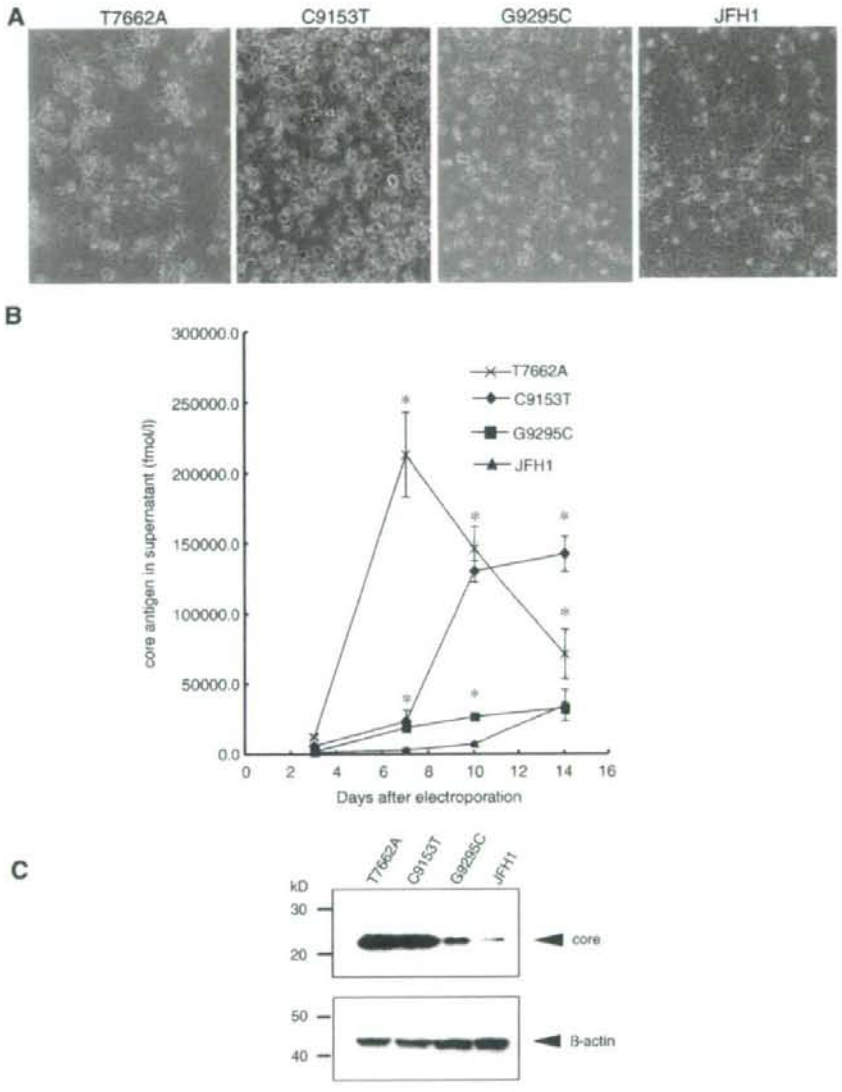

Fig. 9. Introduction of various mutations into the NS5B region of JFH1. The mutations identified in the cytopathic plaque Pl #1; T7662A, C9153T, and G9295C were introduced individually into the parental JFH1. Each JFH1 mutant, T7662A, C9153T, and G9295C, RNA was transfected into Huh-7.5.1 cells by electroporation.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

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

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

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

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

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

Materials and methods

Reagents

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

Cells and cell culture

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

In vitro RNA synthesis and transfection

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

HCV subgenomic replicon constructs

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

Real-time RT-PCR analysis

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

Quantification of HCV core antigen in the culture supernatant

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

Western blotting

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

Immunocytochemistry

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

Plaque assay

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

Sequence analyses

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

Establishment of mutant JFH1 clones

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

Statistical analyses

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

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CLINICAL STUDIES

Association of interferon regulatory factor-7 gene polymorphism with liver cirrhosis in chronic hepatitis C patients

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Keywords
chronic hepatitis C – interferon regulatory factor 7 – liver cirrhosis – polymorphism

Abbreviations
AFP, α-fetoprotein; ALT, alanine aminotransferase; CI, confidence interval; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN, interferon; IRF, interferon regulatory factor; ISRE, interferon-stimulated response element; IU, international unit; OR, odds ratio; PCR, polymerase chain reaction; PT, prothrombin time; SNP, single nucleotide polymorphism.

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Abstract

Background and aims: Interferon (IFN) regulatory factor 7 (IRF-7) has been shown to play an essential role in the transcriptional activation of virus-inducible cellular genes, especially IFN genes. Polymorphisms of the IRF-7 gene may probably affect both the quality and the quantity of IRF-7. We investigated the role of IRF-7 polymorphisms in Japanese patients with chronic hepatitis C virus (HCV) infection. *Methods:* We studied a total of nine polymorphisms of the IRF-7 gene including SNP1047A/G (Lys/Glu) and SNP2157A/G (Gln/Arg) using the Taqman allelic discrimination and sequencing techniques in 406 Japanese patients with chronic HCV infection. We further performed functional analysis of SNP1047 and SNP2157 by transcriptional activation of the IFNA promoter. *Results:* We found that SNP1047AG and SNP2157AG genotypes were in complete linkage disequilibrium and were present in a significantly higher proportion in HCV-infected patients with cirrhosis (5.6%) than in those without cirrhosis (1.7%) ($P = 0.03$). Multivariate analysis also revealed that SNP1047 and SNP2157 were independently associated with cirrhosis at an odds ratio of 2.5. Functional analysis revealed that SNP1047G and SNP2157G alleles increased IFNA expression. *Conclusion:* SNP1047AG and SNP2157AG genotypes were strongly associated with cirrhosis. SNP1047G and SNP2157G alleles might be used as markers of host factors associated with a higher risk of cirrhosis in Japanese patients with chronic HCV infection.

It has been estimated that hepatitis C virus (HCV) infects at least 170 million people worldwide (1), which often leads to the dreadful sequels of liver cirrhosis, end-stage liver disease and hepatocellular carcinoma (HCC) (1–4). The risk of HCC development increases with the severity of inflammation and liver fibrosis (5–8). Several factors, such as alcohol intake, older age at the time of infection, male gender and co-infection with the hepatitis B virus or human immunodeficiency virus, are known to accelerate disease progression in HCV infection (6, 8, 9). In addition, host genetic factors have been reported to affect the outcome of HCV infection (10–17). Actually, we have reported previously that genetic polymorphisms including single nucleotide polymorphisms (SNPs) in interleukin-1β, UDP glucuronosyltransferase 1A7, SCYB14, GFRA1, CRHR2 and MDM2 genes were associated with the development of HCC in Japanese patients with chronic HCV infection (14–17).

The endogenous production of interferons (IFNs) serves as an initial defence mechanism against viral infection. IFN regulatory factor 7 (IRF-7), one of the IFN-stimulated genes synthesized, regulates the transcription of IFNA genes. IFN-β produced in response to a viral challenge (18) induces transcription of IRF-7, and IRF-7 then binds and induces the promoter of IFNA genes (19). The transcription effecter action of IRF-7 promotes IFNA subtype expression and diversification of the IFN-stimulated gene response, establishing a positive-feedback loop that amplifies IFN production and antiviral action (20). The importance of murine IRF-7 in the Type I IFN gene expression was demonstrated by Taniguchi's group, who showed that virus-activated transcription of Type I IFN genes

depends on the synthesis of IRF-7 (21). There are many reports showing that induction of the human IFNA subtypes by viral infection requires IRF-7 (22–26). A recent study using IRF-7 knockout mice has demonstrated that transcription of both IFN-α and IFN-β is dependent on IRF-7, indicating that IRF-7 is a master regulator of type I IFNs (20). Polymorphisms of the IRF-7 gene may probably affect both the quality and the quantity of IRF-7.

In this study, we investigated the role of IRF-7 SNPs in Japanese patients with chronic HCV infection.

Methods

Patients

We studied 406 consecutive Japanese patients with chronic HCV infection who consulted at the outpatient clinic of the University of Tokyo Hospital between August 2001 and June 2003 (227 men and 179 women; ages 22–84 years; median age 62 years; 178 with cirrhosis). The genomic DNA of these patients was made available after obtaining written informed consent for genotyping. Approval was obtained from the institutional ethics committee (No. 400), and all the procedures followed institutional guidelines (27). Patients selected for this study were those who tested positive for the HCV antibody by the second-generation enzyme immunoassay (Ortho Diagnostics, Tokyo, Japan), and HCV RNA was measured using the Amplicor HCV assay version 1 (Roche, Tokyo, Japan). All patients were negative for the hepatitis B surface antigen (Abbott Laboratories, North Chicago, IL, USA). HCV genotypes were determined using a genotyping assay (SRL Laboratory Co., Tokyo, Japan). Patients with an ethanol intake of ≥ 80 g/day for > 10 years were considered to have a positive history of alcohol abuse. The following clinical variables were obtained for each patient at the time of whole-blood collection: age, gender, serum albumin level, serum total bilirubin level, serum alanine aminotransferase (ALT) level, serum α-fetoprotein (AFP) level, prothrombin time (PT), platelet count and serum viral load. The diagnosis of cirrhosis was made based on liver histology according to the criteria of Desmet (28) and Scheuer *et al.* (29). In patients without biopsy specimens, the diagnosis of cirrhosis was based on the presence of clinical manifestations of portal hypertension (e.g. varices, encephalopathy or ascites), biochemical abnormalities (elevated serum bilirubin, decreased serum albumin or prolonged PT) and obvious morphological change of the liver detected by hepatic imaging (e.g. ultrasonography, computed tomography, arteriography or magnetic resonance imaging).

Polymorphism genotyping

Genomic DNA was extracted from 100 μl whole blood as described previously (16). We studied two polymorphisms, 2157A/G (Gln/Arg) and 1047A/G (Lys/Glu), that are nonsynonymous polymorphisms. To determine the 2157A/G polymorphism, we could identify other three SNPs that located very close: 2068C/A, 2173A/G and 2200C/G. Furthermore, we selected four polymorphisms, −667C/T, −486A/G, −206C/G and −424–425, located close to the IFN-stimulated response element (ISRE) in the promoter region. Finally, we studied total nine polymorphisms of the IRF-7 gene (Table 1).

Genetic polymorphisms in the promoter region of IRF-7 were determined by direct sequencing of amplified gene fragments. We used two sets of primers: the outer primer, forward primer 5′-ccctccactcctccctact c-3′ and backward primer 5′-gtgtcacaggtgtccacagg-3′ for first polymerase chain reaction (PCR) and the inner primers, forward primer 5′-tcctccctactccgcgc tgg-3′ and backward primer 5′-gctgcctcggtatggatct c-3′ for nested PCR to amplify the specific IRF-7

Table 1. Interferon regulatory factor 7 gene polymorphisms studied

dbSNP rs#	Gene position	Alleles	Role	Amino acid	Method
rs10902179	−667	C/T	Promoter	–	Sequencing
rs2277270	−486	A/G	Intron	–	Sequencing
rs3832720	−424–425	−/GCCTCC	Intron	–	Sequencing
rs11544076	−206	C/G	Intron	–	Sequencing
rs1061502	1047	A/G	Nonsynonymous	Lys/Glu	Real time PCR
rs1061505	2068	C/A	Synonymous	–	Sequencing
rs3178010	2157	A/G	Nonsynonymous	Gln/Arg	Sequencing
rs12422022	2173	A/G	Intron	–	Sequencing
rs1051390	2200	C/G	Intron	–	Sequencing

SNP, single nucleotide polymorphism.

promoter fragment that covered all four polymorphisms. For the first PCR, a 617 bp fragment of the IRF-7 promoter was amplified by PCR using 20 ng of extracted genomic DNA template. PCR was performed with 5 × Green GoTaq Reaction Buffer and GoTaq DNA Polymerase (Promega, Madison, WI, USA). The thermocycling conditions were as follows: 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 65 °C for 30 s and 72 °C for 1 min then 72 °C for 5 min. To verify the size of the PCR product, amplified products were visualized on a 2% agarose gel with the appropriate size marker. Nested PCR was performed only for samples that did not give clear bands using 4 μl of the first PCR product. The thermocycling conditions of nested PCR were as follows: 95 °C for 2 min followed by 35 cycles of 95 °C for 30 s, 67 °C for 30 s and 72 °C for 1 min then 72 °C for 5 min. The 561 bp fragment of the nested PCR product was verified on 2% agarose gel with the appropriate size marker. For SNP determination, direct sequencing was performed bidirectionally using 10 ng of QIAquick Spin (Qiagen, Hilden, Germany)-purified PCR product, either the forward or backward PCR primer, and the Big Dye Terminator Cycle Sequencing Ready Reaction (PE Applied Biosystems, Foster City, CA, USA) followed by detection on an ABI 310 automated sequencer (PE Applied Biosystems).

We studied the SNP2157A/G genotype using direct sequencing of amplified gene fragments. We used the following primers: forward primer 5′-gctacacggaggaa ctgctg-3′ and backward primer 5′-ggctctggtctcacctt cac-3′ to amplify the specific IRF-7 fragment that covered other three SNPs: 2068C/A, 2173A/G and 2200C/G. A 428 bp fragment of IRF-7 was amplified by PCR using 20 ng of extracted genomic DNA template. PCR was performed with the same protocol as mentioned above. The thermocycling conditions were as follows: 95 °C for 2 min followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 40 s then 72 °C for 5 min. To verify the size of the PCR product, amplified products were visualized on a 2% agarose gel with the appropriate size marker. For SNP determination, sequencing was performed in the same way as mentioned above.

We performed SNP1047A/G genotype using the Taqman SNP genotyping assays and ABI 7000 sequence detection system, which were previously described in Dharel *et al.* (16).

Plasmids and site mutagenesis

pIFNA-pGL3 (pIFNA2 and pIFNA4) having Firefly luciferase gene downstream of the IFNA promoter and pFLAG-CMV-2IRF-7A expressing IRF-7 were generous gifts of Prof. John Hiscott (McGill University, Montreal, Canada). The plasmids were transformed in competent *Escherichia coli* and purified using a High Pure Plasmid Isolation Kit (Roche Diagnostics, Mannheim, Germany). Mutation was introduced into the IRF-7 gene of pFLAG-CMV-2IRF-7A using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Introduction of mutation was verified by dye-terminator cycle sequencing with an automated 310 DNA sequencer (Applied Biosystems).

Cell cultures, transient transfection and luciferase assay

Transfections for luciferase assays were carried out with human embryonic kidney 293 cells grown in Dulbecco's modified Eagle medium (GIBCO BRL, Gaithersburg, MD, USA) supplement with 10% foetal bovine serum and antibiotics. Subconfluent cells were transfected with 20 ng of pRL-TK expressing Renilla luciferase for internal control to adjust transfection efficiency, 600 ng of the pIFNA2 or pIFNA4, and 1200 ng of pFLAG-CMV-2IRF-7A using FuGene6 transfection reagent (Roche Diagnostics) in a six-well plate. At 48 h after transfection, the reporter gene activities were measured by a dual-luciferase reporter assay according to the manufacturer's instructions (Promega). All experiments were independently repeated for at least three times. Relative luciferase activities were calculated for each transfectant and compared with that of a blank pFLAG-CMV-2 expression vector (Sigma, St Louis, MO, USA).

Statistical analysis

The association between the clinical variables and the presence of cirrhosis was evaluated using the two-tailed *t*-test, the Wilcoxon test and the χ^2 test where appropriate. The association between different SNP genotypes and the presence of cirrhosis was evaluated using the χ^2 test. Possible confounding effects among the variables were adjusted using a multivariate logistic regression model (30), and odds ratios (ORs) and 95% confidence intervals (95% CI) were calculated. All data analyses were done using JMP software (version 5.1.2, SAS Institute Inc., Cary, NC, USA). The Hardy–Weinberg equilibrium of alleles at individual loci was evaluated using HWE. For all tests, $P < 0.05$ was considered significant.

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Results

Patient characteristics

The characteristics of the total 406 patients with chronic HCV infection involved in this study are shown in Table 2.

One hundred seventy-eight patients (43.8%) had cirrhosis. There was no significant difference in the proportion of gender, HCV genotype, serum viral load, alcohol drinking > 50 g/day, and serum ALT level between patients with and without cirrhosis. In patients with cirrhosis, age, presence of HCC, serum total bilirubin level and serum AFP level were higher than in patients without cirrhosis; however, serum albumin level, PT and platelet count were lower than in patients without cirrhosis.

Interferon regulatory factor-7 gene polymorphisms in hepatitis C virus-infected Japanese patients

Table 3 shows the distributions of the of IRF-7 gene polymorphisms. Since IRF-7 SNP1047, SNP2068,

Table 2. Patient demographics

Variable*	Total ($n = 406$)	Without cirrhosis ($n = 228$)	With cirrhosis ($n = 178$)	*P*-value
Sex (male)	227 (55.91%)	127 (55.70%)	100 (56.18%)	0.92†
HCC	177 (43.60%)	51 (22.37%)	126 (70.79%)	< 0.0001†
Age (year)	62 (22–84)	60 (22–83)	65 (37–84)	< 0.0001‡
Platelet count ($\times 10^4/\mu l$)	12.7 (1.7–38.67)	16 (4.1–38.67)	9.2 (1.7–27.1)	< 0.0001‡
Albumin (g/dl)	3.9 (2.3–5)	4.1 (2.8–5)	3.6 (2.3–4.8)	< 0.0001‡
ALT (U/L)	65 (9–429)	63.5 (9–341)	66 (13–429)	0.09‡
Total bilirubin (mg/dl)	0.8 (0.2–6.7)	0.7 (0.2–6.7)	0.9 (0.3–3.5)	< 0.0001‡
AFP (μg/L)	12 (1–21 700)	6 (1–3222)	25 (2–21 700)	< 0.0001‡
PT (%)	77.8 (7.3–100)	85 (12.6–100)	71.3 (7.3–100)	< 0.0001‡
HCV load (KIU/ml)	429 (0.5–1910)	453 (7.1–1910)	409.5 (0.5–1407)	0.3‡
Alcohol > 50 g/day	50 (12.92%)	24 (11.32%)	26 (14.86%)	0.36†
HCV genotype 1	259 (75.73%)	143 (75.26%)	116 (76.32%)	0.8†

*Age, albumin, total bilirubin, alanine aminotransferase, AFP, prothrombin time, platelet count and HCV load are shown as median (range). Male, cirrhosis, alcohol and HCV serotype 1 are shown as frequency (%).

†Calculated using the χ^2 test.

‡Calculated using the Wilcoxon test.

AFP, α-fetoprotein; ALT, alanine aminotransferase; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; PT, prothrombin time.

KIU/ml (= 1000 HCV/ml).

Table 3. Interferon regulatory factor 7A single nucleotide polymorphism genotype frequencies in hepatitis C virus-infected patients

SNP	Gene position	Function	Homozygote	Heterozygote	Homozygote
rs10902179	−667C/T	Promoter	355 (87.44%) CC	50 (12.31%) CT	1 (0.25%) TT
rs3832720	−424–425 −/GCCTCC	Intron	224 (55%) (4 Repeat)	153 (37.6%)	30 (7.4%) (3 Repeat)
rs2277270	−486A/G	Intron	371 (91.38%) AA	35 (8.62%) AG	0 (0%) GG
rs11544076	−206C/G	Intron	406 (100%) CC	0 (0%) CG	0 (0%) GG
rs1061502	1047A/G	Nonsynonymous (Lys/Glu)	392 (96.55%) AA	14 (3.45%) AG	0 (0%) GG
rs1061505	2068A/C	Synonymous	392 (96.55%) AA	14 (3.45%) AC	0 (0%) CC
rs3178010	2157A/G	Nonsynonymous (Gln/Arg)	392 (96.55%) AA	14 (3.45%) AG	0 (0%) GG
rs12422022	2173A/G	Intron	392 (96.55%) AA	14 (3.45%) AG	0 (0%) GG
rs1051390	2200C/G	Intron	392 (96.55%) CC	14 (3.45%) CG	0 (0%) GG

SNP, single nucleotide polymorphism.

SNP2157, SNP2173 and SNP2200 were in complete linkage disequilibrium, only SNP1047 was described in this haplotype analysis. Concerning SNP 1047, the proportion of patients with heterozygosity (AG) was only 3.45% among Japanese HCV-infected patients. In addition, we could not find homozygote of rare alleles (GG) in our patients. When the HCV-infected patients were segregated into two groups based on the presence or absence of cirrhosis, the genotype frequency and allelic frequency of SNP1047 were significantly different between patients with and without cirrhosis. The proportion of the SNP1047AG genotype was significantly different between the groups of patients with and without cirrhosis (5.62 vs. 1.75%; $P = 0.034$) (Table 4). In other words, a higher number of patients with cirrhosis had AG genotype compared with the patients without cirrhosis. Having a G allele increased the proportion of patients with cirrhosis with an OR of 3.27 (95% CI: 1.02–10.5, $P = 0.036$).

We examined the four polymorphisms in the promoter region and first intron using the direct sequencing technique in all 406 patients. We found only three polymorphisms and the distribution of −424–425 polymorphism was common (Table 3). SNP-206 was absent in all of the 406 patients. Nevertheless, there was no association between polymorphisms in this region and cirrhosis.

Table 4. Association of SNP1047 with liver cirrhosis

	Patient with HCV			
SNP1047	Without cirrhosis ($n = 228$)	With cirrhosis ($n = 178$)	OR (95% CI)	*P*-value
Genotype				
AA	224 (98.25%)	168 (94.4%)	1	0.034
AG	4 (1.75%)	10 (5.6%)	3.34 (1.03–10.8)	
GG	0 (0%)	0 (0%)		
Allele				
A	456 (99%)	350 (96%)	1	0.036
G	4 (1%)	14 (4%)	3.27 (1.02–10.5)	

CI, confidence interval; HCV, hepatitis C virus; OR, odds ratio; SNP, single nucleotide polymorphism.

Factors associated with the presence of cirrhosis in hepatitis C virus-infected patients

The following factors significantly associated with the presence of cirrhosis according to univariate analysis (Table 5) including SNP1047 ($P = 0.03$, OR = 3.34), age > 60 years ($P < 0.0001$), serum albumin < 3.3 g/dl ($P < 0.0001$), serum total bilirubin > 0.7 mg/dl ($P < 0.0001$), serum AFP > 20 μg/L ($P < 0.0001$) and platelet count $< 12.5 \times 10^4/\mu l$ ($P < 0.0001$). To evaluate the effects of SNP1047 polymorphism on the presence of cirrhosis, a multivariate logistic regression analysis was carried out with six variables. Only five variables (SNP1047 genotypes, age > 60 years, serum AFP > 20 μg/L, serum total bilirubin > 0.7 mg/dl and platelet count $< 12.5 \times 10^4/\mu l$ were included in the final model with OR (95% CI) of 2.5 (1.2–5.6; AA vs. AG), 1.5 (1.2–1.9), 1.7 (1.3–2.2), 2.0 (1.5–2.6) and 2.7 (2.1–3.5) respectively.

Evaluation of transcriptional activity of SNP1047 and SNP2157 of interferon regulatory factor-7

To evaluate the functional significance of SNP1047 and SNP2157 of IRF-7, transcriptional activity was examined by an IFNA promoter luciferase reporter assay. A complete linkage was observed in IRF-7 SNP1047, SNP2068, SNP2157, SNP2173 and SNP2200; nonetheless, only SNP1047 and SNP2157 were nonsynonymous SNPs that had amino acid change. Hence, we made mutation at these two points to compare the effect against IFNA promoter activity *in vitro* between SNP1047A/SNP2157A and SNP1047G/SNP2157G. We performed mutagenesis at

Table 5. Factors associated with liver cirrhosis in univariate and multivariate analyses

		Univariate analysis			Multivariate analysis		
Factor	Category	OR	95% CI	*P*-value	OR	95% CI	*P*-value
Age	> 60 years	2.6	1.7–3.9	< 0.0001	1.5	1.2–1.9	0.003
Albumin	< 3.3 g/dl	5.9	2.8–12.7	< 0.0001	–	–	–
AFP	> 20 μg/L	4.8	3.1–7.5	< 0.0001	1.7	1.3–2.2	< 0.0001
Platelet count	$< 12.5 \times 10^4/\mu l$	10.7	6.7–17.1	< 0.0001	2.7	2.1–3.5	< 0.0001
Total bilirubin	> 0.7 mg/dl	4.8	3.0–7.8	< 0.0001	2.0	1.5–2.6	< 0.0001
SNP1047	A/A	1			1		
	A/G	3.34	1.03–10.8	0.03	2.5	1.2–5.6	0.02

AFP, α-fetoprotein; CI, confidence interval; HCC, hepatocellular carcinoma; OR, odds ratio; SNP, single nucleotide polymorphism.

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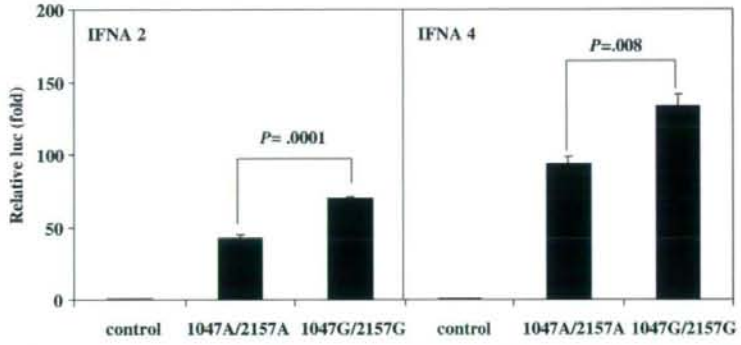

Fig. 1. Activation of luciferase reporter gene having IFNA2 or IFNA4 promoter by IRF-7 1047G/2157G and 1047A/2157A. Human embryonic kidney 293 cells were transfected with the pRL-TK (a control plasmid for transfection efficiency), pIFNA2 or pIFNA4 (reporter constructs containing luciferase gene) and IRF-7 expression plasmids, and luciferase activity was analysed at 48 h post-transfection. Relative luciferase activity was measured as fold activation (relative to the basal level for the reporter gene in the presence of the pFlag-CMV-2 plasmid after normalization to cotransfected relative light unit activity); the values represent the average of at least triplicate experiments. Standard error bars are shown. IFN, interferon; IRF, interferon regulatory factor.

both sites, and then we cotransfected SNP1047A/SNP2157A or SNP1047G/SNP2157G with pIFNA2 or pIFNA4 reporter plasmid. In addition, we used pRL-TK for the internal control of transfection efficiency. The relative luciferase activity was significantly higher in SNP1047G/SNP2157G than in SNP1047A/SNP2157A: the relative luciferase activity increased from 40 to 70 and from 90 to 130 when using pIFNA2 and pIFNA4 reporter plasmids respectively ($P = 0.0001$ and 0.008 respectively) (Fig. 1). IFNA4 promoter was significantly more highly activated than IFNA2 promoter by both SNP1047G/SNP2157G and SNP1047A/SNP2157A. We also performed Western blot analysis to confirm almost equal amounts of IRF-7 expression between SNP1047G/SNP2157G and SNP1047A/SNP2157A (data not shown).

Discussion

In this study, we identified a potential genetic marker for susceptibility to cirrhosis in chronic hepatitis C. We evaluated the relationships between the IRF-7 SNPs and the outcome of chronic HCV infection. Our results showed an effect of the nonsynonymous IRF-7 SNP1047 and SNP2157 on the presence of cirrhosis after controlling for other confounding clinical variables. The proportion of patients with cirrhosis was greater in SNP1047 and SNP2157 AG genotype than in AA genotype. The multivariate model confirmed an independent association between the SNP1047 and SNP2157 AG genotype and the presence of cirrhosis.

We studied the SNP1047 and SNP2157 because they are only two nonsynonymous SNPs that had been validated by multiple, independent submissions to the refSNP cluster and by frequency or genotype data: minor alleles were observed in at least two chromosomes. We studied the function of these SNPs by making two points of mutation because they were in complete linkage disequilibrium. We found that 1047G/2157G induced significantly higher expression of IFNA than 1047A/2157A did. As a result, A-to-G substitution at both SNPs increased their function of transcriptional activation. The structure of IRF-7 gene shows that SNP1047 is located in the constitutive activation domain and SNP2157 is located in the inhibitory domain (23, 31–33). The activation domain of IRF-7 contained acidic region, so changing amino acid from glutamine to lysine at SNP1047 might modify the transcription activity of IRF-7.

SNP1047G and SNP2157G that conferred higher activity to induce IFNA expression associated with cirrhosis in HCV-infected patients. This circumstance was possibly delineated by two postulates. Firstly, the role of IRF-7 was also implied in virus-mediated induction of IFNB and RANTES (regulated on activation normal T cell expressed and secreted; CCL5) genes (24, 34, 35), and it was a strong transactivator of RANTES chemokine gene expression (35). RANTES serves as a key ligand for CCR5 and plays an important role in attracting T cells to the portal area of the liver infected with HCV, and its expression is significantly elevated, especially in periportal and lobular areas that have the most lymphocytic infiltration (36, 37). RANTES has been suggested to be involved in the progression of chronic hepatitis C to advanced forms of liver disease (38–40). The deletion

mutation of inhibitory domain of IRF-7 activated the IFN and RANTES promoter more than the wild type of IRF-7 (40). Hence, overexpression of IFNA from SNP1047G and SNP2157G may also increase the expression of RANTES, and it might be involved in the progression of chronic hepatitis C to advanced forms of liver disease. Nonetheless, we could not see the difference of transaminase level between 1047G/2157G and 1047A/2157A. To attest these arguments, we should compare the transcriptional activity of RANTES promoter among SNP1047G/SNP2157G and SNP1047A/SNP2157A. Secondly, besides type I IFNs, IRF-7 also displayed an increment of the expression of genes encoding tumor necrosis factor-related apoptosis-inducing ligand, interleukin-12, interleukin-15 and CD80 (41). In addition to the role in innate immunity, IRF-7 modulated the expression of a large number of cellular genes. Most of them were upregulated gene especially the categories of signal transduction, transcription factors and apoptosis (42). Furthermore, IRF-7 was found to be a macrophage differentiation factor (43). These postulates might account for the increasing rate of cirrhosis in IRF-7 gene 1047G/2157G.

In our study, we also studied the gene function of IRF-7 in both IFNA2 and IFNA4. IFN type I genes can be subdivided into two groups: (i) immediate-early genes (IFNB and murine IFNA4, which is equivalent to human IFNA1); and (ii) delayed-type genes (which include the other IFNA subtypes) (26, 32). IRF-7 was used to induce the IFNA promoter; at least five IFNA promoters (1, 2, 4, 7, 14), and they were strongly transactivated by wild-type IRF-7 (50-fold induction of IFNA7 to 100-fold induction of IFNA4) (40). So we used IFNA4 because it was most strongly transactivated by IRF-7 among IFNA subtypes, and we used IFNA2 because IFNA2 was the treatment of choice in HCV infection. The result confirmed that IRF-7 could induce both IFNA2 and IFNA4 promoter. In addition, IFNA4 were significantly more highly transactivated than IFNA2. In particular, 1047A/2157A (wild-type) IRF-7 transactivated about 90-fold induction of IFNA4, and it was nearly the same as the previous report (40).

Interferon regulatory factor-7 is essential for the induction of type I IFN via virus-mediated (20). IFN activation of the ISGF3 complex results in the transcriptional upregulation of IRF-7 by binding to the ISRE within promoter region (44). So we tried to identify the role of promoter SNPs of IRF-7 in HCV-infected patients. However, we could not identify any association between promoter polymorphisms and the outcome of chronic HCV infection.

Our analyses showed a prominent effect of the IRF-7 nonsynonymous SNPs on the risk of developing cirrhosis. Although SNP1047 and SNP2157 G alleles were found in a minority of Japanese patients with chronic HCV infection, these polymorphisms might be used as markers of host factors associated with higher risk of cirrhosis in patients with chronic HCV infection.

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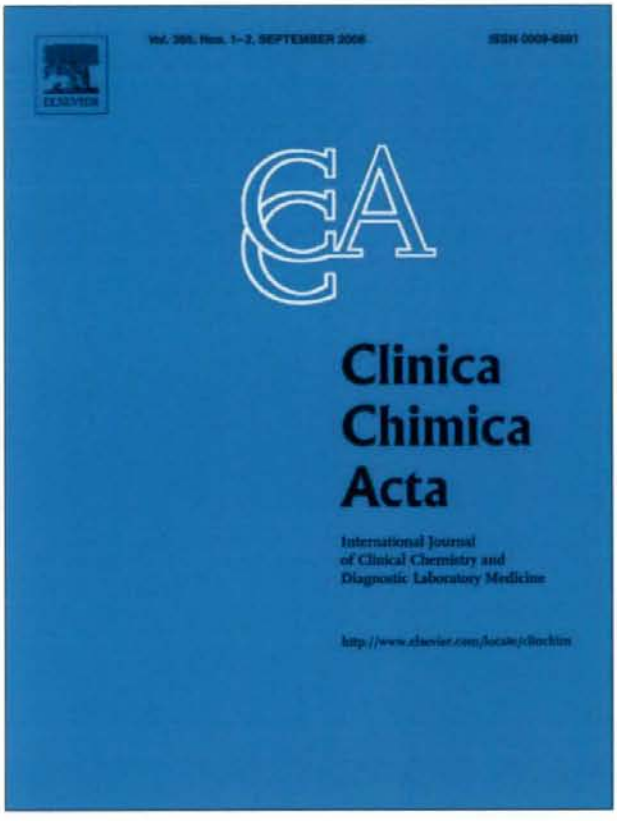

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Rapid detection of the hepatitis B virus YMDD mutant using TaqMan-minor groove binder probes

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ABSTRACT

Background: TaqMan-minor groove binder (MGB) probes were used in a real-time PCR-based assay for the rapid and accurate detection of hepatitis B virus (HBV) YMDD mutants.
Methods: TaqMan-MGB probes were designed to distinguish between wild-type (YMDD) and mutant (YVDD and YIDD) strains of HBV. The detection limit and sensitivity of the assay were determined using a dilution series of a mixture of wild-type and mutant plasmids. Serum samples collected from four patients with chronic mutant HBV infections during lamivudine therapy were analyzed using this method.
Results: The detection limit for YVDD and YIDD was 10 and 50 copies, respectively, whereas the sensitivity was 10% within a mixed virus population. In the clinical samples, mutant strains of HBV could be detected at levels <2.6 log copies/ml of HBV DNA. While 15 of the 21 samples tested by this method were positive for the YMDD mutant, direct sequencing and a reverse hybridization line probe assay (INNO-LiPA HBV DR v2) detected the mutant strain in only 11 and 9 samples, respectively. Moreover, the data for 6 samples analyzed by TA cloning were fully consistent with our TaqMan PCR results.
Conclusions: We successfully established a sensitive and accurate assay for the YMDD mutant of HBV. This method may be useful for monitoring patients treated with lamivudine.

1. Introduction

Chronic hepatitis B virus (HBV) infections have serious effects on human health, with 1 million people worldwide dying from HBV-related hepatic failure, liver cirrhosis, and hepatocellular carcinoma annually [1]. Nucleoside analogs such as lamivudine are currently approved for use as antiviral drugs, but lamivudine monotherapy promotes the emergence of resistant viral strains. The principal mutations identified in HBV as a result of lamivudine treatment are located at codon 204, within the tyrosine–methionine–aspartate–aspartate (YMDD) motif of the virus' reverse transcriptase, leading to the replacement of methionine (M) by valine (V) or isoleucine (I) (M204V/I). For detecting the YMDD mutant, direct sequencing is the most accurate method, but its sensitivity is limited (approximately 20% of the total virus population). Other simpler and more accurate molecular techniques have been developed to overcome some of the limitations of DNA sequencing, including restriction fragment length polymorphism analysis [2], 5' nuclease assays [3], melting point analysis [4], line probe assays [5], and peptide nucleic acid-mediated PCR clamping [6]. Real-time PCR using mutation-specific primers [7] or TaqMan probes [8] can be used to rapidly detect and quantify lamivudine-resistant mutations, but the sensitivity of these methods is not sufficiently high to detect the mutants at levels below 2.6 log copies/ml of HBV DNA.

In this study, we established a novel rapid, sensitive, and accurate assay for mutations in HBV based on real-time PCR using TaqMan-MGB probes. TaqMan-MGB probes have a fluorophore at the 5'-end and a nonfluorescent quencher at the 3'-end that produce very low background and strong hybridization-triggered fluorescence, resulting in high levels of accuracy and sensitivity [9]. This assay allowed us to rapidly detect HBV mutants in clinical samples with high sensitivity and accuracy, and may be useful for monitoring lamivudine-resistant mutations during lamivudine therapy.

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2. Methods

2.1. Plasmid DNA controls

Three plasmids (wild-type: 204 M [ATG], mutant: 204 V [GTG] and 204I [ATT]) were used to validate the specificity and sensitivity of the assay [10].

2.2. Patients and samples

Patients whose viral titers increased during lamivudine therapy were examined for the presence of YMDD mutations. Twenty-one serum samples from 4 patients were analyzed. Informed consent was obtained from all patients, and the appropriate institutional review board approved this study. The relative amount of HBV DNA was determined using a Roche Amplicor Monitor test (Roche Diagnostics, Tokyo, Japan) with a detection limit of 2.6 log copies/ml, which is equivalent to about 400 copies/ml.

HBV DNA was extracted from the serum samples using a QIAamp UltraSens Virus Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. For each sample, 1 ml of serum was used and the extracted DNA was eluted in 60 μl of elution buffer. The extracted DNA was stored at −20 °C until use.

2.3. TaqMan PCR using TaqMan-MGB probes

The samples were amplified using an ABI 7000 Real Time PCR System (Applied Biosystems, Foster City, CA, USA), and the results were analyzed using 7000 system SDS software. The primers and probes used were as follows: Forward primer, 5'-GGGCTTTCCCCCACTGTT-3'; reverse primer, 5'-GTACAGACTTGGCCCCCAATAC-3'; YMDD probe, 5'-FAM-CTTTCAGTTA-TATGGATGATG-MGB-3'; YVDD probe, 5'-VIC-CTTTCAGTTATGTGGATGAT-MGB-3'; and YIDD probe, 5'-VIC-CTTTCAGTTATATTGATGATGTG-MGB-3'. Two separate reaction mixtures were prepared to detect the YVDD and YIDD mutants. TaqMan PCR amplification was performed in a 96-well plate in a 25-μl volume containing 1 μl of the extracted HBV DNA, 0.9 μmol/l primers, 0.2 μmol/l each probe (YMDD and YVDD or YIDD), and 12.5 μl of Universal PCR Master Mix (Applied Biosystems). All of the reactions were performed in triplicate. The program used included 40 cycles at 95°C for 15 s followed by 62°C for 1 min.

2.4. Direct sequencing and TA cloning

Direct sequencing and TA cloning were carried out as described previously [11]. PCR was performed using 5'-TGGGCCTCAGTCCGTTTCTC-3' (HBV nucleotides [nt] 647–666) and 5'-GGACTCAAGATGTTGTACAG-3' (HBV nt 786–767) as the sense and antisense primers, respectively.

2.5. Reverse hybridization line probe assay

An *in vitro* reverse hybridization line probe assay was performed using an INNO-LiPA HBV DR v2 Kit (Innogenetics NV, Gent, Belgium) with human serum or plasma samples in accordance with the manufacturer's instructions [12].

3. Results

3.1. Determination of the specificity of the TaqMan-MGB probes

Cross-reactivity tests were performed to ensure that the probes specifically bound their targets. A dilution series of each plasmid (10^8–10^2 copies) was used as the template for TaqMan PCR. A negative control was included to avoid nonspecific amplification. Each single YMDD, YVDD, and YIDD probes were able to detect only the corresponding plasmid between 10^8 and 10^2 copies. Furthermore, mixtures containing the wild-type (YMDD) and mutant (YVDD or YIDD) probes also detect only its corresponding plasmid between 10^8 and 10^2 copies, thereby showing specificity for their target sequences (data not shown).

3.2. Determination of the sensitivity and detection limits of the TaqMan-MGB probes

Mixtures containing the wild-type (YMDD) and mutant (YVDD or YIDD) plasmids were used to determine the sensitivity of the assay. The wild-type and mutant plasmids were mixed at a ratio of 100:0, 90:10, 80:20, 50:50, 20:80, 10:90, or 0:100, corresponding to a final concentration of 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, and 10^2 copies, respectively. Using the plasmid mixture as a template, real-time TaqMan PCR was performed. In all cases, 10% of the YVDD mutants among 10^2 copies present in the mixture were detected using this assay. With regard to YIDD, however, 10% of 500 copies were successfully detected (data not shown). These results suggest that the detection limits for the YVDD and YIDD mutants were 10 and 50 copies, respectively, whereas the level of sensitivity was 10% within a mixed virus population. The detection limit for direct sequencing was 20% when the mutant and wild-type plasmids were mixed at the same ratios (100:0, 90:10, 80:20, 50:50, 20:80, 10:90, and 0:100) at a concentration of 10^5 copies (data not shown), suggesting that our PCR-based method is superior to direct sequencing for detecting mutant strains present at a low copy number.

Table 1
HBV mutant at codon 204 determined by TaqMan PCR, sequencing, INNO-LIPA HBV DR v2 assay and TA cloning

Patient/sample		Time of sampling[a] (weeks)	HBV DNA[b] (Log copies/ml)	ALT(IU/L)	Taqman PCR[c]	Direct sequencing	INNO-LIPA HBV DR v2	Sequencing of TA cloning
A								
	1	Pretreatment	7.5	264	M	M	M	
	2	On treatment 20	<2.6	39	M/I	M	N/A[d]	M/I
	3	On treatment 60	4.3	26	M/I	M	M	M/I
	4	On treatment 68	4.4	34	M/I	M/I	I	
	5	On treatment 76	6.3	30	I	I		
B								
	1	Pretreatment	6.3	52	M	M	M	
	2	On treatment 12	<2.6	33	M/I	M	N/A[d]	M/V/I
	3	On treatment 56	5.1	50	V/I	V/I	M/V/I	
	4	On treatment 84	6	44	V/I	V/I	M/V/I	
	5	On treatment 100	6.9	60	V/I	V/I	M/V/I	
C								
	1	Pretreatment	>7.6	60	M	M	M	
	2	On treatment 4	6.5	63	M	M	M	
	3	On treatment 20	5.4	28	M	M	N/A[d]	
	4	On treatment 40	6.8	27	M/V/I	M/V/I	M/V/I	M/V/I
	5	On treatment 44	>7.6	48	M/V/I	M/V/I	M/V/I	
D								
	1	Pretreatment	>7.6	61	M	M	M	
	2	On treatment 12	3.4	28	M/I	M	M	M/I
	3	On treatment 36	3.2	17	M/I	M/I	M	
	4	On treatment 60	2.7	10	M/I	M/I	M	
	5	On treatment 84	3	12	M/V/I	M/I	M/V/I	
	6	On treatment 104	5.2	12	M/V/I	V	M/V/I	M/V/I

[a] Number of weeks relative to treatment.
[b] Measured by Cobas Amplicor Monitor.
[c] M, methionine; V, valine; I, isoleucine.
[d] N/A, Not amplified (PCR failure).

3.3. Comparison of real-time TaqMan PCR and direct sequencing

We next examined HBV clinical samples using both our PCR-based assay and direct sequencing. As shown in Table 1, the results produced by real-time TaqMan PCR and direct sequencing were fully concordant in 14 samples (14/21, 66.7%). In the remaining seven samples, however, the results were different. In all of the discordant results, real-time TaqMan PCR identified an extra mutant.

3.4. Real-time TaqMan PCR versus a reverse hybridization line probe assay

To further validate the accuracy and sensitivity of our real-time TaqMan PCR-based method, all of the samples were tested by reverse hybridization line probe assay using an INNO-LiPA HBV DR v2 Kit. As shown in Table 1, the results for 10 samples (47.6%) were fully concordant between the 2 assays. In four of the samples (A-3, D-2, D-3, and D-4), however, in which the level of HBV DNA ranged from 2.7 to 4.4 log copies/ml, only the wild-type sequence was detected by the reverse hybridization line probe assay. Neither the wild-type nor the mutant sequence was detected in 2 samples (A-2 and B-2) in which the level of HBV DNA was below 2.6 log copies/ml due to PCR failure. No amplification was observed with a third sample (C-3), but its HBV DNA level was 5.4 log copies/ml.

3.5. Validation of the discordant TaqMan PCR, direct sequencing, and reverse hybridization line probe assay results by subcloning and sequencing

To further evaluate these 3 methods, 6 of the samples (A-2, A-3, B-2, C-4, D-2, and D-6) were cloned following PCR amplification. After subcloning, 20 clones per sample were picked at random and sequenced. As shown in Tables 1 and 2, our real-time TaqMan PCR results were fully concordant with those produced by subcloning and sequencing, except for those involving sample B-2, in which the sequence GTG was detected only by TA cloning. Thus, those mutant viral strains that were present in minor amounts in the mixtures were more likely to be detected by real-time TaqMan PCR. Although the HBV DNA level was below 2.6 log copies/ml in samples A-2 and B-2, the YIDD mutant was detected only by real-time TaqMan PCR, and not by direct sequencing or the line probe assay.

Table 2
Sequences obtained by TA cloning in six HBV samples

Sample/codon 204[a]	Sequence obtained by TA cloning	Number of clones obtained
A-2		
M	ATG	17/20 (85.0%)
V	–	–
I	ATC	3/20 (15.0%)
A-3		
M	ATG	19/20 (95.0%)
V	–	–
I	ATC	1/20 (5.0%)
B-2		
M	ATG	18/20 (90.0%)
V	GTG	1/20 (5.0%)
I	ATC	1/20 (5.0%)
C-4		
M	ATG	18/20 (90.0%)
V	GTG	1/20 (5.0%)
I	ATT	1/20 (5.0%)
D-2		
M	ATG	11/20 (55.0%)
V	–	–
I	ATC	9/20 (45.0%)
D-6		
M	ATG	1/20 (5.0%)
V	GTG	18/20 (90.0%)
I	ATA	1/20 (5.0%)

[a] M, methionine; V, valine; I, isoleucine.

4. Discussion

In the present study, we established a novel rapid, sensitive, and accurate method for detecting YMDD mutants using TaqMan-MGB probes. The detection limit of this method is 10 copies for YVDD and 50 copies for YIDD with 10% sensitivity within a mixed virus population.

The assay was compared with direct sequencing and a reverse hybridization line probe assay by analyzing clinical samples. The results produced using our PCR-based method were correlated with those obtained by direct sequencing and the reverse hybridization line probe assay; however, in some samples containing a mixed population of wild-type and mutant virus (e.g., A-3), the minor mutant clone was detected only by real-time TaqMan PCR. The reverse hybridization line probe assay is also a sensitive method for detecting lamivudine-resistant mutations, but its sensitivity seems to depend on the viral load. To validate the accuracy of our TaqMan PCR-based assay, 6 samples were selected for subcloning and sequencing. All of the results were concordant with those produced by real-time TaqMan PCR, suggesting the accuracy and superiority of our novel method.

Our assay is similar to the method of Malmstrom et al. [8], which is a modification of a previously reported method using the LightCycler [13]. In that study, three types of probes labeled with different fluorophores were mixed and used for analysis, but the authors did not report the sensitivity and specificity of their assay. In the present study, a mixture of three probes resulted in reduced specificity and sensitivity, and was therefore not suitable for analysis (data not shown). Thus, we employed a mixture of only two probes. Note that the detection limit in the aforementioned study for a minor population was 10%, which is similar to that obtained using our method. Based on these results, we propose that our assay is superior to theirs.

In conclusion, we established a novel rapid, simple, and sensitive assay to detect lamivudine-related HBV mutants. This method will be useful for the early detection and monitoring of resistant mutants during lamivudine therapy.

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ORIGINAL ARTICLE

Genetic variability and diversity of intracellular genome-length hepatitis C virus RNA in long-term cell culture

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Abstract Hepatitis C virus (HCV) is known to circulate persistently in vivo as a complex population of different but closely related viral variants. To understand the quasispecies nature of HCV, we performed genetic analysis of intracellular HCV RNAs obtained in long-term cell culture of genome-length HCV-RNA-replicating cells. The results revealed that genetic mutations in HCV RNAs accumulated in a time-dependent manner, and that the mutation rates of HCV RNAs were 3.5–4.8 $\times$ 10^{-3} base substitutions/site/year. The mutation rates of nonstructural regions that are essential for RNA replication were lower than those of structural regions. The genetic diversity of HCVs was also enlarged in a time-dependent manner. Furthermore, we found that the GC content of HCV RNA was increased in a time-dependent manner. These results suggest that an HCV-RNA-replicating cell culture system would be useful for analysis of the evolutionary dynamics and variations of HCV.

Introduction

Hepatitis C virus (HCV) infection frequently causes chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma. Such persistent infection has now become a serious health problem, with more than 170 million people worldwide currently infected with HCV [23]. HCV is an enveloped, positive single-stranded RNA (9.6 kb) virus belonging to the family *Flaviviridae*, and the HCV genome encodes a large polyprotein precursor of approximately 3,000 amino acid (aa) residues [9]. This polyprotein is cleaved by a combination of the host and viral proteases into at least 10 proteins in the following order: core, envelope 1 (E1), E2, p7, non-structural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B [5, 6, 8].

The most characteristic feature of the HCV genome is its remarkable diversity and variation. To date, more than six genotypes and multiple subtypes, which show more than 20% difference at the nucleotide level compared with any of the other subtypes, have been identified worldwide [4, 19]. An approximately 5–8% difference at the nucleotide level is observed within a single genotype [8]. Furthermore, an approximately 1% difference at the nucleotide level is also observed among HCV genomes in an individual [20]. Regarding variations of the HCV genome, three reports using specimens from chimpanzees [16, 18] and a human patient [17] have estimated that the mutation rate of the HCV genome was 1.4–1.9 $\times$ 10^{-3} base substitutions/site/year. Since the selective pressure of the immune system functions in vivo [10, 24], an experimental system of HCV replication is needed to define the actual mutation frequency of HCV RNA.

We considered that the cell-culture-based HCV replicon system developed in 1999 [15] would be useful as an experimental system for analysis of the genetic variations and diversity of HCV, since it has been shown that HCV subgenomic RNA (so-called replicon RNA) containing the NS3–NS5B regions could autonomously and efficiently replicate in a human hepatoma cell line, HuH-7, using this

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