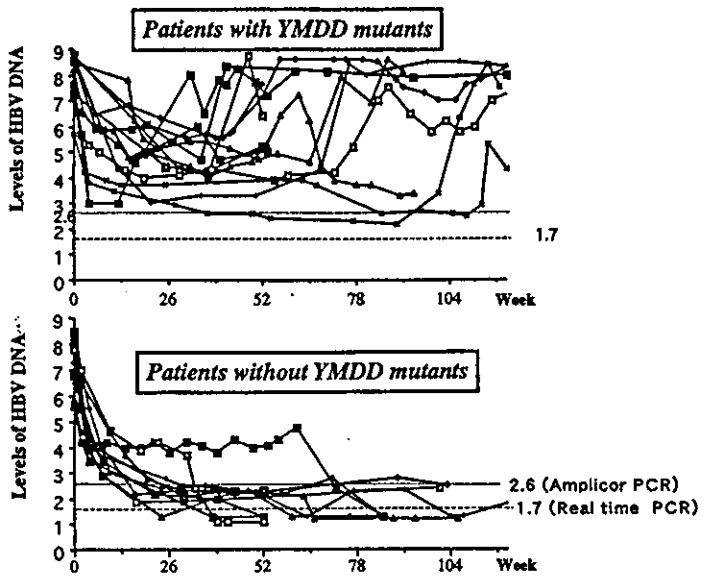

Fig. 4. Kinetics of HBV DNA levels during lamivudine therapy

Table 2. Patients' background before therapy and emergence of YMDD mutants according to the lowest levels of HBV DNA reached during therapy

Minimum level of DNA (log copies/ml)	<2.6	2.5-1.7	<1.7	P*value
No. of patients	11	5	8	
HBV DNA (LGE/ml)*	8.3±0.6	7.4±1.2	7.3±0.8	P<0.05[b]
ALT (IU/L)*	170±118	130±100	196±101	P=0.29[b]
HBeAg +/-*	10 / 1	4 / 1	7 / 1	P=0.7[c]
Cirrhosis +/-	5 / 6	1 / 4	4 / 4	P=0.44[c]
Dose (mg/day)	146±56	124±22	133±38	P=0.46[b]
Treatment period (week)	90±40	104±39	89±35	P=0.83[b]
YMDD mutant (%)	11 (100)	2 (40)	0 (0)	P<0.001[c]
Time of Re-increase of HBV DNA (week) (No. of patients)	49.6±18.4 n=11	107,115 n=2	n=0	

[a]≧2.6 and 2.5 - 1.7 vs. < 1.7 [b]Mann-Whitney U-test [c]Fisher's exact probability test
*Values befone treatment

Future Therapies

Various combination therapies, for example prednisolone [4], IFN [5,6], and HB vaccine are tried in order to obtain greater efficacy with short-term lamivudine therapy. However, these trials has a sufficient number of cases for the analysis.

Clinical trials on other antiviral agents such as adefovir dipivoxil and entecavir, used either in combination or alone, are now on-going. Treatment modalities for HBV-related liver cirrhosis are mainly supportive. Effect of lamivudine is now under trial [10,11].

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Gastrointestinal, Hepatobiliary and Pancreatic Pathology

Hepatitis C Virus Down-Regulates Insulin Receptor Substrates 1 and 2 through Up-Regulation of Suppressor of Cytokine Signaling 3

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The pathogenesis of hepatitis C virus (HCV)-associated insulin resistance remains unclear. Therefore, we investigated mechanisms for HCV-associated insulin resistance. Homeostasis model assessment for insulin resistance was increased in patients with HCV infection. An increase in fasting insulin levels was associated with the presence of serum HCV core, the severity of hepatic fibrosis and a decrease in expression of insulin receptor substrate (IRS) 1 and IRS2, central molecules of the insulin-signaling cascade, in patients with HCV infection. Down-regulation of IRS1 and IRS2 was also seen in HCV core-transgenic mice livers and HCV core-transfected human hepatoma cells. Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal, a potent proteosomal proteolysis inhibitor, blocked down-regulation of IRS1 and IRS2 in HCV core-transfected hepatoma cells. In human hepatoma cells, HCV core up-regulated suppressor of cytokine signaling (SOCS) 3 and caused ubiquitination of IRS1 and IRS2. HCV core-induced down-regulation of IRS1 and IRS2 was not seen in SOCS3$^{-/-}$ mouse embryonic fibroblast cells. Furthermore, HCV core suppressed insulin-induced phosphorylation of p85 subunit of phosphatidylinositol 3-kinase and Akt, activation of 6-phosphofructo-2-kinase, and glucose uptake. In conclusion, HCV infection changes a subset of hepatic molecules regulating glucose metabolism. A possible mechanism is that HCV core-induced SOCS3 promotes proteosomal degradation of IRS1 and IRS2 through ubiquitination. *(Am J Pathol 2004, 165:1499–1508)*

Chronic liver diseases are associated with glucose intolerance called hepatogenous diabetes.[1] Glucose intolerance impairs sustained response rate to anti-viral therapy in patients with chronic hepatitis C virus (HCV) infection[2] and is a risk factor for development of hepatocellular carcinoma[3] as well as long-term survival in patients with cirrhosis.[4] Several epidemiological studies have revealed an association between HCV infection and type 2 diabetes mellitus (DM) in cirrhotic patients.[5–13] Case-cohort analysis confirms an increased risk for type 2 DM in cirrhotic patients with HCV infection.[14] Cirrhotic patients with HCV infection are twice as likely to have type 2 DM than patients with hepatitis B virus (HBV) infection.[6,7,12] Thus, epidemiological data show that HCV infection antedates type 2 DM. It is, however, difficult to prove that HCV itself triggers glucose intolerance in patients with liver cirrhosis. Various factors such as reduced glucose uptake,[15] porto-systemic shunting,[16] and impaired glucagon metabolism[17] are also involved in glucose metabolism in patients with liver cirrhosis. Although glucose intolerance may occur even in the early stage of HCV infection, changes in glucose metabolism in noncirrhotic patients are not evident. To ascertain if HCV infection

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directly causes glucose intolerance, changes in glucose metabolism in noncirrhotic patients with various hepatobiliary disorders were investigated.

The liver plays a major role in regulation of glucose metabolism because it is the main source of endogenous glucose and the major site involved in insulin metabolism.[18,19] Thus, hepatic factors may be involved in HCV-associated glucose intolerance. However, the pathogenic mechanisms for HCV-associated glucose intolerance remain unclear. Insulin exerts many biological effects through insulin receptor substrate (IRS) 1 and IRS2. Disruption of IRS1 results in insulin resistance, but not DM, because of compensatory hyperinsulinemia.[20,21] Disruption of IRS2 results in severe DM because of insulin resistance and disturbance of insulin secretion.[22] Thus, IRS1 and IRS2 are the molecules that augment the specificity of the insulin-signaling cascade and play a central role in insulin-mediated glucose metabolism.

HCV chronically infects hepatocytes. HCV may escape from the host immune response by suppressing cytokine signaling. We recently showed that HCV core up-regulates suppressor of cytokine signaling (SOCS) 3 expression.[23] Although SOCS3 is known to be a negative regulator for cytokine signaling such as interleukin-6, growth hormone, and interferon-α, the role of SOCS3 on HCV-associated glucose intolerance has never been investigated. The aims of this study were to investigate changes in glucose metabolism in noncirrhotic patients with various hepatobiliary disorders and the molecular mechanisms for HCV-associated glucose intolerance.

Materials and Methods

Materials

All reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan) unless otherwise indicated. Affinity-purified polyclonal rabbit anti-SOCS3 antibody was generated against synthetic peptide SKFPAAGMSRPLDTSLRL (Immuno-Biological Laboratories, Gunma, Japan).

Patients

A total of 357 patients with chronic hepatitis C (n = 158), chronic hepatitis B (n = 54), autoimmune hepatitis (AIH) (n = 36), fatty liver (n = 40), primary biliary cirrhosis (PBC) (n = 49), or histologically normal livers (CON; n = 20) were studied retrospectively during the period from January 1997 to August 2003 at Kurume University Hospital. All of the patients were untreated and hospitalized for diagnostic liver biopsy. All of the diagnoses were based on clinical, serological, and histological evidence. Domestic data were collected at the time of liver biopsy including age, sex, and alcohol use. Body mass index (BMI) was calculated as body weight in kg divided by the square of height in meters (kg/m^2). Some liver diseases such as AIH and PBC show gender differences, and it is also possible that HCV infection affects BMI. Therefore, age, sex, BMI, and biochemical parameters were not matched among the groups to reduce selection bias. Patients with other causes of liver disease, in particular those known to be involved in the pathogenesis of diabetes such as hemochromatosis or alcoholic liver disease (on the basis of histology or a history of excessive alcohol consumption) were excluded, as were those who had been taking corticosteroids or with a history of, or evidence of, pancreatitis or a pancreatic tumor. The study protocol was approved by the institutional review board, and informed consent for participation in the study was obtained from each subject. None of the patients was institutionalized.

Laboratory Determinations

Venous blood samples were taken in the morning after a 12-hour overnight fast. Plasma glucose levels were measured by a glucose oxidase method. Serum insulin levels were measured by using a sandwich enzyme immunoassay kit (Eiken Chemical, Tokyo, Japan). β-Cell function and insulin resistance were calculated on the basis of fasting levels of plasma glucose and insulin, according to the homeostasis model assessment (HOMA) method.[24] The formulas for the HOMA model are as follows: β-cell function (HOMA-β) = fasting insulin (μU/ml) × 360/(fasting glucose (mg/dl) − 63); insulin resistance (HOMA-IR) = fasting glucose (mg/dl) × fasting insulin (μU/ml)/405.

Determination of HCV Genotype and Measurement of HCV Core

HCV genotype was determined by polymerase chain reaction with type-specific primers and HCV genotypes were classified according to classification system of Simmonds and colleagues.[25] Unselected serum samples (n = 58) were assayed for HCV core by using a newly developed HCV core antigen enzyme-linked immunosorbent assay test system (Ortho-Clinical Diagnostics K.K., Tokyo, Japan) as previously described.[26] This assay has high stability and reproducibility under all conditions and the detection limit is 44 fmol/L.

Histological Data

For each patient, a liver biopsy specimen was fixed in 10% formalin buffer and stained with hematoxylin and eosin. Liver biopsy specimens were evaluated by a single experienced pathologist who was unaware of the patients' clinical and laboratory data. The specimens were scored according to the METAVIR scoring system, which is suited for evaluation of chronic hepatitis C.[27] Activity was graded according to the intensity of necroinflammatory lesions: 0 , no activity; 1, mild activity; 2, moderate activity; and 3, severe activity. The stage of fibrosis was scored as follows: 0, no fibrosis; 1, portal fibrosis without septa; 2, portal fibrosis with few septa; 3, portal fibrosis with many septa; and 4, cirrhosis.

Immunohistochemistry

Paraffin-embedded liver sections from patients with HCV infection were deparaffinized and subjected to immunohistochemical staining using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) with an anti-human IRS1 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or an anti-human IRS2 polyclonal antibody (Santa Cruz Biotechnology), and developed with 3,3′-diaminobenzidine. The primary antibodies for IRS1 and IRS2 were used at a 1:100 dilution. The specificity of IRS1 and IRS2 staining was confirmed by immunization using an excess amount of the N-terminal peptide of IRS1 and IRS2.

Immunoblotting

Immunoblotting was performed as previously described[28,29] using antibodies against the following: IRS1, IRS2, insulin receptor (Chemicon, Temecula, CA), SOCS-3, Myc (Santa Cruz Biotechnology), phospho-(Tyr) p85 subunit of phosphatidylinositol 3-kinase (PI3K; Cell Signaling Technologies, Beverly, MA), phospho-(Ser 473)-Akt (Cell Signaling Technologies), signal transducer and activation of transcription (STAT) 5 (Santa Cruz Biotechnology), or ubiquitin (Santa Cruz Biotechnology). Equal amounts of protein (40 μg) from liver homogenates or cell extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 7.5% acrylamide gel. The resolved proteins were transferred electrophoretically onto polyvinylidene difluoride membranes (Amersham Int., Buckinghamshire, UK). The membranes were incubated with the primary antibodies indicated in each figure, and were subsequently incubated by the secondary antibodies: a horseradish peroxidase-conjugated goat anti-mouse IgG (Amersham Int.) and a horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham Int.). The membranes were then incubated with chemiluminescence reagents (ECL kit; Amersham Int.) and immediately exposed on radiograph film. In the experiment for ubiquitination of IRS1 and IRS2 (Figure 4, b and c), cell extracts were immunoprecipitated with anti-IRS1 or anti-IRS2 antibodies and then immunoblotted with anti-ubiquitin antibody as previously described.[23]

Core and HCV cDNA

The HCV core region (573 nucleotides) was amplified by reverse transcriptase- polymerase chain reaction, using HCV RNA as a template extracted from the serum of a patient with HCV (genotype Ib) infection. The nucleotide sequence is 98% identical and the amino acid sequence is 100% identical to those of HCV strain MD7-1.[30] The HCV core region was subcloned into expression vector pcDNA3 with the NH_2-terminal Myc tag. Expression of all HCV proteins, including the core, envelope proteins (E1and E2), and nonstructural proteins (NS1, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) were confirmed by immunoblotting in our previous study.[23]

HCV Core-Transgenic Mice

The transgenic mouse was generated using a construct carrying the HCV core cDNA (genotype 1b) fused to the promoter of the HBV X gene.[23] Transgenic mice were developed using conventional methods (C57BL/6 × DBA/2). F1 mice were used to obtain fertilized eggs and those founder mice were mated with C57BL/6 mice for more than five generations. The expression of HCV core was expressed in various tissues including brain, heart, lung, kidney, thymus, and liver. All animal experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the University of Kurume Institutional Animal Care and Use Committee.

Cells and Transfection

HepG2 cells derived from a human hepatoblastoma and retaining many of the differentiated features of mature hepatocytes,[31] Huh 7 and HLF cells derived from hepatocellular carcinomas,[32] and primary mouse embryonic fibroblast (MEF) cells from heterozygous ($SOCS3^{-/-}$) SOCS3 knockout mice and from wild-type ($SOCS3^{+/+}$) mice were cultured in Dulbecco's modified Eagle's medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% dialyzed fetal bovine serum (Life Technologies, Gaithersburg, MD). The expression vector carrying the HCV core region was transfected using synthetic liposomes (Lipofectamine 2000; Life Technologies) in Opti-MEM I (Life Technologies) as previously described.[33,34] Cell extracts for each experiment were prepared 24 hours after transfection. In some experiments, 10 μmol/L of carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG132; Peptide Institute, Osaka, Japan), a proteosomal proteolysis inhibitor, was mixed with cDNA of Myc-tagged HCV core and incubated for 1 hour. To examine the effects of HCV core on insulin signaling, stable transfectants of HCV core in the HLF were used.

Assay for 6-Phosphofructo-2-Kinase (EC 2.7.1.1; Fru 6-P,2-Kinase)

The activity of Fru 6-P,2-kinase was assayed by measuring formation of fructose 2,6-bisphosphate as described previously.[35]

Statistical Analysis

All data are expressed as mean ± SD. Differences between two groups were analyzed using the Mann-Whitney *U*-test. Statistical comparisons among multiple groups were performed by analysis of variance followed by Scheffé's post hoc test using StatView Power PC version for Macintosh (version 5.1; SAS Institute, Cary, NC). *P* values <0.05 were considered significant.

Table 1. Characteristics of All Patients

	Control	CH-C	CH-B	AIH	Fatty liver	PBC
Number	20	158	54	36	40	49
Age (yr)	52 ± 10	53 ± 8	42 ± 9	50 ± 11	44 ± 7	51 ± 8
Sex						
Female	12 (60.0%)	63 (39.9%)	29 (53.7%)	31 (86.1%)	23 (57.5%)	41 (83.7%)
Male	8 (40.0%)	95 (60.1%)	25 (46.3%)	5 (13.9%)	17 (42.5%)	8 (16.3%)
Body mass index (kg/m^2)	22.3 ± 1.9	22.8 ± 2.0	22.0 ± 1.5	22.2 ± 1.8	23.2 ± 3.0	22.5 ± 1.8
Aspartate aminotransferase (U/l)	25 ± 16	70 ± 29	86 ± 57	71 ± 39	35 ± 37	23 ± 12
Alanine aminotransaminase (U/l)	28 ± 18	77 ± 36	94 ± 56	85 ± 50	43 ± 33	33 ± 13
Albumin (g/dl)	3.8 ± 0.3	3.8 ± 0.3	3.7 ± 0.3	3.7 ± 0.4	4.0 ± 0.4	3.7 ± 0.3
Total bilirubin (mg/dl)	0.6 ± 0.2	0.7 ± 0.2	0.6 ± 0.3	1.0 ± 0.8	0.6 ± 0.2	0.7 ± 0.3

Note. Data are expressed as mean ± SD or number of patients. All patients were Japanese. All the diagnoses are based on clinical, serological, and histological evidences.
CH-C, chronic hepatitis C; CH-B, chronic hepatitis B; AIH, autoimmune hepatitis; PBC, primary biliary cirrhosis.

Results

Characteristics of All Patients

We enrolled 337 patients with noncirrhotic chronic hepatobiliary diseases and 20 controls (histologically normal liver). Clinical and laboratory data for these patients are summarized in Table 1. All patients were Japanese. Patients with HBV infection and fatty liver were younger than the other groups and females constituted more than 80% of the group of patients with AIH and PBC. Serum aspartate aminotransferase and alanine aminotransferase levels were increased in all of the groups except for controls. There was no significant difference in BMI among all of the groups. Serum albumin levels and bilirubin levels were normal in all of the groups.

Changes in Glucose Metabolism in Patients with Various Chronic Liver Diseases

Fasting glucose levels were within normal range in all of the groups and showed no significant differences among the groups. However, fasting insulin levels were ~1.5 times higher in patients with HCV infection compared to the other groups (Figure 1, a and b). β-Cell function and insulin resistance were evaluated by HOMA-β and HOMA-IR, respectively. HOMA-β levels were increased in patients with HCV infection compared to controls (Figure 1c). HOMA-IR levels were significantly higher in patients with HCV infection compared to the other groups (Figure 1d).

The Involvement of Virological Factors in HCV-Associated Hyperinsulinemia

All serum samples from patients with HCV infection were HCV-RNA-positive. HCV genotypes were classified according to the classification system of Simmonds and colleagues.[25] Genotype 1a and 3 were not found in any sample. Three samples were excluded in this analysis because of mixed HCV genotypes or undetermined HCV genotype. There was no significant difference in fasting insulin levels among different HCV genotypes (Figure 2a). On the other hand, in patients with >44 fmol/L of HCV core, fasting insulin levels were significantly elevated compared to patients with undetectable levels (<44 fmol/L) of HCV core (Figure 2b).

Histological Parameters and Fasting Insulin Levels

Liver specimens were evaluated according to the METAVIR system.[27] There was no significant difference in fasting insulin levels among different activities

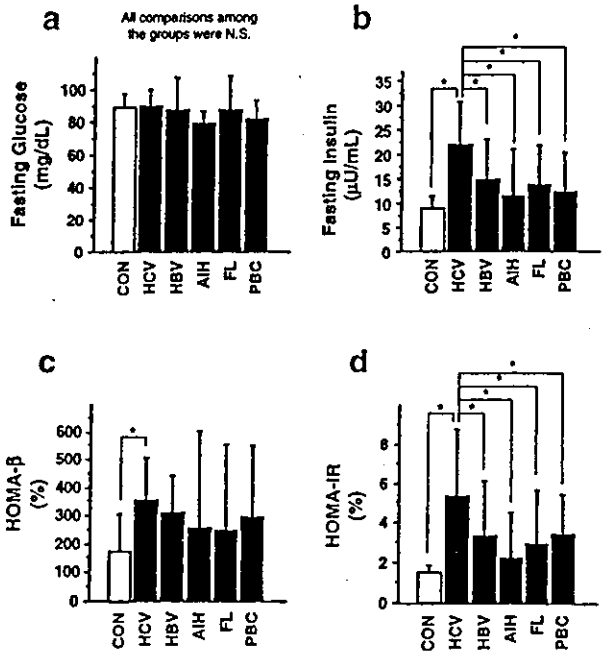

Figure 1. Fasting glucose and insulin levels, β-cell function, and insulin resistance in patients with various chronic liver diseases. Fasting plasma glucose (a) and fasting serum insulin (b) were measured. HOMA-β (c) and HOMA-IR (d) were calculated (see Materials and Methods). Values were expressed as mean ± SD. The comparisons between the groups were made using analysis of variance with Scheffé's post hoc test. N.S., not significant. *, $P < 0.05$. CON, histologically normal livers as controls ($n = 20$); HCV, chronic hepatitis C virus infection ($n = 158$); HBV, chronic hepatitis B virus infection ($n = 54$); AIH, autoimmune hepatitis ($n = 36$); FL, fatty liver ($n = 40$); PBC, primary biliary cirrhosis ($n = 49$).

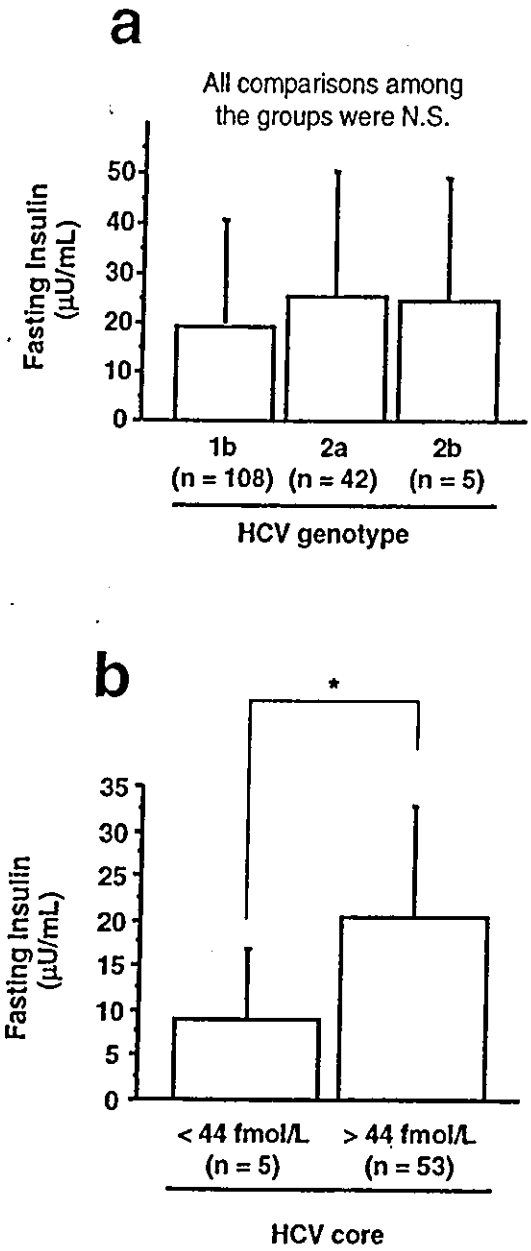

Figure 2. The involvement of virological factors in HCV-associated hyperinsulinemia. a: HCV genotypes and fasting insulin levels. Three cases that showed mixed or undetermined HCV genotypes were excluded. Values were expressed as mean ± SD. The comparisons between the groups were made using analysis of variance with Scheffé's post hoc test. N.S., not significant. b: HCV core and fasting insulin levels. Values were expressed as mean ± SD. The comparison between the two groups was made using the Mann-Whitney *U*-test. *, $P < 0.05$.

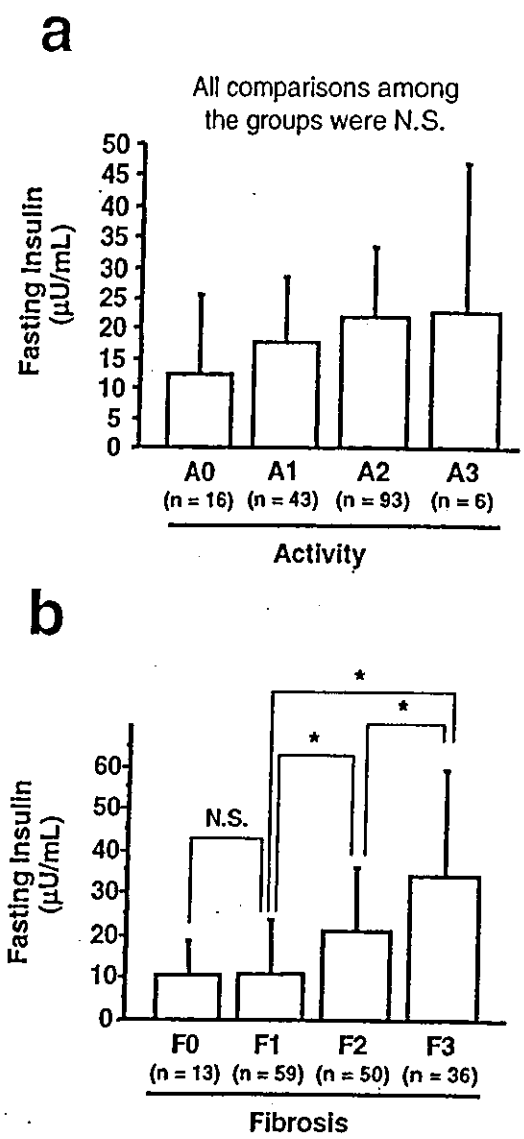

Figure 3. Histological parameters and fasting insulin levels in patients with HCV infection. The liver specimens were evaluated according to the METAVIR system (see Materials and Methods). a: Activity and fasting insulin levels. b: Fibrosis and fasting insulin levels. Values were expressed as mean ± SD. The comparisons between groups were made using analysis of variance with Scheffé's post hoc test. N.S., not significant. *, $P < 0.05$.

(Figure 3a). Although no significant difference was seen in fasting insulin levels between F0 and F1, fasting insulin levels were significantly increased in F2 compared to those of F0 and F1. Fasting insulin levels in F3 were elevated more than those of F0, F1, and F2 (Figure 3b). A similar relationship was also found between HOMA-IR levels and the degree of hepatic fibrosis (data not shown).

Protein Expression Levels of IRS1 and IRS2 in the Liver from Patients with HCV Infection and HCV Core-Tg Mice

The protein expression levels of IRS1 and IRS2 in liver samples from controls and patients with HCV infection were examined by immunostaining and immunoblotting. In control livers, immunostaining demonstrated that periportal hepatocytes, rather than perivenular hepatocytes, highly expressed both IRS1 and IRS2, showing lobular heterogeneity of IRS1 and IRS2 expression (Figure 4a). Decreased IRS1 and IRS2 expression levels along with progression of hepatic fibrosis were seen in periportal hepatocytes. Immunoblotting showed that IRS1 and IRS2 expression levels decreased with the progression of hepatic fibrosis in livers from patients with HCV infection

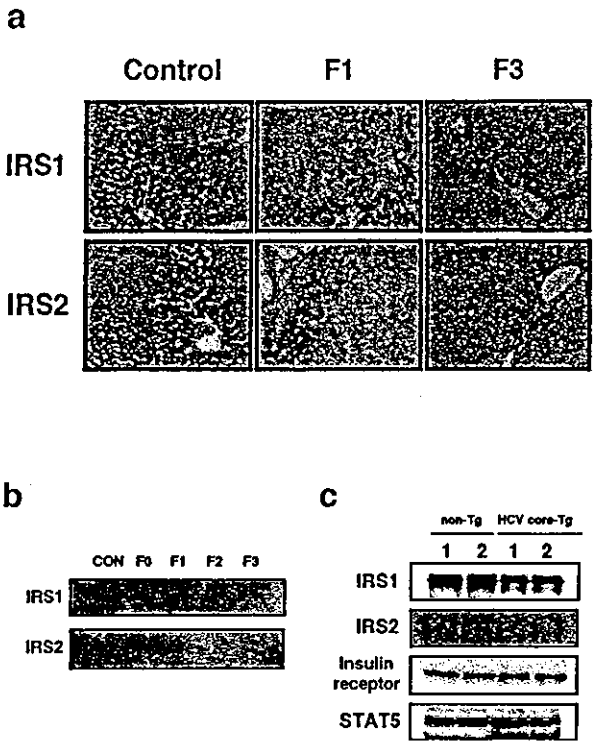

Figure 4. Protein expression levels of IRS1 and IRS2 in livers from patients with HCV infection and HCV core-transgenic (Tg) mice. **a:** Immunostaining for IRS1 and IRS2. IRS1 (**top column**) and IRS2 (**bottom column**) staining of liver sections from control (**left column**), F1 (**middle column**), and F3 (**right column**). Expression of IRS1 and IRS2 were visualized by 3,3′-diaminobenzidine (brown). **Arrow** indicates portal vein. **b:** Immunoblotting for IRS1 and IRS2. Proteins (40 μg) in liver extracts from control and patients with HCV infection were immunoblotted with anti-IRS1 antibodies (**top column**) or anti-IRS2 antibodies (**bottom column**). **c:** Hepatic IRS1 and IRS2 expression in HCV core-Tg mice. Non-Tg littermates and HCV core Tg-mice livers (8 weeks old) were subjected to immunoblotting for IRS1, IRS2, insulin receptor, and STAT5 (as a reference protein). These experiments were repeated three times and representative immunoblotting and immunostaining images are shown. Original magnifications, ×400.

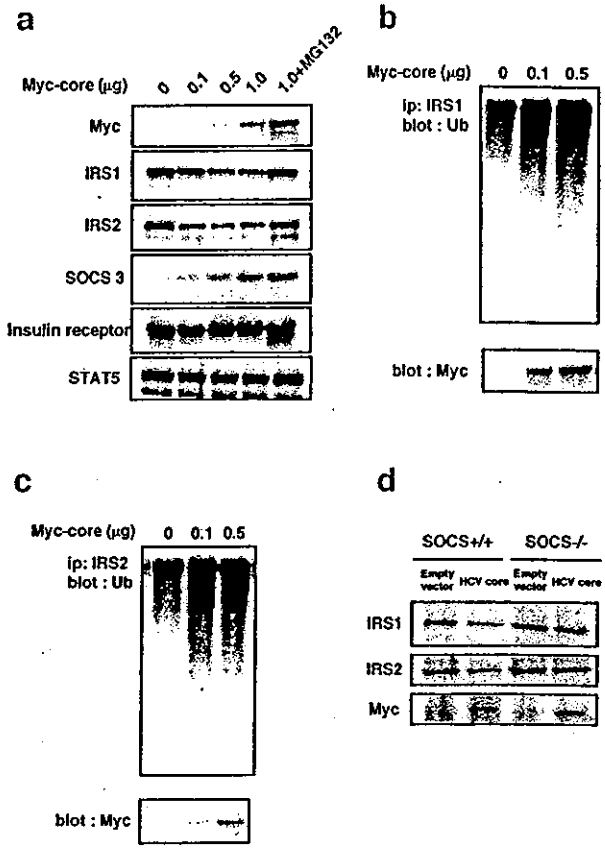

Figure 5. The effect of HCV core on insulin signaling molecules. **a:** The effect of HCV core on IRS1, IRS2, SOCS3, and insulin receptor expression. Myc-tagged HCV core was transiently expressed in HepG2 cells. Twenty-four hours after transfection the cell extracts were immunoblotted with the indicated antibodies. MG132 (10 μmol/L) was added with cDNA of Myc-tagged HCV core and incubated for 1 hour. STAT5 was used as a reference protein. **b** and **c:** The identification of ubiquitinated IRS1 and IRS2. Whole-cell extracts (40 μg of crude extract) were subjected to immunoprecipitation with IRS1 or IRS2 and followed by immunoblotting using anti-ubiquitin monoclonal antibody. **d:** The effect of HCV core on IRS1 and IRS2 expression in SOCS3$^{-/-}$ MEF cells. Twenty-four hours after transfection of HCV core, MEF cell extracts were immunoblotted with anti-IRS1 antibodies or anti-IRS2 antibodies. Representative immunoblotting images from three separate experiments are shown.

(Figure 4b), confirming results of immunostaining. We developed HCV core-Tg mice in which the HCV core protein is expressed ubiquitously. The HCV core-Tg mouse is an informative animal model for studying the pathogenesis of HCV infection.[36,37] In two independent HCV core-Tg mice, decreased expression of hepatic IRS1 and IRS2, but not of insulin receptor, were also demonstrated compared to two independent wild-type littermates (Figure 4c).

The Effect of HCV Core on IRS1, IRS2, SOCS3, and Insulin Receptor Expression

The effects of HCV core on IRS1, IRS2, SOCS3, and insulin receptor expression were examined in HepG2 and Huh7 cells prepared by transient transfection with Myc-tagged HCV core and HLF cells with stable transfection of Myc-tagged HCV core. Expression of Myc-tagged HCV core was confirmed by immunoblotting for Myc in both cells. HCV core dose dependently decreased IRS1 and IRS2 expression in HepG2 cells (Figure 5a). In contrast, SOCS3 expression was dose dependently increased by transient transfection with Myc-tagged HCV core in HepG2 cells. No changes in insulin receptor expression and STAT5 (used as a reference protein) were seen in HepG2 cells transfected with Myc-tagged HCV-core (Figure 5a). We also analyzed the effects of a proteosomal proteolysis inhibitor, MG132. The treatment with MG132 caused an increase in expression levels of IRS1 and IRS2 (Figure 5a). Similar results were obtained in Huh7 cells with transient transfection of HCV core and HLF cells with stable transfection of HCV core (data not shown).

Role of Ubiquitination in the Regulation of IRS1 and IRS2 Expression

To investigate the involvement of ubiquitination in down-regulation of IRS1 and IRS2 in HepG2 cells transfected with HCV core, whole-cell extracts were immunoprecipitated with anti-IRS1 or anti-IRS2 antibodies and immunoblotted with anti-ubiquitin monoclonal antibodies. HCV

core caused an accumulation of ubiquitin-conjugated IRS1 (Figure 5b) and IRS2 (Figure 5c). Expression of Myc-tagged HCV core was confirmed by immunoblotting for Myc (Figure 5, b and c).

Role of SOCS3 in the Regulation of IRS1 and IRS2 Expression

Because SOCS3$^{-/-}$ mice display embryonic lethality, we examined the association between SOCS3 and regulation of IRS1 and IRS2 by using SOCS3$^{-/-}$ MEF cells. HCV core down-regulated IRS1 and IRS2 in SOCS3$^{+/+}$ MEF cells. On the other hand, HCV core did not cause down-regulation of IRS1 and IRS2 in SOCS3$^{-/-}$ MEF cells (Figure 5d).

The Effects of HCV Core on Insulin Signaling

HLF cells with stable transfection of HCV core were treated with insulin (100 ng/ml) from 0 minutes to 60 minutes and phosphorylation of p85 subunit of PI3K and Akt were determined. Insulin-induced phosphorylation of p85 subunit of PI3K and Akt was observed in HLF cells transfected with empty vector. On the other hand, HCV core decreased phosphorylation of p85 subunit of PI3K and Akt at the base line (0 minutes) and inhibited insulin-induced phosphorylation of p85 subunit of PI3K and Akt (Figure 6a). STAT5 protein (reference protein) levels were unchanged by insulin stimulation. Insulin activated Fru 6-P, 2-kinase, a downstream of Akt signal and one of the potent regulators of glycolysis, and decreased medium glucose levels (Figure 6, b and c). HCV core suppresses insulin-induced activation of Fru 6-P, 2-kinase and decreases in medium glucose levels (Figure 6, b and c).

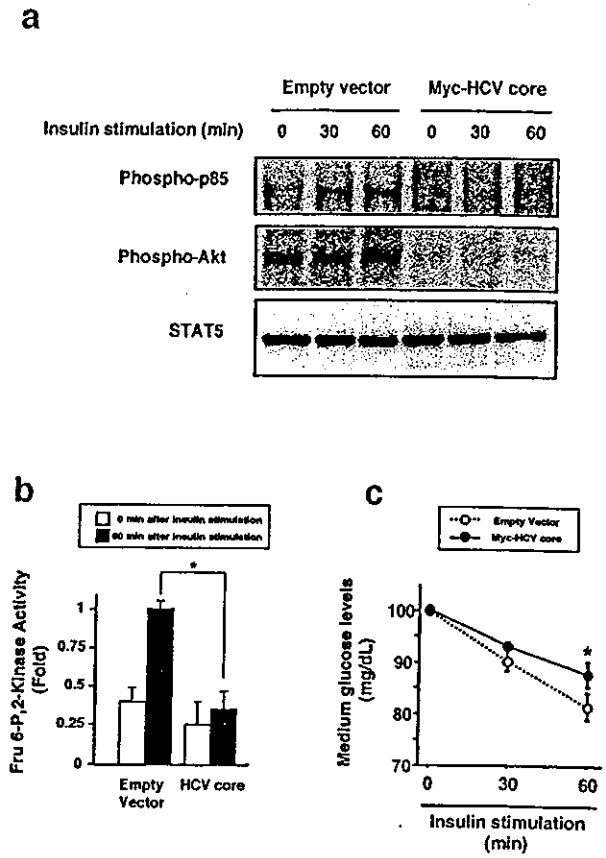

Figure 6. The effects of HCV-core on insulin signaling. HLF cells with transfection of empty vector or Myc-HCV core were incubated in the presence of insulin (100 ng/ml) for 60 minutes. a: The effect of HCV-core on insulin-induced phosphorylation of p85 subunit of phosphatidylinositol 3-kinase (PI3K) and Akt. Whole-cell lysates were subjected to immunoblotting for phospho-p85 subunit of PI3K and phospho-Akt. STAT5 was used as reference protein. Representative immunoblotting images from three separate experiments are shown. b: The effect of HCV-core on insulin-induced activation of Fru 6-P, 2-kinase. Sixty minutes after insulin stimulation, Fru 6-P, 2-kinase activity in whole-cell lysates was assayed. Values were expressed as mean ± SD. The comparison between the two groups was made using the Mann-Whitney *U*-test. *, $P < 0.05$. c: The effect of HCV-core on insulin-induced activation of glucose uptake. Glucose levels in culture medium were measured at 0, 30, and 60 minutes after insulin stimulation. Values were expressed as mean ± SD. The comparisons between groups were made using analysis of variance with Scheffé's post hoc test. N.S., not significant. *, $P < 0.05$.

Discussion

In this study, we showed that more severe insulin resistance was present in noncirrhotic patients with HCV infection than in patients with other hepatobiliary diseases. Insulin resistance was associated with the presence of serum HCV core, the severity of hepatic fibrosis and decreased expression of hepatic IRS1 and IRS2 in patients with HCV infection. HCV core down-regulated the expression of IRS1 and IRS2 in human hepatoma cell lines as well as in whole animals. These findings suggest that HCV causes changes in specific hepatic molecules regulating glucose metabolism and results in severe insulin resistance. A possible mechanism is that HCV core-induced SOCS3 promotes proteosomal degradation of IRS1 and IRS2 through ubiquitination.

Although fasting glucose levels were similar among all of the groups, fasting insulin and HOMA-IR levels, a indicator of insulin resistance, were significantly increased in patients with HCV infection compared to the other hepatobiliary disorders. β-Cell function evaluated by HOMA-β was also increased in patients with HCV infection compared to controls. These findings indicate that HCV infection induced insulin resistance and fasting glucose levels were compensated by hyperinsulinemia. Mangia and colleagues[38] reported no association between HCV infection and DM in noncirrhotic patients and that the prevalence of DM in noncirrhotic patients is comparable to the expected prevalence in the general population. These results are not in accord with our results and this discrepancy may be explained by different evaluation methods for glucose intolerance. They evaluated glucose intolerance by fasting glucose levels and only patients who had >126 mg/dl of glucose levels were considered as abnormal glucose metabolizers. Because fasting glucose levels are compensated by hyperinsulinemia (Figure 1, a and b), cryptic changes in glucose metabolism can be evaluated by measuring fasting insulin or HOMA-IR levels.[24] In our own study, we provide convincing evidence that more severe insulin resistance is present in noncirrhotic patients with HCV infection than

in patients with other hepatobiliary disorders. Unique mechanisms may underlie HCV-associated severe insulin resistance.

HCV genotype 2a is specifically linked with extrahepatic manifestations such as cryoglobulinemia and benign monoclonal gammopathy.[39,40] Mason and colleagues[6] also reported an association between genotype 2a and DM, whereas no association was found between fasting insulin levels and HCV genotypes in our study. The limited number of patients may prevent drawing definite conclusions in both studies. HCV core can modulate cell signaling.[23] Therefore, we investigated the relationship between HCV core and fasting insulin levels. In patients with undetectable levels of HCV core, fasting insulin levels were within the normal range. In contrast, in patients with detectable levels of HCV core, fasting insulin levels were increased. Thus, HCV core seems to play a crucial role in HCV-associated insulin resistance.

Then, we examined an association between histological parameters and insulin resistance. Although no significant association was found between activity and insulin resistance, serum insulin levels and HOMA-IR levels were significantly increased with the severity of hepatic fibrosis. These data are in good agreement with recent report by Hui and colleagues.[13] HOMA-IR is independently associated with an increased rate of fibrosis progression.[13,41] Insulin stimulates hepatic stellate cells to proliferate and secrete extracellular matrix.[42] Thus, it appears that insulin resistance contributes to fibrotic progression in patients with HCV infection. To innovate therapies for prevention of fibrotic progression, it is important to investigate the molecular mechanisms for HCV-induced insulin resistance.

IRS1 and IRS2 act as important mediators of insulin action and down-regulation of hepatic IRS1 and IRS2 results in an increase in hepatic insulin resistance. Knockout of the IRS1 gene induces insulin resistance and subsequent compensatory hyperinsulinemia.[20] Knockout of the IRS2 gene causes severe diabetes as a consequence of insulin resistance and disturbance of insulin secretion.[22] We showed that decrease in expression of IRS1 and IRS2 was associated with the progression of hepatic fibrosis. Down-regulation of IRS1 and IRS2 was also seen in livers from HCV core-Tg mice. These data suggest that down-regulation of IRS1 and IRS2 is responsible for compensatory hyperinsulinemia and progression of hepatic fibrosis.

The effects of HCV core on the expression of IRS1 and IRS2 were investigated by simplified *in vitro* experiments. HCV is a positive-strand RNA virus consisting of a putative structure (core, E1, E2/p7) and at least six nonstructural proteins (NS2, NS3, NS4A, NS5A, NS5B). HCV core is implicated in cellular transformation.[37] In this study, HCV core decreased expression of IRS1 and IRS2 in human hepatoma cell lines. These *in vitro* findings add weight to the results of our human studies.

One of the negative modulation mechanisms of IRS1 and IRS2 is proteosomal degradation.[43] On the other hand, we previously reported that HCV core induced SOCS3 in mouse fibroblast NIH 3T3 cells.[23] In the current study, HCV core-induced SOCS3 was also validated in HepG2 cells as well as Huh7 cells and HLF cells. The SOCS family of proteins has similar structural characteristics referred to collectively as a "SOCS box," a unique NH_2-terminal domain of variable length, a central Src-homology 2 domain, and a COOH-terminal, with this structural resemblance reflecting functional similarities among SOCS proteins. The SOCS box acts as an adaptor to facilitate the ubiquitination of signaling proteins and their subsequent targeting to the proteosome by complexing with Elongins B and C.[43,44] These facts and our findings led to the assumption that HCV core-induced SOCS3 promoted proteosomal degradation of IRS1 and IRS2 through ubiquitination. To test this hypothesis, HCV core-transfected cells were incubated with MG132, a potent proteosomal proteolysis inhibitor. MG132 blocked HCV core-induced decrease of IRS1 and IRS2 in HepG2. Ubiquitination of IRS1 and IRS2 was increased by transfection of HCV core. Moreover, HCV core did not cause down-regulation of IRS1 and IRS2 in SOCS3$^{-/-}$ MEF cells. All of these data support our hypothesis. Transient overexpression of SOCS3 in mouse liver induces fasting hyperglycemia, and fasting hyperinsulinemia. These changes are returned to normal as SOCS3 expression subsided.[43] Thus, studies in whole animals lend added credence to our hypothesis that HCV core-induced SOCS3 promotes proteosomal degradation of IRS1 and IRS2 through ubiquitination.

To verify the biological significance of these studies on IRS1 and IRS2, the effects of HCV core on insulin signaling were examined. Insulin phosphorylates the p85 subunit of PI3K and Akt, which are downstream components of IRS in liver.[45] Akt activates Fru 6-P,2-kinase, one of the key enzymes of glycolysis, and glucose uptake.[46] HCV core inhibited insulin-induced phosphorylation of p85 subunit of PI3K and Akt, activation of Fru 6-P,2-kinase, and glucose uptake. Thus, HCV core-transfected HLF cells were resistant to insulin stimulation compared with empty vector-transfected HLF cells, suggesting biological significance of HCV core-induced down-regulation of IRS1 and IRS2.

Recently, Aytug and colleagues[47] studied changes in the upstream insulin-signaling molecules in the liver specimens obtained from patients with chronic HCV infection and showed impairments of insulin-stimulated PI3K activity and Akt phosphorylation, which were in good agreements with our findings. However, our data for IRS1 differed from those reported by Aytug and colleagues.[47] They showed increased IRS1 expression with reduction in tyrosine phosphorylation. Although the reason for this discrepancy remains unclear, one possibility is that their HCV-infected patients show higher BMI values than those of ours. Obesity is a well-recognized risk factor for the development of insulin resistance. Adipocytes secrete a large number of factors with diverse functions. Tumor necrosis factor-α and free fatty acids are secreted by adipocytes and are known to impair insulin signaling by reducing IRS1 tyrosine phosphorylation.[48,49] Increased IRS1 expression may be for an adaptation to reduction of IRS1 tyrosine phosphorylation. Moreover, leptin, which is also secreted by adipocytes, is involved in the development of HCV-associated insulin

resistance.[50] Thus, there may exist several molecular mechanisms for HCV-associated insulin resistance.

In conclusion, more severe insulin resistance was present in noncirrhotic patients with HCV infection than in patients with other hepatobiliary diseases. We uncovered a unique mechanism of HCV-associated insulin resistance. HCV core-induced SOCS3 may promote proteosomal degradation of IRS1 and IRS2 through ubiquitination.

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MAJOR ARTICLE

Predicting Relapse after Cessation of Lamivudine Monotherapy for Chronic Hepatitis B Virus Infection

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There have been reports of relapse after cessation of lamivudine monotherapy for hepatitis B virus (HBV) infection. The aim of this study was to examine factors that predict posttreatment relapse. Comparison 22 patients who experienced relapse with 11 who did not after cessation of therapy showed that predictive factors for nonrelapse were hepatitis B e antigen seroconversion and duration of undetectable HBV DNA load (<0.7 log IU/mL), as determined by HBV real-time detection direct testing. However, 7 of 12 patients with seroconversion experienced relapse after cessation of therapy. Multivariate analysis revealed that the duration of an undetectable HBV DNA load was the only independent predictive factor for nonrelapse (odds ratio, 0.50; 95% confidence interval, 0.27–0.9). More-prolonged lamivudine therapy is required after seroconversion, and persistent duration of an HBV DNA level of <0.7 log IU/mL for >6 months can more accurately aid in the decision of when to stop lamivudine therapy.

Hepatitis B virus (HBV) infection is a serious clinical problem worldwide that can lead to progressive liver disease and hepatocellular carcinoma [1]. Patients with genotype C infection have a more aggressive clinical phenotype than do those with genotype B infection, leading to poorer clinical outcomes [2].

Lamivudine monotherapy is currently considered to be the best therapeutic option for patients with chronic hepatitis B, irrespective of hepatitis B e antigen (HBeAg) status [3]. It is generally accepted that the probability of viral resistance or virologic breakthroughs increases with the prolongation of lamivudine therapy by drug-resistant HBV mutants [4–7], possibly followed by acute hepatitis flares, hepatic decompensation, and possibly fatal hepatic failure [8–10].

The Asia-Pacific Consensus on the Prevention and Management of Chronic Hepatitis B and C has recommended that full HBeAg seroconversion, defined as undetectable HBeAg and HBV DNA with reappearance of antibody to HBeAg (anti-HBe), may be considered a suitable end point for lamivudine therapy. On the basis of currently available data, it is also recommended that lamivudine therapy be maintained for 4–6 months after achieving HBeAg seroconversion to decrease the chance of posttreatment relapse [11]. This study evaluated the posttreatment durability of lamivudine and the predictive factors for cessation of lamivudine therapy without relapse for patients infected with HBV genotype C.

PATIENTS, MATERIALS, AND METHODS

Patients. We analyzed 33 Japanese patients (24 men and 9 women; mean age ± SD, 42.1 ± 9.6 years) with

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chronic HBV infection after cessation of lamivudine therapy. All patients were administered 100 mg of lamivudine per day for ≥6 months (median, 12 months; range, 6–25 months). Once an undetectable serum HBV DNA level (<3.7 log genome equivalents [LGE]/mL), as measured by transcription-mediated amplification-hybridization protection assay (TMA-HPA; Chugai Diagnostics Science), and a serum alanine aminotransferase (ALT) level of <40 IU/L (i.e., the upper limit of normal) had both been maintained for ≥6 months, lamivudine therapy was stopped prospectively in this study. In addition, for HBeAg-positive patients, HBeAg seroconversion was used as another criterion for stopping lamivudine therapy.

The patients sought care at Chukyo Hospital (Nagoya, Japan), Nagoya City University Hospital (Nagoya), and Kurume University Hospital (Kurume, Japan) during the period of 1996–2002. All patients provided written informed consent. Table 1 shows the pretreatment demographic and clinical characteristics of these 33 patients, who were positive for serum hepatitis B surface antigen (HBsAg). We investigated only patients who were infected with HBV genotype C. Five (15%) of 33 patients had liver cirrhosis, and 28 patients (85%) had chronic hepatitis. All patients were then clinically observed after cessation of lamivudine monotherapy (median duration of posttreatment monitoring, 13 months; range, 9–26 months).

Methods. Presence of serum HBsAg, HBeAg, and anti-HBe (as measured by chemiluminescent enzyme immunoassay [Ortho Clinical Diagnostics]) and levels of HBV DNA and ALT were tested every month. HBV DNA was mainly measured using the TMA-HPA assay, which has a detection range of 3.7–8.7 LGE/mL; when the HBV DNA level was undetectable (<3.7 LGE/mL), we performed a highly reproducible and sensitive HBV real-time detection direct test (HBV RTD-Direct test [SRL Inc.]) retrospectively, which combines the use of a DNA extraction system based on magnetic bead–coated polyclonal anti-HBsAg. The HBV RTD-Direct test has a dynamic range of 0.7–8.0 log IU/mL. This procedure has a good correlation with the TMA-HPA assay and a higher sensitivity than TMA-HPA by ~100 times [12].

The end point was relapse of infection after cessation of therapy. Relapse was defined as reappearance of serum HBV DNA (>3.7 LGE/mL, as measured using the TMA-HPA assay) plus a reactivation in the serum ALT to >80 U/L, as well as HBeAg seroreversion, while receiving treatment. Possible predictive factors were then analyzed.

Statistical evaluation. Data are expressed as mean ± SD or median (range). The primary focus of this analysis was to compare patients who experienced relapse (after cessation of lamivudine therapy, reappearance of serum HBV DNA [assessed by TMA-HPA] plus ALT level reactivation [i.e., a level of >80 IU/L], as well as HBeAg seroconversion while receiving treatment) with those who did not experience relapse. The Mann-Whitney *U* test and the χ^2 test were used to analyze the data. The effects of age, sex, prevalence of cirrhosis, presence of HBeAg, pretreatment ALT level, pretreatment HBV DNA level, duration of lamivudine therapy, duration of an HBV DNA level of <3.7 LGE/mL, and duration of an HBV DNA level of <0.7 log IU/mL were assessed by logistic regression analysis. The cumulative relapse rate was calculated by Kaplan-Meier method, and the difference was determined by the log-rank test. $P < .05$ was considered to be statistically significant. For statistical analysis, we used Stata statistical software, version 7.0 (Stata).

RESULTS

All patients tested positive for HBV DNA and HBsAg before treatment; 19 patients (58%) tested positive for HBeAg. All patients also had abnormal ALT levels before receiving treatment. Nineteen patients (58%) had suppressed, undetectable HBV DNA levels (<0.7 log IU/mL), as measured by HBV RTD-

Table 1. Characteristics of 33 hepatitis B virus (HBV)–infected patients after cessation of lamivudine therapy.

Characteristic	Value
Age, mean years ± SD	42.1 ± 9.6
Male sex, no. (%) of patients	24 (73)
Presence of cirrhosis, no. (%) of patients	5 (15)
HBeAg positive, no. (%) of patients	19 (58)
Seronegative	13 (68)
Seroconversion	12 (63)
Pretreatment ALT level, median U/L (range)	152 (56–671)
Pretreatment HBV DNA level, median LGE/mL (range)	7.4 (4.1–8.9)
Duration of lamivudine therapy, median months (range)	12 (6–25)
Posttreatment follow-up duration, median months (range)	13 (9–26)

NOTE. ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen; LGE, log genome equivalents.

Table 2. Comparison of the characteristics of hepatitis B virus (HBV)–infected patients who experienced relapse and those who did not.

Characteristic	Patients who experienced relapse (n = 22)	Patients who did not experience relapse (n = 11)	P
Age, mean years ± SD	42.5 ± 8.6	41.4 ± 11.7	NS
Male sex, no. (%) of patients	16 (73)	8 (73)	NS
Presence of cirrhosis, no. (%) of patients	4 (18)	1 (9)	NS
HBeAg positive, no. (%) of patients	14 (64)	5 (45)	NS
Seronegative	8 (57)	5 (100)	NS
Seroconversion	7 (50)	5 (100)	.047
Pretreatment ALT level, mean U/L ± SD	193.3 ± 142.3	181.4 ± 112.3	NS
Pretreatment HBV DNA level, mean LGE/mL ± SD	7.5 ± 1.1	7.1 ± 0.8	NS
Duration of lamivudine therapy, mean months ± SD	11.6 ± 4.0	12.3 ± 5.4	NS
Duration of HBV DNA level of <3.7 LGE/mL, months			NS
Mean ± SD	7.7 ± 4.7	9.5 ± 4.9	
Median (range)	6.5 (2–18)	9 (5–22)	
Duration of HBV DNA level of <0.7 log IU/mL, months			.0067
Mean ± SD	1.2 ± 1.7	5.6 ± 4.9	
Median (range)	0.5 (0–5)	5 (0–16)	

NOTE. ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; LGE, log genome equivalents.

Direct testing during treatment. During this period, 13 (68%) of 19 patients tested HBeAg seronegative, and 12 (63%) of 19 HBeAg-positive patients had seroconversion.

After cessation of lamivudine therapy, 22 patients (67%) experienced relapse and 11 (33%) did not. In a univariate comparison of patients who experienced relapse with those who did not, predictive factors for the absence of relapse after cessation of lamivudine were HBeAg seroconversion (7 [50%] of 14 vs. 5 [100%] of 5; P = .047) and duration of an HBV DNA level of <0.7 log IU/mL, as measured using the HBV RTD-Direct test (1.2 ± 1.7 vs. 5.6 ± 4.9 months; P = .0067). Duration of an HBV DNA level of <3.7 LGE/mL (as measured using the TMA-HPA assay), total duration of lamivudine therapy, age, sex, presence of cirrhosis, presence of HBeAg, seronegative status, pretreatment ALT level, and pretreatment HBV DNA level were not significant (table 2).

Table 3. Comparison of the characteristics of patients who experienced relapse with those for patients who did not among hepatitis B e antigen–positive, hepatitis B virus (HBV)–infected patients.

Characteristic	Patients who experienced relapse (n = 14)	Patients who did not experience relapse (n = 5)	P
Age, mean years ± SD	41.3 ± 8.2	37.8 ± 10.4	NS
Male sex, no. (%) of patients	12 (86)	3 (60)	NS
Presence of cirrhosis, no. (%) of patients	2 (14)	0 (0)	NS
Seronegative, n/N (%)	8/14 (57)	5/5 (100)	NS
Seroconversion, n/N (%)	7/14 (50)	5/5 (100)	.047
Pretreatment ALT level, mean U/L ± SD	196.1 ± 102.7	159.4 ± 97	NS
Pretreatment HBV DNA level, mean LGE/mL ± SD	8.0 ± 0.7	7.2 ± 0.5	.021
Duration of lamivudine therapy, mean months ± SD	11.6 ± 4.5	13.0 ± 7.2	NS
Duration of HBV DNA level of <3.7 LGE/mL, months			NS
Mean ± SD	6.9 ± 4.7	10.4 ± 6.7	
Median (range)	6 (2–18)	8 (6–22)	
Duration of HBV DNA level of <0.7 log IU/mL, months			.0057
Mean ± SD	0.7 ± 1.3	6.2 ± 6.9	
Median (range)	0 (0–4)	5 (0–16)	

NOTE. ALT, alanine aminotransferase; LGE, log genome equivalents.

Table 4. Biochemical and virologic changes in persons who experienced hepatitis B e antigen seroconversion before and after cessation of lamivudine therapy.

Patient	Relapse	Sex	Age, years	Pretreatment laboratory value: ALT level, IU/L	Pretreatment laboratory value: HBV DNA level, LGE/mL	Duration of therapy, months	Duration of undetectable HBV DNA level, months: <3.7 LGE/mL	Duration of undetectable HBV DNA level, months: <0.7 log IU/mL	Duration of undetectable HBV load after cessation of therapy, months	Pretreatment HBeAg level, IU/L	Pretreatment anti-HBe value, % inhibition	Minimum HBeAg level,[a] IU/L	Maximum anti-HBe value,[b] % inhibition
1	Yes	F	48	84	6.4	16	10	4	11	220	0	0.2	92
2	Yes	M	43	85	7.9	16	13	1	9	130	0	0.2	86
3	Yes	M	46	339	8.5	12	10	1	14	92.3	0	0.9	96
4	Yes	M	47	161	8.3	9	5	0	11	49.9	0	0.9	94.8
5	Yes	M	24	352	8.4	6	4	0	20	31.1	0.7	0.9	89.3
6	Yes	F	28	160	8.6	6	2	0	23	13.4	55.4	0.9	88.9
7	Yes	M	47	62	7.3	11	3	0	16	5.1	82.4	1	77.1
8	No	M	29	320	7	13	10	10	11	130	0	0.2	100
9	No	M	47	95	7	25	22	16	9	145	0	0.4	86.5
10	No	F	48	152	7.2	8	6	5	10	26.4	0	0.6	97
11	No	M	40	72	6.8	12	8	0	21	19.7	0	0.7	91.7
12	No	F	25	158	8	7	6	0	18	195.2	0	0.8	96.4

NOTE. ALT, alanine aminotransferase; anti-HBe, antibody to hepatitis B e antigen; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; LGE, log genome equivalents.

[a] Minimum value of HBeAg after cessation of lamivudine therapy.

[b] Maximum value of anti-HBe after cessation of lamivudine therapy.

To further elucidate the relationship between presence of HBeAg and relapse, a univariate analysis was performed using the data for the 19 HBeAg-positive patients. Predictive factors for absence of posttreatment relapse after cessation of lamivudine therapy in HBeAg-positive patients were HBeAg seroconversion (7 [50%] of 14 vs. 5 [100%] of 5 patients; $P = .047$), duration of an HBV DNA level of <0.7 log IU/mL (0.7 ± 1.3 vs. 6.2 ± 6.9; $P = .0057$), and pretreatment HBV DNA level (8.0 ± 0.7 vs. 7.2 ± 0.5 U/L; $P = .021$) (table 3). Twelve patients (63%) had seroconversion to anti-HBe during treatment; however, 7 of these patients experienced relapse after cessation of lamivudine therapy. Among the patients who experienced HBeAg seroconversion, comparison of 7 patients who experienced relapse with 5 who did not revealed no significant differences; however, patients who experienced relapse had higher pretreatment HBV DNA levels, shorter durations of an HBV DNA level of <0.7 log IU/mL, and lower anti-HBe titers at the end of therapy (table 4).

Multivariate analysis involving 8 factors indicated that a duration of an HBV DNA level of <0.7 log IU/mL (OR, 0.50; 95% CI, 0.27–0.93; $P = .027$) remained the only independent predictive factor for absence of posttreatment relapse. It is interesting to note that this analysis suggests that, when the duration of an HBV DNA level of <0.7 log IU/mL is extended by an additional month, the risk of relapse is reduced by one-half (table 5).

To analyze the relationship between the duration of an HBV DNA level of <0.7 log IU/mL and relapse in more detail, we classified patients into 2 groups: group A, consisting of 28 patients with a <6-months duration of HBV DNA levels of <0.7 log IU/mL before cessation of lamivudine therapy; and group B, consisting of 5 patients with a ≥6-months duration of HBV DNA levels of <0.7 log IU/mL before cessation of lamivudine therapy. The cumulative relapse rate was significantly higher in group A than in group B (79% vs. 0%; $P = .006$) (figure 1).

DISCUSSION

Several clinical trials have shown that lamivudine is effective for improvement of transaminase levels and liver histology findings; however, the end point of the treatment has remained unclear because of relapse after cessation of therapy. In fact, 22 (67%) of 33 patients experienced relapse after cessation of lamivudine therapy in this study. Univariate analysis showed that HBeAg seroconversion and duration of an HBV DNA level of <0.7 log IU/mL was a prognostic indicator of no relapse; multivariate analysis showed that the duration of an HBV DNA level of <0.7 log IU/mL was a prognostic indicator of no relapse.

The TMA-HPA assay, which can detect HBV DNA levels of 3.7–8.7 LGE/mL, is widely used for measuring HBV DNA levels in Japan; however, it was not enough to predict relapse after cessation of lamivudine therapy. By contrast, measurement of HBV DNA levels of 0.7–8.0 log IU/mL by using the HBV RTD-Direct test, which combines the use of a DNA extraction based on magnetic beads and real-time PCR and is also a more sensitive method, revealed that a persistent low titer of HBV DNA

Table 5. ORs of relapse for predictive factors in multivariate analyses.

Factor	OR (95% CI)[a]	*P*
Age, years	0.99 (0.86–1.15)	.964
Male sex, %	2.65 (0.09–77.31)	.572
Cirrhosis, %	0.11 (0.01–5.58)	.268
Presence of HBeAg, %	0.14 (0.01–4.02)	.251
Pretreatment ALT level, U/L	1.00 (0.99–1.02)	.878
Pretreatment HBV DNA level, LGE/mL	0.66 (0.15–2.98)	.591
Duration of lamivudine therapy, months	0.99 (0.43–2.26)	.979
Duration of HBV-DNA level of <3.7 LGE/mL, months	1.95 (0.75–5.08)	.171
Duration of HBV-DNA level of <0.7 log IU/mL, months	0.50 (0.27–0.93)	.027[b]

NOTE. ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; LGE, log genome equivalents.

[a] Each OR is adjusted for other variables in the table.

[b] Statistically significant.

suppressed by lamivudine could lead to a lower relapse rate after cessation of treatment. Use of such a diagnostic kit is in line with the recommendations of previously published reports [13–15].

In this study, the rate of HBe seroconversion was 63%, which is much higher than expected (it was 16% in an Asian study [16]); the most likely reason for the high HBeAg seroconversion rate for the patients was the high baseline ALT level (median, 152 U/L), because it is well documented that high ALT levels predispose patients to earlier HBeAg seroconversion [17]. Although HBeAg seroconversion was predictive of absence of relapse, 7 (58%) of the 12 patients who had HBeAg seroconversion experienced relapse. This observation contrasts with the results from Western countries, in which IFN-α– and lamivudine-induced seroconversion is usually maintained after the cessation of therapy [18–24]. Song et al. [25] suggested that the high relapse rate may be caused by immune tolerance, which is caused by a long-standing viral infection. HBeAg seroconversion does not ensure prolonged suppression of HBV infection in areas where HBV is endemic and where parental transmission is common [26, 27]. Another factor may be the prevalence of different HBV genotypes among geographic regions. Genotypes C and D are associated with lower rates of response to IFN therapy than are genotypes B and A [2, 28]. In addition, the subtype *adw* is reported to be associated with a higher risk of lamivudine resistance than is the *ayw* subtype [2, 28].

Recently, Dienstag et al. [18, 19] suggested that lamivudine

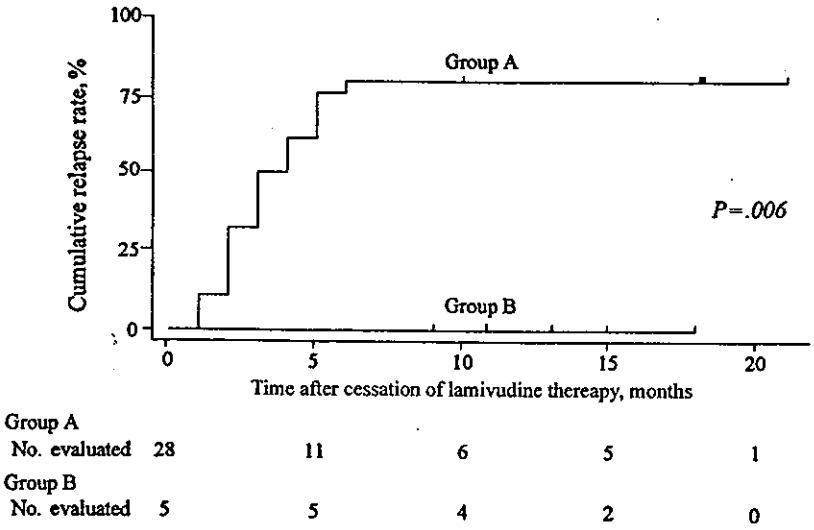

Figure 1. Graph illustrating cumulative relapse rates among 28 patients with <6 months' duration of a hepatitis B virus (HBV) DNA level of <0.7 log IU/mL (group A) and 5 patients with ≥6 months' duration of an HBV DNA level of <0.7 log IU/mL (group B) before cessation of lamivudine therapy. The relapse rate was significantly higher in group A than in group B ($P = .006$).

therapy could be stopped after confirmed HBeAg loss or HBeAg seroconversion. However, our results indicate that more-prolonged lamivudine therapy is needed after seroconversion; the duration of lamivudine therapy should be modified by using results from the highly sensitive HBV RTD-Direct test and by determining pretreatment levels of serum HBV DNA, because these were predictive factors for relapse. On the basis of the results of this study, we suggest that, for patients in whom HBeAg seroconversion and persistent duration of an HBV DNA level of <0.7 log IU/mL of ≥6 months' duration, and in HBeAg-negative patients for whom the duration of an HBV DNA level of <0.7 log IU/mL is ≥6 months, cessation of therapy can be considered. For optimal monitoring for HBV viremia, the sensitive HBV RTD-Direct test could also have therapeutic implications, because it may allow not only early recognition of a virologic breakthrough, but also prediction of an early subsequent biochemical breakthrough.

In summary, our data indicate that more-prolonged lamivudine therapy is probably required after HBeAg seroconversion, and duration of an undetectable HBV DNA level using the highly sensitive HBV RTD-Direct test, as opposed to other methods of HBV DNA detection, can more helpfully aid in the decision of when to stop lamivudine therapy.

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LIVER

Benefit of interferon therapy in hepatocellular carcinoma prevention for individual patients with chronic hepatitis C

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Background: An increase in the incidence of hepatocellular carcinoma (HCC) in Japan since the 1980s suggests an imminent outbreak in other countries where viral spread occurred more recently. Interferon therapy for chronic hepatitis C, in general, has been shown to prevent HCC.
Aims: To determine the scale of benefit in individual patients.
Subjects: Histologically proven chronic hepatitis C patients in the Inhibition of Hepatocarcinogenesis by Interferon Therapy (IHIT) cohort (*Ann Intern Med* 1999;131:174), as updated in March 2003.
Methods: The lifetime risk for HCC was calculated based on HCC incidence rates, stratified by sex, age, fibrosis stage, and outcome of interferon therapy. The gain in HCC free survival was defined as the difference between expected HCC free survival with sustained virological response and that without.
Results: The gain in HCC free survival was greater when a patient was younger and fibrosis was more advanced. For example, a 30 year old male with F3 fibrosis gained 12.4 years by attaining sustained response while a patient with F1 fibrosis older than 60 years gained less than one year. For a treatment protocol with a given sustained response rate, prior estimation of the gain can be obtained by multiplying the calculated HCC free survival for responders by the response rate.
Conclusions: The gain in HCC free survival may serve as an indicator of the benefit of interferon therapy in terms of HCC prevention and be useful in the consideration of indication and selection of treatment protocol for individual patients.

Various studies have indicated that a nationwide spread of hepatitis C virus (HCV) took place in Japan in the 1950s and 1960s.[1 2] As a result, there has been a rapid increase in hepatocellular carcinoma (HCC) incidence since 1980, now claiming more than 30 000 deaths each year. Simultaneously, there was a decline in the number of deaths assigned to cirrhosis. The decline may be partly due to advances in HCC diagnosis but the major cause seems to lie in the increasing risk of HCC as patients are getting older. The time lag of 30 years between the peaks of infection spread and HCC incidence corresponds to the observed interval between the time of blood transfusion and carcinogenesis in typical HCC patients.[3] These data strongly suggest an imminent outbreak of HCC incidence in other countries, including the USA, where HCV infection is thought to have been spread more recently.[4 5]

Shortly after the discovery of HCV in 1989,[6 7] interferon therapy was confirmed to be effective against HCV infection.[8–10] We and other groups have shown that interferon therapy significantly reduces the risk of HCC development among chronic hepatitis C patients.[11–15] In our previous study, 50% relative risk reduction, compared with untreated patients, was observed for conventional interferon monotherapy that showed an overall sustained virological response (SVR) rate of 33%, and a relative risk reduction to 20% was revealed among patients who achieved SVR.[15] As the antiviral efficacy of interferon therapy has been improved by recent advances such as combination with ribavirin[16–18] and introduction of pegylated interferons,[19–22] we can expect that the efficacy on HCC prevention has also been strengthened.

We have also shown that cirrhosis gradually resolves once SVR is achieved,[23] suggesting that interferon therapy will also prevent death due to liver failure or variceal rupture. However, HCC is clearly the dominant cause of death in patients with chronic hepatitis C, at least among our cohort in Japan where the average age is over 50 years and most patients abstain from heavy alcohol consumption.[24] Thus we have focused on HCC prevention as the primary object of interferon therapy.

Considering the current status of therapeutics, interferon therapy is clearly recommended only in a selected group of patients.[25] Since the benefit of antiviral therapy differs among individual patients, the indication as well as the choice of regimen should be decided based on the expected benefit for each patient. Quantification of benefit requires reasonable assessment of the lifetime risk of HCC and the expected reduction in it with treatment. In this study, we propose an indicator, the gain in HCC free survival, to quantify the benefit specific to individual patients. The value is calculated based on both life expectancy and individualised risk of HCC, and applicable to distinct protocols with varying efficacy. It may serve as the gold standard for the benefit of antiviral therapy in terms of HCC prevention.

MATERIALS AND METHODS

Incidence rates of HCC

Crude data were obtained from the IHIT (Inhibition of Hepatocarcinogenesis by Interferon Therapy) database,[15 23 24] as updated on 31 March 2003. Every patient underwent liver biopsy in 1990 or later, and liver fibrosis was staged according to the classification system of Desmet and colleagues.[26] Patients had no history of HCC, and were positive for HCV antibody and negative for hepatitis B surface antigen. We excluded those who developed HCC or dropped out of surveillance within one year after liver biopsy, and the start

Abbreviations: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; SVR, sustained virological response; NNT, number needed to treat

of observation was set at exactly one year after liver biopsy. Entry into the cohort was closed in 1999. The cohort population analysed in this study consisted of 2392 patients who received interferon monotherapy within one year of liver biopsy, and 395 patients who did not. Among 2392 interferon treated patients, 836 (34.9%) showed SVR, as determined six months after cessation of interferon administration. After undergoing liver biopsy, 90% of patients abstained from alcohol except for infrequent social occasions, and only 2% continued drinking alcohol (>80 g daily).

Patients underwent abdominal ultrasonography every 3–6 months, and contrast enhanced computed tomography was also performed every 6–12 months in patients with advanced fibrosis. A final diagnosis was made based on haemodynamic patterns on contrast enhanced computed tomography, abdominal angiography, or computed tomography during angiography. Ultrasound guided tumour biopsy was performed in ambiguous cases. The SVR group showed 27 events of HCC development during an observation period of 4767 person years; in the non-SVR group, 214 events in 9922 person years; and in the untreated group, 67 events in 2168 person years.

HCC incidence rates, stratified by age, sex, and fibrosis stage at entry, were calculated in each group by the person year method. Risk ratios were analysed using Cox proportional hazard regression. Age, as ranked by 10 years, and fibrosis stage were represented by dummy variables in the analysis. Adjusted HCC incidence rates were calculated so that the sum of squares of differences between the adjusted and observed values, weighted by the number of patients in each category, was minimised while conserving the risk ratios obtained by proportional hazard regression.

HCC free survival

The probability that a patient remains free of HCC at nth year of observation was calculated as:

$$(1-Q1)(1-P1)\times(1-Q2)(1-P2)\times(1-Q3)\times (1-P3)\times \ldots \times(1-Qn)(1-Pn)$$

where Qi is the age and sex specific death rate in the general population and Pi is the annual incidence of HCC specific to the patient in the ith year. Age and sex specific death rates were those published by the Ministry of Health, Welfare, and Labour of Japan for the vital statistics surveyed in 2000.[27] The gain in HCC free survival by interferon therapy was defined as the area between the cumulative HCC free survival curves. This model is based on an assumption that fibrosis stage remains constant with time (see model limitations in the results section).

Statistics

Values are expressed as mean (SD) unless otherwise specified. All statistical analyses were performed with SAS Software version 6.12. We used an original program coded in Object Pascal to calculate cumulative HCC free survival.

RESULTS

Incidence rates of HCC

Demographic data of the patients analysed in the current study are summarised in table 1, and observed HCC development and deaths are shown in table 2, illustrating that HCC was the major sequela among the cohort. Crude incidences of HCC did not differ between the untreated group and the non-SVR group in the corresponding category (data not shown). Patients with advanced fibrosis (stages F3 or F4) in the non-SVR group or in the untreated group showed a very high incidence rate. In fact, values obtained were greater than those found in 1999,[15] suggesting that the risk of HCC has increased with time. Fibrosis stage was determined at the time of liver biopsy and had possibly progressed during the observation period. As previously described, HCC incidence rates were substantially lower in the SVR group.

Cox proportional hazard regression analysis revealed that male sex, older age, and advanced fibrosis were associated with a higher risk of HCC, both in the non-SVR groups (table 3) and in the untreated group (data not shown). Multivariate analysis showed that the risk ratio of non-SVR to no treatment was 0.835 (95% confidence interval (CI) 0.625–1.1125; $p = 0.2214$). We previously showed that the risk of HCC was decreased in patients who showed normalised serum alanine aminotransferase levels in spite of continued viraemia after interferon therapy.[24] However, active hepatitis recrudesces not infrequently in a short period[28] and the effect on HCC prevention in those patients appears to decline in the long run. Thus we assumed that interferon therapy without attaining SVR had no effect on HCC prevention. Table 4 shows the incidence rates of HCC, as fitted to the crude data by the least squares method. These values were used in modelling of the estimated HCC free survival of individual patients.

HCC free survival

Using the fitted HCC incidence rates and the age and sex specific death rates, we estimated the lifetime cumulative HCC free survival with or without SVR. Figure 1 shows an example of a 30 year old male patient with chronic hepatitis C with stage F3 fibrosis. The area under the curve indicates the expected HCC free survival and the area between the two curves is the gain in HCC free survival when the patient achieves SVR. The gain, or the area, was calculated to be 12.4 years in this case.

We similarly calculated the gain in HCC free survival under various conditions (see fig 2, table 5). By definition, these values are applicable only after SVR has been achieved. The gain in HCC free survival that can be expected before the virological outcome is known is the product of the value in table 5 and the prior probability of achieving SVR.

The gain in HCC free survival was greater when a patient was younger or fibrosis was more advanced. Judging by the expected gain, indications for treatment are questionable in patients with fibrosis stage F0 or F1 and older than 60 years because they would gain less than one year even if they

Table 1 Demographic data for the patients analysed in the current study

		Interferon treated	
	Untreated	SVR	Non-SVR
No of patients	395	836	1556
Age (y)	55.0 (10.7)	47.7 (11.9)	50.5 (6.4)
Sex (M/F)	204/191	583/253	942/614
Fibrosis stage			
(F0-1/F2/F3/F4) (n)	128/141/42/84	278/331/173/54	440/568/381/167
(F0-1/F2/F3/F4) (%)	32/36/11/21	33/40/21/6	28/37/24/11

SVR, sustained virological response.

Table 2 Incidence of hepatocellular carcinoma (HCC) and death in the study cohort

	Untreated	Interferon treated	
		SVR	Non-SVR
No of patients	395	836	1556
Follow up (y)	6.5 (2.8)	6.7 (3.0)	7.4 (2.9)
HCC development (n)	67	27	214
Death (n)	33	11	89
With HCC (n)*	22	6	59
Hepatic deaths			
Without HCC (n)	4	1	8
Non-hepatic deaths (n)	7	4	22

*Includes deaths not directly related to HCC in patients who had developed the cancer.
SVR, sustained virological response.

attained SVR. On the other hand, patients with fibrosis stage F3 or F4 and younger than 50 years will gain more than 10 years with SVR, and more than five years even if the probability of attaining SVR is 50%.

Recently, the efficacy of interferon therapy has been improved by the introduction of peginterferon and ribavirin. However, more effective protocols will be accompanied by an increase in cost and incidence of untoward effects. These must be counterbalanced by an increase in expected benefit. While the increase in cost is the same, that in benefit is directly proportional to the values shown in table 5 and differs in each patient. The SVR rate for type 1b genotype high viral load infection was approximately 7% among the current cohort where six months of interferon monotherapy was the main protocol. The combination of peginterferon and ribavirin for 48 weeks, which is still under clinical trials in Japan, is expected to show a response rate of 40% or better for those patients. This difference (33%) corresponds to five years of the gain in HCC free survival in 40 year old patients with fibrosis stage F4 and to approximately one year in 60 year olds with fibrosis stage F2 (one third of the values given in table 5). Although these values are a hypothetical extrapolation, they may be clinically useful in choosing treatment protocols.

Model limitations

The model described in this article is based on several assumptions. Firstly, we assumed that interferon therapy that failed to achieve SVR had no beneficial effect, as described above, and this may result in underestimation of the benefit. However, the difference cannot be large: a 30 year old male with fibrosis stage F4 has a gain of 16.59 years instead of 15.98 years, and an 80 year old male with fibrosis stage F0/1 has a gain of 0.18 years instead of 0.15 years if we based the calculations on the incidence observed in the untreated group.

Secondly, we assumed that the benefit of interferon therapy was limited to HCC prevention. This is certainly an underestimation: successful interferon therapy improves liver function and may prevent death from liver failure. Several studies, failing to find an effect on HCC incidence, still indicated improvement in liver function with interferon therapy.[29 30] However, hepatic death without developing HCC was rare in the current cohort; one patient in the SVR group (variceal rupture (n = 1)), eight in the non-SVR group (liver failure (n = 4), variceal rupture (n = 3), not specified (n = 1)), and four in the untreated group (liver failure (n = 2), variceal rupture (n = 2)) died of a liver related cause without developing HCC, indicating annual mortality rates of 0.02%, 0.08%, and 0.18%, respectively (table 2). These values were small relative to the observed incidence of HCC.

Thirdly, we assumed that fibrosis stage remained constant, with the risk of HCC unchanged except for the increment due to aging. This may not be true: in fact, we previously indicated fibrosis progression in untreated patients and amelioration in interferon responders.[23] However, we did

Table 3 Annual hepatocellular carcinoma (HCC) incidence rates according to age and sex

Age (y)	F0/1	F2	F3	F4
SVR, male				
<39	0.05% (0/65)	0.09% (0/59)	0.16% (0/14)	0.24% (0/4)
40–49	0.05% (0/57)	0.09% (0/66)	0.16% (1/29)	0.24% (0/9)
50–59	0.39% (0/38)	0.69% (3/62)	1.21% (5/51)	1.86% (1/18)
60+	0.70% (3/29)	1.18% (3/38)	2.01% (4/35)	3.20% (1/9)
SVR, female				
<39	0.02% (0/32)	0.05% (0/38)	0.10% (0/7)	0.15% (0/1)
40–49	0.03% (0/25)	0.05% (0/23)	0.10% (0/3)	0.15% (1/1)
50–59	0.23% (0/20)	0.41% (1/33)	0.73% (1/20)	1.12% (1/6)
60+	0.40% (0/6)	0.71% (1/18)	1.25% (0/14)	1.93% (1/6)
Non-SVR, male				
<39	0.05% (0/83)	0.13% (0/72)	0.28% (2/29)	0.56% (0/6)
40–49	0.35% (2/85)	1.00% (4/101)	2.16% (7/46)	4.26% (10/32)
50–59	0.82% (6/82)	2.33% (19/111)	5.06% (26/74)	10.0% (17/33)
60+	1.03% (4/36)	2.93% (13/59)	6.35% (17/64)	12.5% (15/29)
Non-SVR, female				
<39	0.02% (0/37)	0.07% (0/21)	0.14% (0/10)	0.29% (0/2)
40–49	0.18% (0/41)	0.51% (2/44)	1.10% (3/18)	2.17% (0/6)
50–59	0.42% (1/53)	1.19% (8/96)	2.57% (19/80)	5.08% (5/32)
60+	0.52% (1/23)	1.49% (11/64)	3.23% (10/60)	6.37% (12/27)

The percentages indicate the annual incidence rates fitted by the least squares method using the risk ratios shown in table 4. Numbers in parentheses are the observed events/number at risk in each category.
SVR, sustained virological response; F0–F4, fibrosis stage.