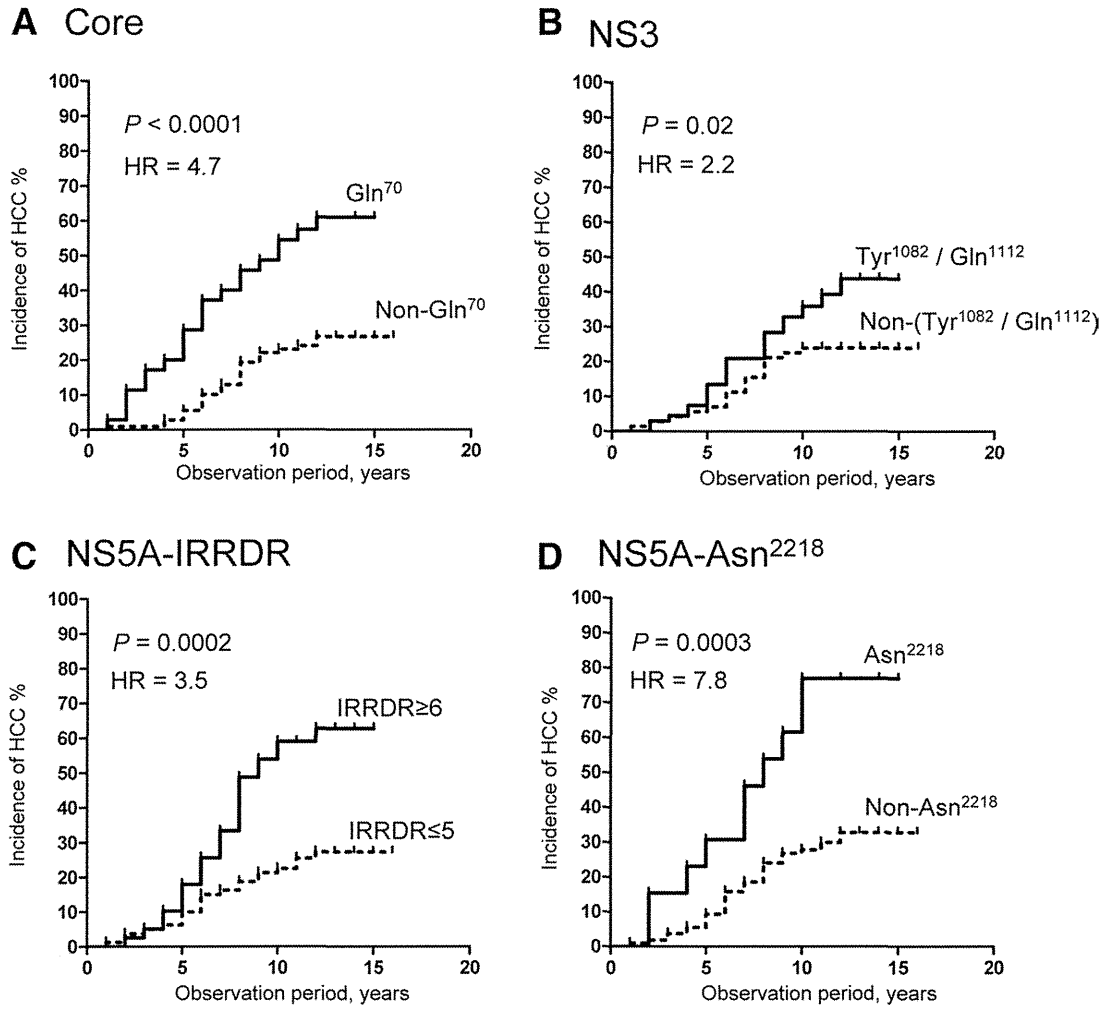

Fig. 1. Cumulative HCC incidence on the basis of HCV-1b sequence patterns. (A) Position 70 of the core protein. The numbers of core-Gln^{70} and non-Gln^{70} analyzed were 34 and 109, respectively. (B) Positions 1082 and 1112 of NS3. The numbers of NS3-(Tyr^{1082}/Gln^{1112}) and non-(Tyr^{1082}/Gln^{1112}) 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.

conserved in 95% (41/43) and 100% (43/43), respectively, of the sequence pairs analyzed.

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

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.

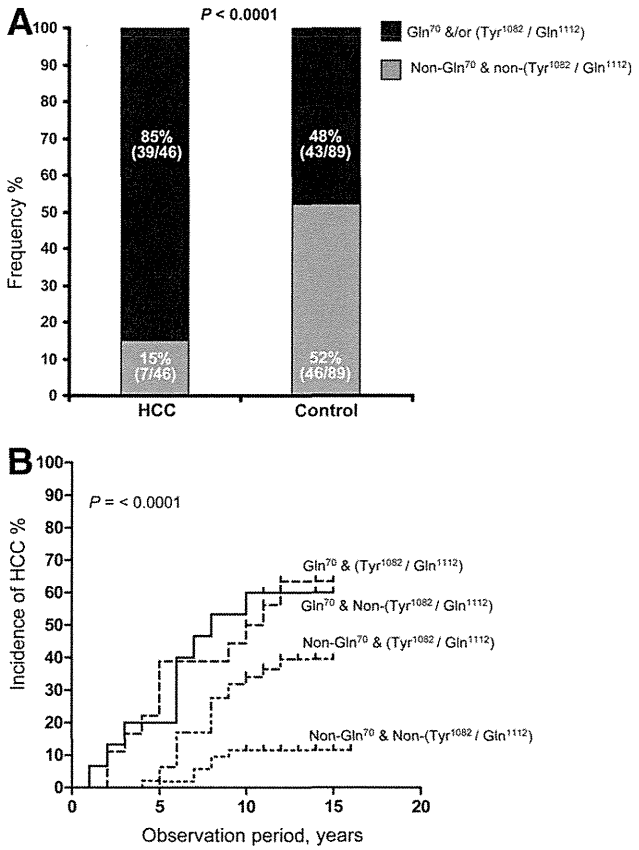

Fig. 2. (A) Proportions of HCV-1b isolates of the HCC high-risk group (core-Gln^{70} and/or NS3-[Tyr^{1082}/Gln^{1112}]) and the low-risk group (non-Gln^{70} and non-[Tyr^{1082}/Gln^{1112}]) among HCC and control groups. (B) Cumulative HCC incidence on the basis of different combined sequence patterns 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≥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 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

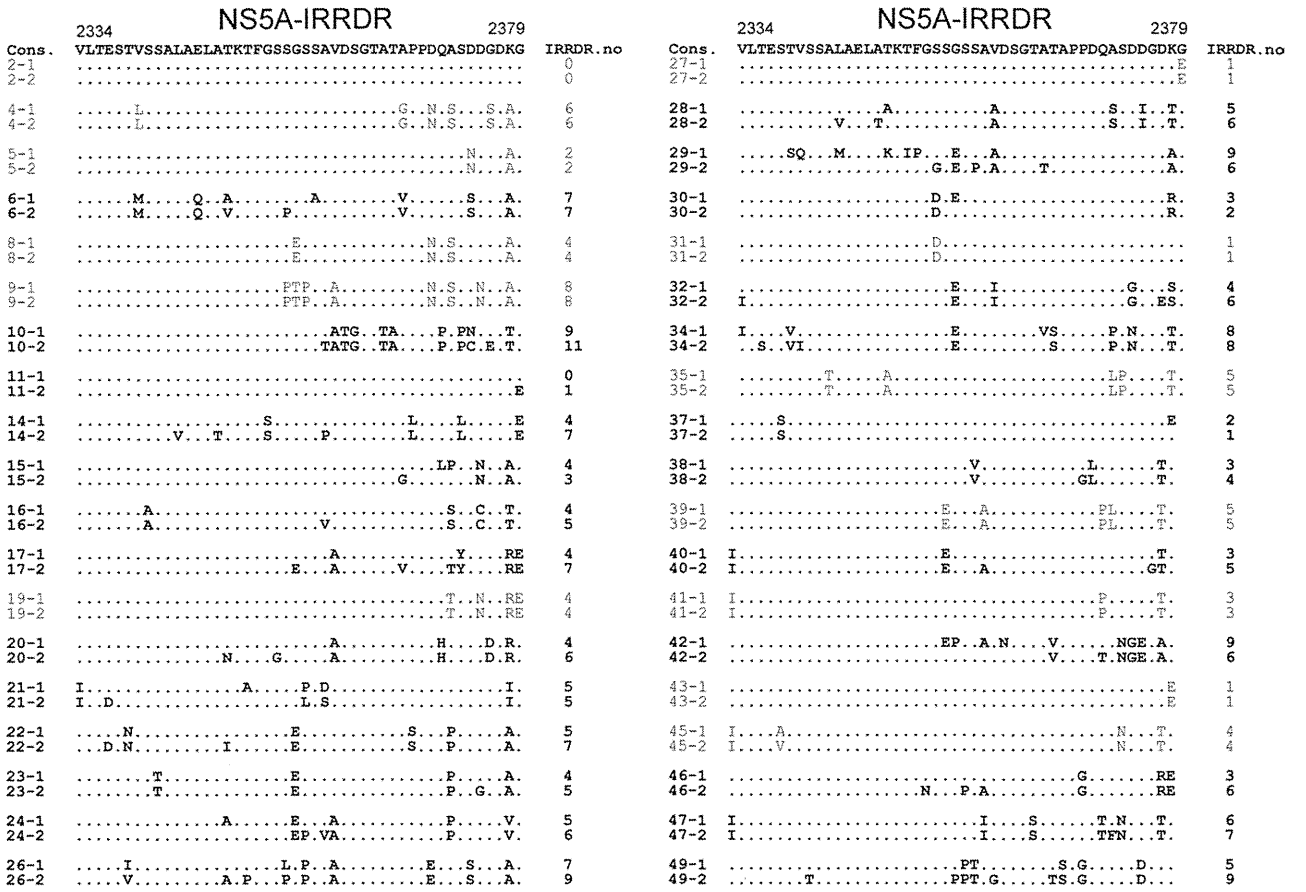

Fig. 3. Pairwise comparison of IRRDR sequences of HCV-1b during the follow-up period between chronic hepatitis and HCC development. Sequence pairs that differ between pre-HCC (numbered with -1) and post-HCC isolates (numbered with -2) are shown. The consensus sequence (Cons.) is shown at the top. The 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.

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$\geq$6) was closely associated with HCC development (Table 2). We previously reported that IRRDR$\geq$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}).

References

1. Lauer GM, Walker BD. Hepatitis C virus infection. N Engl J Med 2001;345:41-52.
2. Niederau C, Lange S, Heintges T, Erhardt A, Buschkamp M, Hurter D, et al. Prognosis of chronic hepatitis C: results of a large, prospective cohort study. HEPATOLOGY 1998;28:1687-1695.
3. Ikeda K, Saitoh S, Suzuki Y, Kobayashi M, Tsubota A, Koida I, et al. Disease progression and hepatocellular carcinogenesis in patients with chronic viral hepatitis: a prospective observation of 2215 patients. J Hepatol 1998;28:930-938.
4. Yoshida H, Shiratori Y, Moriyama M, Arakawa Y, Ide T, Sata M, et al. Interferon therapy reduces the risk for hepatocellular carcinoma: national surveillance program of cirrhotic and noncirrhotic patients with chronic hepatitis C in Japan. IHIT Study Group. Inhibition of hepatocarcinogenesis by interferon therapy. Ann Intern Med 1999;131: 174-181.
5. Lok AS, Seeff LB, Morgan TR, di Bisceglie AM, Sterling RK, Curto TM, et al. Incidence of hepatocellular carcinoma and associated risk factors in hepatitis C-related advanced liver disease. Gastroenterology 2009;136:138-148.
6. Miki D, Ochi H, Hayes CN, Abe H, Yoshima T, Aikata H, et al. Variation in the DEPDC5 locus is associated with progression to hepatocellular carcinoma in chronic hepatitis C virus carriers. Nat Genet 2011; 43:797-800.
7. Banerjee A, Ray RB, Ray R. Oncogenic potential of hepatitis C virus proteins. Viruses 2010;2:2108-2133.
8. Marusawa H, Hijikata M, Chiba T, Shimotohno K. Hepatitis C virus core protein inhibits Fas- and tumor necrosis factor alpha-mediated apoptosis via NF-κB activation. J Virol 1999;73:4713-4720.
9. Ray RB, Meyer K, Ray R. Suppression of apoptotic cell death by hepatitis C virus core protein. Virology 1996;226:176-182.
10. Chang J, Yang SH, Cho YG, Hwang SB, Hahn YS, Sung YC. Hepatitis C virus core from two different genotypes has an oncogenic potential but is not sufficient for transforming primary rat embryo fibroblasts in cooperation with the H-*ras* oncogene. J Virol 1998;72: 3060-3065.
11. Ray RB, Lagging LM, Meyer K, Ray R. Hepatitis C virus core protein cooperates with ras and transforms primary rat embryo fibroblasts to tumorigenic phenotype. J Virol 1996;70:4438-4443.
12. Moriya K, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, Ishibashi K, et al. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. Nat Med 1998;4:1065-1067.
13. Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, et al. Amino acid substitutions in the hepatitis C virus core region are the important predictor of hepatocarcinogenesis. HEPATOLOGY 2007;46: 1357-1364.
14. Akuta N, Suzuki F, Hirakawa M, Kawamura Y, Sezaki H, Suzuki Y, et al. Amino acid substitutions in hepatitis C virus core region predict hepatocarcinogenesis following eradication of HCV RNA by antiviral therapy. J Med Virol 2011;83:1016-1022.
15. Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, et al. Substitution of amino acid 70 in the hepatitis C virus core region of genotype 1b is an important predictor of elevated alpha-fetoprotein in patients without hepatocellular carcinoma. J Med Virol 2008;80: 1354-1362.
16. Kobayashi M, Akuta N, Suzuki F, Hosaka T, Sezaki H, Kobayashi M, et al. Influence of amino-acid polymorphism in the core protein on progression of liver disease in patients infected with hepatitis C virus genotype 1b. J Med Virol 2010;82:41-48.
17. El-Shamy A, Shoji I, Saito T, Watanabe H, Ide YH, Deng L, et al. Sequence heterogeneity of NS5A and core proteins of hepatitis C virus and virological responses to pegylated-interferon/ribavirin combination therapy. Microbiol Immunol 2011;55:418-426.
18. El-Shamy A, Kim SR, Ide YH, Sasase N, Imoto S, Deng L, et al. Polymorphisms of hepatitis C virus non-structural protein 5A and core protein and clinical outcome of pegylated-interferon/ribavirin combination therapy. Intervirology 2012;55:1-11.
19. Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, et al. Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and non-virological response to interferon-ribavirin combination therapy. Intervirology 2005;48: 372-380.
20. Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, et al. Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b: amino acid substitutions in the core region and low-density lipoprotein cholesterol levels. J Hepatol 2007;46: 403-410.
21. Sakamuro D, Furukawa T, Takegami T. Hepatitis C virus nonstructural protein NS3 transforms NIH 3T3 cells. J Virol 1995;69:3893-3896.
22. Zemel R, Gerechet S, Greif H, Bachmatove L, Birk Y, Golan-Goldhirsh A, et al. Cell transformation induced by hepatitis C virus NS3 serine protease. J Viral Hepat 2001;8:96-102.
23. Fujita T, Ishido S, Muramatsu S, Itoh M, Hotta H. Suppression of actinomycin D-induced apoptosis by the NS3 protein of hepatitis C virus. Biochem Biophys Res Commun 1996;229:825-831.

24. Macdonald A, Harris M. Hepatitis C virus NS5A: tales of a promiscuous protein. J Gen Virol 2004;85:2485-2502.
25. Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, et al. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. N Engl J Med 1996;334:77-81.
26. El-Shamy A, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, Hotta H. Sequence variation in hepatitis C virus nonstructural protein 5A predicts clinical outcome of pegylated interferon/ribavirin combination therapy. HEPATOLOGY 2008;48:38-47.
27. Kim SR, El-Shamy A, Imoto S, Kim KI, Ide YH, Deng L, et al. Prediction of response to pegylated interferon/ribavirin combination therapy for chronic hepatitis C genotype 1b and high viral load. J Gastroenterol 2012;47:1143-1151.
28. Ikeda K, Saitoh S, Arase Y, Chayama K, Suzuki Y, Kobayashi M, et al. Effect of interferon therapy on hepatocellular carcinogenesis in patients with chronic hepatitis type C: A long-term observation study of 1,643 patients using statistical bias correction with proportional hazard analysis. HEPATOLOGY 1999;29:1124-1130.
29. Okanoue T, Itoh Y, Minami M, Sakamoto S, Yasui K, Sakamoto M, et al. Interferon therapy lowers the rate of progression to hepatocellular carcinoma in chronic hepatitis C but not significantly in an advanced stage: a retrospective study in 1148 patients. Viral Hepatitis Therapy Study Group. J Hepatol 1999;30:653-659.
30. Benvegnu L, Chemello L, Noventa F, Fattovich G, Pontisso P, Alberti A. Retrospective analysis of the effect of interferon therapy on the clinical outcome of patients with viral cirrhosis. Cancer 1998;83:901-909.
31. Okamoto H, Sugiyama Y, Okada S, Kurai K, Akahane Y, Sugai Y, et al. Typing hepatitis C virus by polymerase chain reaction with type-specific primers: application to clinical surveys and tracing infectious sources. J Gen Virol 1992;73:673-679.
32. El-Shamy A, Sasayama M, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, et al. Prediction of efficient virological response to pegylated interferon/ribavirin combination therapy by NS5A sequences of hepatitis C virus and anti-NS5A antibodies in pre-treatment sera. Microbiol Immunol 2007;51:471-482.
33. Ogata S, Nagano-Fujii M, Ku Y, Yoon S, Hotta H. Comparative sequence analysis of the core protein and its frameshift product, the F protein, of hepatitis C virus subtype 1b strains obtained from patients with and without hepatocellular carcinoma. J Clin Microbiol 2002;40:3625-3630.
34. Ogata S, Florese RH, Nagano-Fujii M, Hidajat R, Deng L, Ku Y, et al. Identification of hepatitis C virus (HCV) subtype 1b strains that are highly, or only weakly, associated with hepatocellular carcinoma on the basis of the secondary structure of an amino-terminal portion of the HCV NS3 protein. J Clin Microbiol 2003;41:2835-2841.
35. Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Sugimura T, et al. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc Natl Acad Sci U S A 1990;87:9524-9528.
36. Delhem N, Sabile A, Gajardo R, Podevin P, Abadie A, Blaton MA, et al. Activation of the interferon-inducible protein kinase PKR by hepatocellular carcinoma derived-hepatitis C virus core protein. Oncogene 2001;20:5836-5845.
37. Love RA, Parge HE, Wickersham JA, Hostomsky Z, Habuka N, Moomaw EW, et al. The crystal structure of hepatitis C virus NS3 proteinase reveals a trypsin-like fold and a structural zinc binding site. Cell 1996;87:331-342.
38. Saiz JC, Lopez-Labrador FX, Ampurdanes S, Dopazo J, Forns X, Sanchez-Tapias JM, et al. The prognostic relevance of the nonstructural 5A gene interferon sensitivity determining region is different in infections with genotype 1b and 3a isolates of hepatitis C virus. J Infect Dis 1998;177:839-847.
39. Sarrazin C, Berg T, Lee JH, Teuber G, Dietrich CF, Roth WK, et al. Improved correlation between multiple mutations within the NS5A region and virological response in European patients chronically infected with hepatitis C virus type 1b undergoing combination therapy. J Hepatol 1999;30:1004-1013.

PLOS | ONE

Isolation of Human Monoclonal Antibodies to the Envelope E2 Protein of Hepatitis C Virus and Their Characterization

Yohko K. Shimizu[1]*, Minako Hijikata[2], Masamichi Oshima[3], Kazufumi Shimizu[4], Harvey J. Alter[5], Robert H. Purcell[6], Hiroshi Yoshikura[7], Hak Hotta[1]

1 Division of Microbiology, Center for Infectious Diseases, Kobe University Graduate School of Medicine, Kobe, Japan, **2** Department of Respiratory Diseases, Research Institute, National Center for Global Health and Medicine, Tokyo, Japan, **3** Department of Immunology, National Institute of Infectious Diseases, Tokyo, Japan, **4** Department of Gynecology, Nihon University School of Medicine, Tokyo, Japan, **5** Department of Transfusion Medicine, Warren Grant Magnuson Clinical Center, National Institutes of Health, Bethesda, Maryland, United States of America, **6** Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, United States of America, **7** Food Safety Division, Ministry of Health Labor and Welfare, Tokyo, Japan

Abstract

We isolated and characterized two human monoclonal antibodies to the envelope E2 protein of hepatitis C virus (HCV). Lymphoblastoid cell lines stably producing antibodies were obtained by immortalizing peripheral blood mononuclear cells of a patient with chronic hepatitis C using Epstein-Barr virus. Screening for antibody-positive clones was carried out by immunofluorescence with Huh7 cells expressing the E2 protein of HCV strain H (genotype 1a) isolated from the same patient. Isotype of resulting antibodies, #37 and #55, was IgG1/kappa and IgG1/lambda, respectively. Epitope mapping revealed that #37 and #55 recognize conformational epitopes spanning amino acids 429 to 652 and 508 to 607, respectively. By immunofluorescence using virus-infected Huh7.5 cells as targets both antibodies were reactive with all of the nine different HCV genotypes/subtypes tested. The antibodies showed a different pattern of immuno-staining; while #37 gave granular reactions mostly located in the periphery of the nucleus, #55 gave diffuse staining throughout the cytoplasm. Both antibodies were shown by immuno-gold electron microscopy to bind to intact viral particles. In a neutralization assay (focus-forming unit reduction using chimeric infectious HCV containing structural proteins derived from genotypes 1a, 1b, 2a, 2b, 3a, 4a, 5a, 6a, and 7a), #55 inhibited the infection of all HCV genotypes tested but genotype 7a to a lesser extent. #37 did not neutralize any of these viruses. As a broadly cross-neutralizing human antibody, #55 may be useful for passive immunotherapy of HCV infection.

Citation: Shimizu YK, Hijikata M, Oshima M, Shimizu K, Alter HJ, et al. (2013) Isolation of Human Monoclonal Antibodies to the Envelope E2 Protein of Hepatitis C Virus and Their Characterization. PLoS ONE 8(2): e55874. doi:10.1371/journal.pone.0055874

Editor: Gunnar F. Kaufmann, The Scripps Research Institute and Sorrento Therapeutics, Inc., United States of America

Received September 18, 2012; **Accepted** January 3, 2013; **Published** February 7, 2013

Funding: This work was supported, in part, by the Science and Technology Partnership for Sustainable Development (SATREPS) program of Japan Science and Technology Agency (JST) and Japan International Cooperation Agency (JICA) and the Intramural Research Program of the National Institute of Allergy and Infectious Diseases. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: yshimizu@nih.go.jp

Introduction

Hepatitis C virus (HCV) is a member of the *Flaviviridae* family and contains a 9.6 kb positive-strand RNA genome. The virus has been classified into seven major genotypes. The envelope glycoproteins, E1 and E2, mediate viral entry via cellular co-receptors, including CD81, claudin-1, occludin, and SBR1. The E1 and E2 proteins, located on the surface of viral particles, are the potential targets of neutralizing antibodies. At present, however, neither antibody-based prophylaxis nor an effective vaccine is available.

HCV persists in the presence of circulating antibodies. It has been speculated that this relates to the highly mutable, quasispecies nature of this RNA virus and the continual emergence of neutralization-resistant strains. However, the persistence of HCV in the presence of anti-HCV antibodies can not be fully explained by high variability alone. It has been found that neutralizing activity is detectable in sera from infected patients during both acute and persistent HCV infection [1,2], and that high titers of neutralizing antibodies correlate with natural resolution of chronic hepatitis C [3]. Further, polyclonal hyper-immune antibodies to the E2 protein have been shown to prevent or delay the onset of HCV infection in chimpanzees when administrated before exposure to the virus [4]. The ability of HCV to persist in its host despite the presence of neutralizing antibodies remains unexplained.

With the advent of recently developed systems to study the full cycle of HCV infection [5], various human monoclonal antibodies to the E1 and E2 proteins have been evaluated for their neutralizing activity and some of them were found to contain broadly cross-neutralizing antibodies [6–11]. Passive immunotherapy with such antibodies has preventive and therapeutic potential particularly for preventing HCV re-infection in liver transplant recipients.

During the course of our studies on lymphoblastoid cell lines producing antibodies against HCV, we were able to isolate one clone producing broadly cross-neutralizing antibodies and one

clone producing non-neutralizing antibodies from a well-characterized HCV-carrier (patient H). Isolation and characterization of these human monoclonal antibodies are detailed in this report.

Materials and Methods

Peripheral Blood Mononuclear Cells (PBMC) and Cell Lines

Following written informed consent, the blood sample was obtained in 2000 from patient H who developed chronic HCV infection after transfusion in 1977 [12]. The work was conducted with approval from the Institutional Review Board of the Clinical Center, National Institutes of Health, Bethesda, USA. (IRB # 91-CC-0117). PBMC were isolated by Ficoll-Isopaque (Pharmacia, Uppsala, Sweden), washed three times in phosphate-buffered saline (PBS), re-suspended in Cell Culture Freezing Medium (Life Technologies Japan, Tokyo, Japan), and stored at −80°C until use. Huh 7 cells, a cell line derived from a hepatocellular carcinoma, and highly permissive Huh7.5 cells [13] (provided by C. Rice, Rockefeller University, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Wako, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Nichirei, Tokyo, Japan). Cells were grown at 37°C in a CO_2 incubator.

Immunofluorescence (IF)

After fixation in ice-cold 100% acetone for 5 min, cells were incubated with primary antibody for 30 min at room temperature, washed 3 times in PBS, and incubated with a 1:200 dilution of the AlexaFluor 488 (Invitrogen, Carlsbad, CA, USA) secondary antibody for 30 min at room temperature. The samples were examined under a TE200 fluorescence microscope (Nikon, Tokyo, Japan).

Equilibrium Centrifugation in Sucrose Density Gradient (SDG)

A crude supernatant containing HCV was centrifuged at 2,380×g for 15 min at 4°C, filtered through the 0.45 μm membrane, concentrated approximately 100-fold using Amicon Ultra-15 centrifugal filter unit with Ultracel-100 (100 kD cut-off) membrane (Millipore, Billerica, MA, USA). The concentrated sample (1.5 ml) was overlaid on 6 ml of a discontinuous gradient with 10, 20, 30, 40, 50, and 60% (w/v) sucrose steps and centrifuged at 289,000×g for 20 h at 4°C in a CS 100GXL centrifuge (Hitachi, Tokyo, Japan). Buoyant density of fractions was determined by refractometry and expressed in g/ml.

Immuno-gold Electron Microscopy (EM)

For preparing a concentrated virus sample, fraction 3 obtained from the SDG centrifugation described above was diluted in 6.5 ml PBS and spun down at 215,000×g for 4 h at 4°C in a S58A-0015 rotor (Hitachi, Tokyo, Japan). The resulting pellet was suspended in 50 μl of PBS, mixed with an equal volume of antibody #55, #37, or a control antibody (500 μg/ml), and incubated overnight at 4°C. The mixture was then treated with 10 μl of goat anti-human IgG conjugated with colloidal gold-particles (Jackson Labs, Grove, PA, USA) overnight at 4°C. The sample was placed on a high resolution carbon grid, STEM100Cu (Oken, Tokyo, Japan), negatively stained with 2% uranyl acetate solution, and examined under a JEM-100C transmission electron microscope (JEOL, Tokyo, Japan) at 100 kV.

Reverse-transcription (RT), PCR, and Quantitative PCR (qPCR)

Extraction of RNA, RT, and PCR were carried out as described previously [14]. The amount of HCV cDNA was measured by qPCR using SYBR Premix Ex Taq (Takara, Tokyo, Japan) with an ABI Prism model Fast 7700 instrument (Applied Biosystems, Tokyo, Japan). To determine copy numbers, standard curves were prepared with serial 10-fold dilutions of a known amount of a plasmid bearing the amplified HCV sequence. We used primers that amplified the 5′ non-coding region of the viral genome. The sequences of the primers used were 5′-TTC ACG CAG AAA GCG TCT AG-3′ as a sense primer and 5′-CCC TAT CAG GCA GTA CCA CA-3′ as an anti-sense primer [15]. For detection of RNA encoding the V_H regions of antibodies, we used primer pair CG1z (5′-GCA TGT ACT AGT TTT GTC ACA AGA TTT GGG-3′) and VH6a (5′-CAG GTA CAG CTC GAG CAG TCA GG-3′) for #37, and primer pair CG1z and VH3a (5′-GAG GTG CAG CTC GAG GAG TCT GGG-3′) for #55. The RT-PCR products were cloned into pCR4TOPO (Invitrogen, Carlsbad, CA, USA) and the molecular clones were sequenced with an ABI PRISM^{TM}310 Genetic Analyzer (Applied Biosystems, Tokyo, Japan).

Expression of HCV E2 Proteins

Forns et al., [16] reported that the HCV E2 protein, when expressed on the cell surface, acquired its native conformation more efficiently when truncated at amino acid (aa) 661 of the viral genome. Therefore, we prepared expression vectors encoding truncated forms of E2 (aa 384 to 661) derived from HCV isolates of patient H, obtained in 1977 (strain H77, AF011751) and in 2000 (strain H00) for the screening of antibody-positive clones. For epitope mapping, we prepared vectors encoding various sizes of E2 proteins derived from strain H77. The inserts were amplified by RT-PCR, cloned into pDisplay (Invitrogen, Carlsbad, CA, USA) in frame between a signal sequence and a trans-membrane domain. All clones were sequenced to ensure that the DNA encoded the authentic HCV sequence. Huh 7 cells were grown in Lab-Tek 8-chamber slides (Nagle Nunc, Naperville, IL, USA) until 80% confluent and transfected with the constructs using SuperFect (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. After 48 h, cells were washed, fixed with cold acetone for 5 min, and stored at −80°C until use. Expression of the E2 proteins was verified by IF with rabbit hyperimmune sera raised against various domains of HCV E2.

HCV Plasmids and Generation of Infectious HCV

Plasmid pJFH1 that contains full-length cDNA of HCV strain JFH1 was provided by T. Wakita (National Institute of Infectious Diseases, Tokyo, Japan) [5]. Plasmids pFK-JFH/Con1/C-842-dg, pFK-JFH/J6/C846-dg, and pFK-JFH1/H77/C842-dg to generate chimeric infectious HCV Con1/C3, J6/C3, and H77/C3, respectively, were given by R. Bartenschlager (University of Heidelberg, Heidelberg, Germany) [17]. Plasmids pH77C/JFH1, pJ4/JFH1, pJ6/JFH1, pJ8/JFH1, pS52/JFH1, pED43/JFH1, pSA13/JFH1, pHK6a/JFH1, and pQC69/JFH1 to generate chimeric infectious HCV were provided by J. Bukh (Copenhagen University Hospital, Hvidovre, Denmark) [18–22]. These chimeras are JFH1-based recombinants expressing core-NS2 of genotype 1 to 7 isolates. For the synthesis of HCV RNA, the plasmids were transcribed using a Megascript T7 kit (Ambion, Austin, TX, USA). To generate infectious HCV, the *in vitro* transcribed viral genomic RNA was transfected into

Huh7.5 cells by electroporation using a Gene Pulser system (Bio-Rad, Hercules, CA, USA) or by using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) as described by the manufacturer. The culture supernatants collected at 2–7 days after transfection were centrifuged, passed through a 0.45 μm filter, and inoculated into naïve Huh7.5 cells. After additional passages on naïve cells, the cell-free supernatants containing HCV were concentrated approximately 10-fold using Amicon Ultra-15 (Millipore, Billerica, MA, USA) and measured for their infectivity titers. Aliquots were stored at −80°C until use.

Infectivity Titration

Virus titers were determined by focus-forming units (FFU) assay. Huh7.5 cells were seeded at 2×10^5 cells per well in 24-well plates and cultured overnight. Test samples were diluted serially 10-fold and each dilution was inoculated into the cells. After incubation for 6 h at 37°C, the cells were supplemented with fresh complete DMEM and cultured for 24 h. The cells were then immuno-stained and HCV-positive foci were manually counted under a fluorescence microscope. Each test was performed in duplicate or triplicate. The virus titer was expressed in FFU per ml sample, as determined by the mean number of IF-positive foci detected in a whole well.

Virus Neutralization Assays

Neutralization of HCV infection was assessed by the FFU reduction assay. Two independent assays were performed in the different laboratories. The first method was as follows: A 0.5 ml of serial 5-fold dilutions of #37, #55, or an irrelevant control antibody (human-IgG) (Sigma-Aldrich, St. Louis, MO, USA) was pre-incubated at 4°C overnight with an equal volume of the virus solution containing approximately 300 FFU/ml of HCV. The mixtures were inoculated into Huh7.5 cells (5×10^5/well) cultured on a 15mm-coverglass in 12-well plates. After incubation for 48 h, cells on the glass were fixed with cold 100% acetone and subjected to indirect IF for the detection of infected foci using a serum from patient H followed by the AlexaFluor 488 secondary antibody. IF-positive foci on the whole coverglass were manually counted under a fluorescence microscope. Each test was performed in duplicate. The second method was as follows: A 0.1 ml of the dilutions containing 0.2, 1, 3, 10, 30, 100, 300, or 1000 μg/ml of #55 or a control antibody was pre-incubated at 37°C for 1 h with an equal volume of the virus solution containing 10^2 or 10^3 FFU/0.1 ml of HCV. The mixtures were inoculated onto Huh7.5 cells (10^5 cells/well in 24-well plates). After 3 h of adsorption, the inocula were removed and fresh complete DMEM were added to the wells. At 24 h post-infection, cells were fixed with 4% paraformaldehyde (Wako, Tokyo, Japan) followed by permeabilization with 0.1% Triton-100 (Wako, Tokyo, Japan). The cells were then immuno-stained for the HCV proteins, counterstained with Hoechst 33342 (Invitrogen, Carlsbad, CA, USA), and examined under a BZ-9000 fluorescence microscope (Keyence, Osaka, Japan). The number of HCV infected cells in each well was manually counted. The percent neutralization was calculated as the percent reduction of FFU compared with virus incubated with the control antibody. The NT_{50} value, lowest concentration (μg/ml) of antibody required for 50% reduction of FFU, was determined by curvilinear regression analysis.

Results

Establishment of Human Lymphoblastoid Cell Lines Producing Monoclonal Antibodies to the Envelope E2 Protein of HCV

PBMC obtained from patient H were infected with Epstein-Barr virus, strain B95-8, as we described previously [23], and cultured at 37°C in a 75 ml-flask in medium RPMI1640 (Life Technologies Japan, Tokyo, Japan) containing 10% FBS. Ten days later, the cells were distributed in 96-well plates in an amount of 10^4 cells/0.2 ml/well. After 4 days of cultivation, supernatant from each well was screened for presence of antibodies by IF using Huh 7 cells expressing the E2 protein (aa 384 to 661) derived from HCV strain H77. Cells in the well that gave a positive signal were re-distributed into 96-well plates and the wells were screened again. This procedure was repeated 5 times until all of the tested wells became positive on two successive assays. When cellular RNA was extracted and the V_H region of antibody was amplified by RT-PCR, identical sequence was obtained from three randomly selected wells, suggesting that the cells were clones. Antibody from this clone was designated as #37.

With a similar procedure we obtained antibody #55. For the screening of #55, Huh7 cells expressing the E2 protein derived from HCV strain H00 was employed as a target. **Figure 1** shows deduced amino acid sequences of the V_H regions for #37 and #55. The antibodies were isotyped by IF with Huh7 cells expressing the E2 protein (aa 384–661) of HCV strain H77, using specific secondary antibodies to human IgM, IgG1, IgG2, IgG3, and IgG4 subclasses, and to lambda and kappa light chains (Binding Site Inc., San Diego, CA, USA). As shown in **Figure 2**, #37 was IgG1/kappa and #55 was IgG1/lambda. The IgG was purified from the supernatants using a HiTrap protein G HP column (GE Healthcare, Uppsala, Sweden) and used for further characterization.

Epitope Mapping

Both #37 and #55 failed to react with the E2 protein in the western blot assay. Therefore, these antibodies were considered to recognize conformational epitopes. In order to map epitope sequences, we prepared expression vectors encoding various regions of the E2 protein derived from HCV strain H77. They include the regions aa 384 to 661, aa 411 to 661, aa 429 to 652, aa 429 to 607, aa 508 to 652, aa 429 to 552, aa 508 to 607, aa 552 to 652, aa 508 to 552, and aa 552 to 607. Huh7 cells expressing these regions were tested by IF for reactivity with #37 and #55. As shown in **Figure 3**, #37 was reactive with the expressed form of the E2 protein containing aa 429 to 652, but not with smaller sizes than this region. In contrast, #55 was reactive with the truncated form down to the region aa 508 to 607. These results indicate that the target epitopes of #37 and #55 are located in the regions of aa 429 to 652 and aa 508 to 607, respectively.

Cross-reactivity with Different HCV Genotypes and Binding Ability

Recent development of infectious chimeric HCV [17–22] has made it possible to investigate cross-genotype reactivity of the antibodies utilizing virus-infected cells as a target. We examined the cross-reactivity of #37 and #55 by IF using Huh7.5 cells infected HCV with different genotype E2 proteins. Genotypes tested were 1a, 1b, 2a, 2b, 3a, 4a, 5a, 6a, and 7a. Both #37 and #55 were reactive by IF with all genotypes tested.

Binding ability of #37 and #55 was assessed by measuring the minimum concentration of the antibodies required for an IF-

MAb	FR1	CDR1	FR2
#37	QVQLEQSGGGLVKPGESLRLSCAASGFILS	HYHMS	WFRQAPGKGLEWIA
#55	E----E------Q--G------E----MF-	AGW-H	-V--------V-VS

MAb	CDR2	FR3
#37	DINYSGRTTYEADSVRG	RFTVSRDNAKWSLYLQMNSLRVEDTAMYYCAR
#55	R---D-SS-TYV---K-	----------NT-F-----------V----S

MAb	CDR3	FR4
#37	VGVVASINLMVGRRRSDNWFDL	WGQGTLVTVSS
#55	G-YYSYGPFGD.....	---------P-

Figure 1. Amino acid sequences of the V_H regions of #37 and #55. Residues identical to #37 sequences are indicated by a dash. Dots indicate gaps compared with the sequence of #37. MAb, monoclonal antibody; FR, framework regions; CDR, complementarity-determining regions.
doi:10.1371/journal.pone.0055874.g001

positive reaction using HCV-infected Huh7.5 cells as targets. The HCV inocula tested were strain JFH1 (genotype 2a) and ten chimeric HCV, including H77C/JFH1 (genotype 1a), J4/JFH1 (genotype 1b), Con1/C3 (genotype 1b), J6/JFH1 (genotype 2a), J8/JFH1 (genotype 2b), S52/JFH1 (genotype 3a), ED43/JFH1 (genotype 4a), SA13/JFH1 (genotype 5a), HK6a/JFH1 (genotype 6a) and QC69/JFH1 (genotype 7a). Antibody solutions containing 5 μg/ml of IgG were two-fold serially-diluted and each dilution was tested by IF for a positive reaction. The results are shown in **Table 1**. The minimum concentration of #55 required was 10–78 ng/ml, while that of #37 was 156–1250 ng/ml except for H77C/JFH1 and S52/JFH1, which required 20 ng/ml. The higher ability of #37 in binding to H77C/JFH1 was possibly because this chimeric virus was a JFH1-based recombinant with homologous envelope E2 of strain H77 from the PBMC-donor. The higher reactivity with S52/JFH1 remains unexplained. Overall, more of #37 was needed for a positive reaction compared to #55, indicating that #55 has a higher binding ability than #37.

Distribution of Reacting Antigens in the HCV-infected Cells

Distribution of the antigens reacting with #55 and #37 in the HCV-infected cells was observed by IF-staining. We

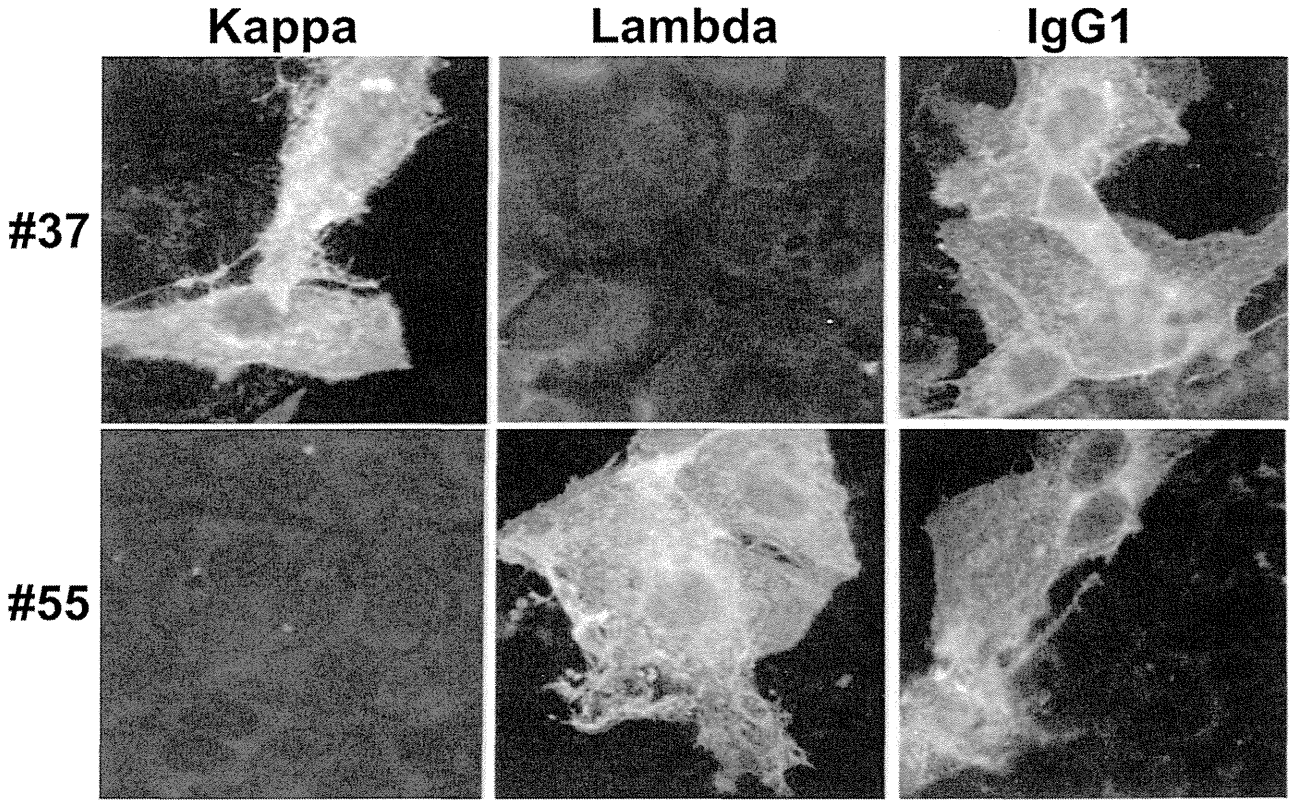

Figure 2. Isotyping revealed that #37 and #55 were IgG1/kappa and IgG1/lambda, respectively. Huh7 cells expressing the HCV E2 (aa 384–661) derived from strain H77 were incubated with #37 or #55, washed, and stained with fluoresceinated anti-human IgG1, anti-human lambda, or anti-human kappa.
doi:10.1371/journal.pone.0055874.g002

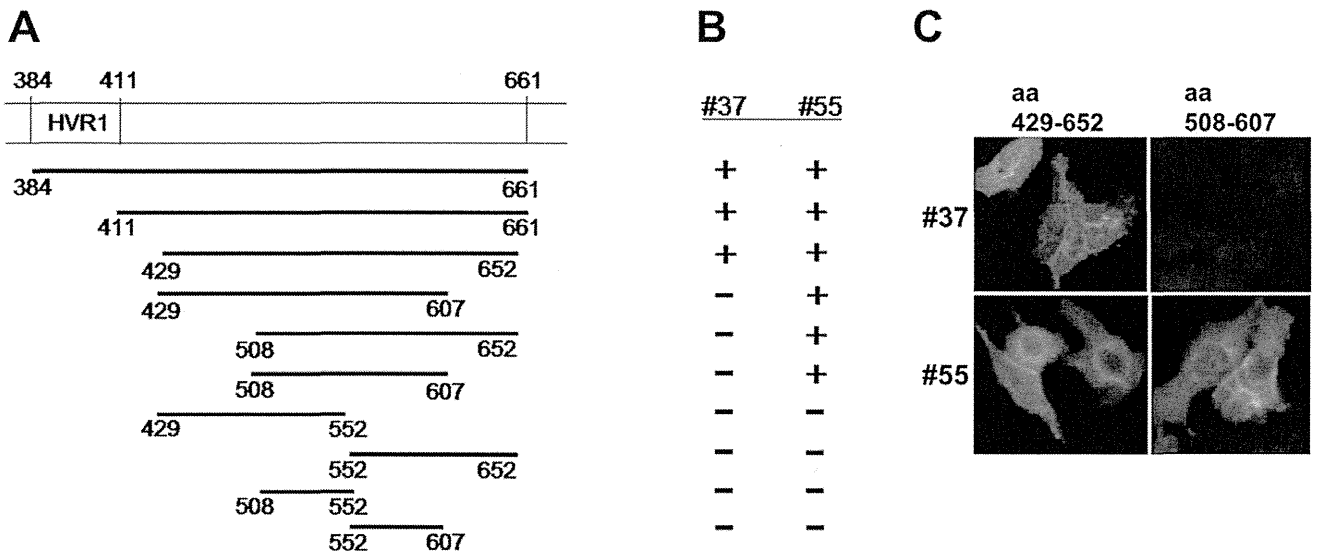

Figure 3. Epitope mapping revealed that #37 and #55 recognized the regions aa 429 to 652 and aa 508 to 607 of the E2 protein, respectively. (**A**). A panel of Huh7 cells expressing various truncations of the E2 protein derived from HCV strain H77 was generated. At the top of the graphic, aa 384 to 661 of the E2 protein is depicted with aa numbers. HVR1, hyper variable region 1. (**B**). Results of the assays for reactivity by IF. +, positive recognition; -, negative recognition. (**C**). IF-reactions of #37 and #55 against the cells expressing aa 429–652 and aa 508–607.
doi:10.1371/journal.pone.0055874.g003

examined Huh7.5 cells infected with 12 different inocula of HCV, including strain JFH1 and eleven chimeric HCV with structural proteins derived from various genotypes. **Figure 4** shows IF positive-staining by #37 and #55 observed in the cells infected with strain JFH1. The antibodies produced a different pattern of staining; while #37 gave coarse granular staining mostly located in the periphery of the nucleus, #55 gave diffuse staining throughout the cytoplasm. Similar patterns of IF-staining were observed for other chimeric HCV tested, including H77C/JFH1, J4/JFH1, J6/JFH1, J8/JFH1, S52/JFH1, ED43/JFH1, SA13/JFH1, HK6a/JFH1, QC69/JFH1, H77/C3, and Con1/C3. An irrelevant control antibody (human IgG) did not give such positive staining in the infected cells. #37, #55, and the control antibody were not reactive with non-infected Huh7.5 cells.

Ability to Recognize HCV Particles

It was recently reported that cell culture-grown HCV particles were pleomorphic, 40–75 nm in diameter, and spherical [24]. To determine whether #37 and #55 are able to recognize intact viral particles, we performed indirect immuno-gold EM using anti-human IgG labeled with colloidal gold particles as a second antibody. As target HCV for this experiment, we employed H77/C3 with homologous envelope E2 of strain H77 from the PBMC-donor. Concentration and purification of viral particles from culture supernatants was carried out by equilibrium SDG centrifugation. **Figure 5A** shows the distribution of HCV RNA measured by RT-qPCR after the centrifugation. Two peaks of viral RNA were obtained at 1.076 g/ml and 1.171 g/ml in fractions 3 and 6, respectively. Copy numbers of HCV RNA were 4.3×10^{5}/0.1 ml for fraction 3 and 7.4×10^{5}/0.1 ml for fraction 6.

Table 1. Binding activity measured by immunofluorescence.

Virus (genotype)	Minimum concentration (ng/ml) required for positive reaction	
	#37	#55
H77C/JFH1 (1a)	20	10
J4/JFH1 (1b)	156	78
J6/JFH1 (2a)	1250	20
J8/JFH1 (2b)	1250	78
S52/JFH1 (3a)	20	10
ED43/JFH1 (4a)	625	20
SA13/JFH1 (5a)	156	78
HK6a/JFH1 (6a)	156	78
QC69/JFH1 (7a)	1250	78
Con1/C3 (1b)	625	78
JFH1 (2a)	313	78

doi:10.1371/journal.pone.0055874.t001

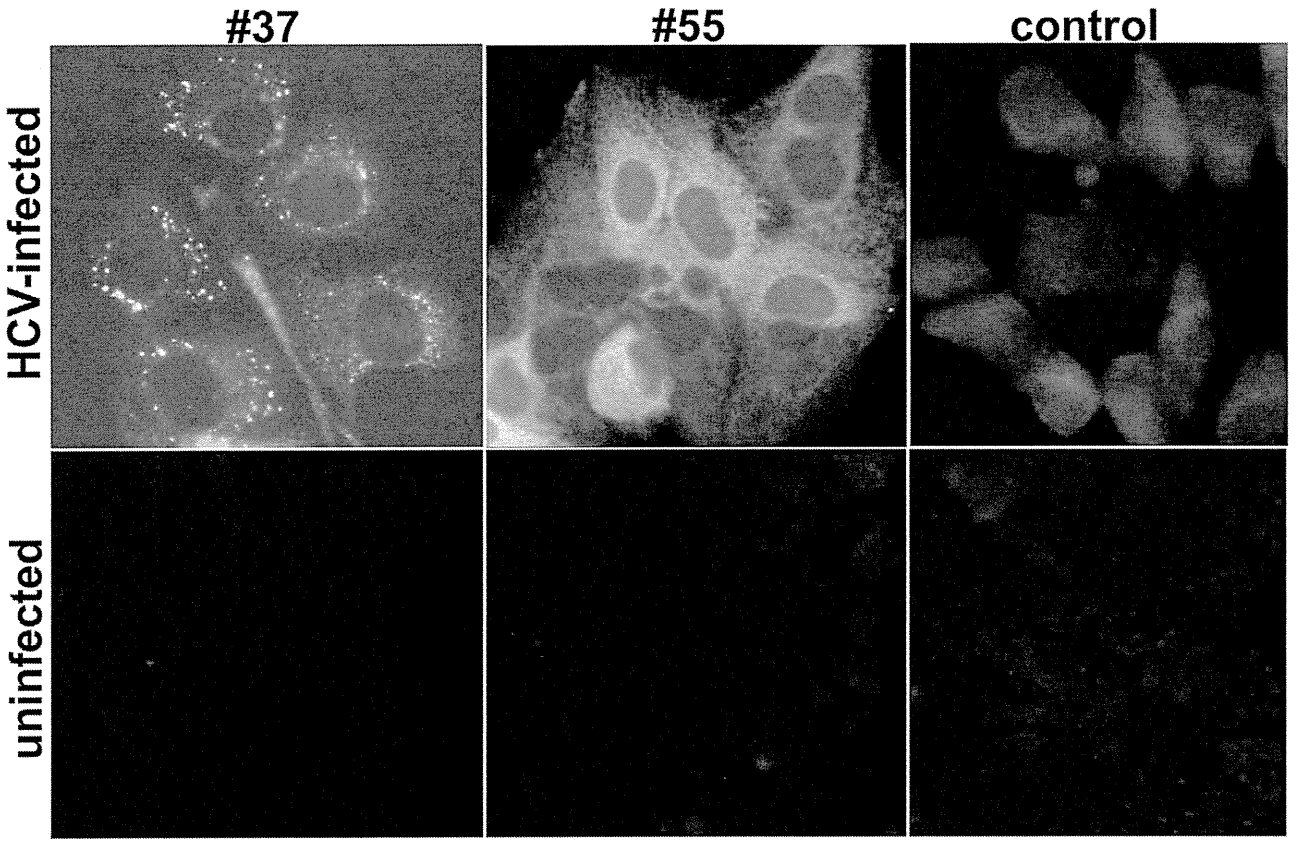

Figure 4. Different pattern of IF-staining by #37 and #55 in the HCV-infected Huh7.5 cells. #37 gave granular IF-reactions scattered in the cytoplasm. #55 gave diffuse staining throughout the cytoplasm. The control antibody (human IgG) gave negative staining. #37, #55, and the control antibody were not reactive with uninfected Huh7.5 cells.
doi:10.1371/journal.pone.0055874.g004

These two fractions were further examined by the FFU assay for their infectivity titers. Fractions 3 and 6 had an infectivity titer of 2.0×10^4 FFU/0.1 ml and 4.7×10^3 FFU/0.1 ml, respectively. Fraction 3 was calculated to have an approximately 9 times higher infectivity titer per HCV RNA than fraction 6 (**Figure 5B**), which was in accordance with our previous observation that the fraction with lower buoyant density was more infectious [25]. Thus, we selected fraction 3 for the EM examination. Fraction 3 was treated with #37 followed by anti-human IgG labeled with colloidal gold-particles, negatively stained, and examined in a transmission electron microscope. We detected HCV-like particles coated with colloidal gold, indicating the binding of #37 to virions. Most of the viral particles reacting with #37 measured approximately 50–60 nm in diameter. **Figure 5C (a)** shows an aggregate of three virions coated with specific gold. These viral particles measured approximately 50 nm in diameter. **Figure 5C (b)** shows two particles; the one on the right (50-nm in diameter) was coated with colloidal gold, indicating the binding of #37. Another particle on the left (35-nm in diameter) was negative for colloidal gold, indicating that #37 was not reactive with this particle. In addition, the presence of such uncoated particle in the same field suggested that colloidal gold did not bind non-specifically. When fraction 3 was reacted with #55 followed by anti-human IgG labeled with colloidal gold particles, larger aggregates of various-sized viral particles were observed, as shown in **Figure 5C (c)**. The viral particles varied in sizes from 40 to 70 nm in diameter. Immuno-gold EM demonstrated that both #37 and #55 can bind to HCV particles. **Figure 5C (d)** shows negative reaction of colloidal gold by an irrelevant control antibody (human IgG).

Neutralizing Activity

To investigate whether #37 and #55 could inhibit HCV infection, we performed an *in vitro* neutralization assay by reduction of FFU. As HCV inocula, we used chimeric H77/C3 (genotype 1a), chimeric Con1/C3 (genotype 1b), and chimeric J6/C3 (genotype 2a) in this assay. A virus sample containing approximately 300 FFU/ml of HCV was pretreated at 4°C overnight with #37, #55, or an irrelevant control antibody at a final concentration of 0.1, 0.5, 2.5, 12.5, 62.5, or 312.5 μg/ml and the mixtures were then inoculated into Huh7.5 cells. After 48 h post-infection, IF-positive foci were manually counted under a fluorescence microscope. Each test was performed in duplicate. As shown in **Figure 6**, compared to the results obtained with an irrelevant control antibody, #55 inhibited the viral infection in dose-dependent manner for all of the 3 samples tested. Inhibition by #37 was not observed.

Since #55 was found to have a neutralizing activity as shown above, further examination by the FFU reduction assay was conducted with HCV strain JFH1 (genotype 2) and various chimeric HCV containing the E2 proteins from 9 different genotypes. Chimeric viruses tested included H77C/JFH1 (genotype 1a), J4/JFH1 (genotype 1b), J6/JFH1 (genotype 2a), J8/JFH1 (genotype 2b), S52/JFH1 (genotype 3a), ED43/JFH1 (genotype 4a), SA13/JFH1 (genotype 5a), HK6a/JFH1 (genotype 6a), and QC69/JFH1 (genotype 7a). Two different concentrations of target