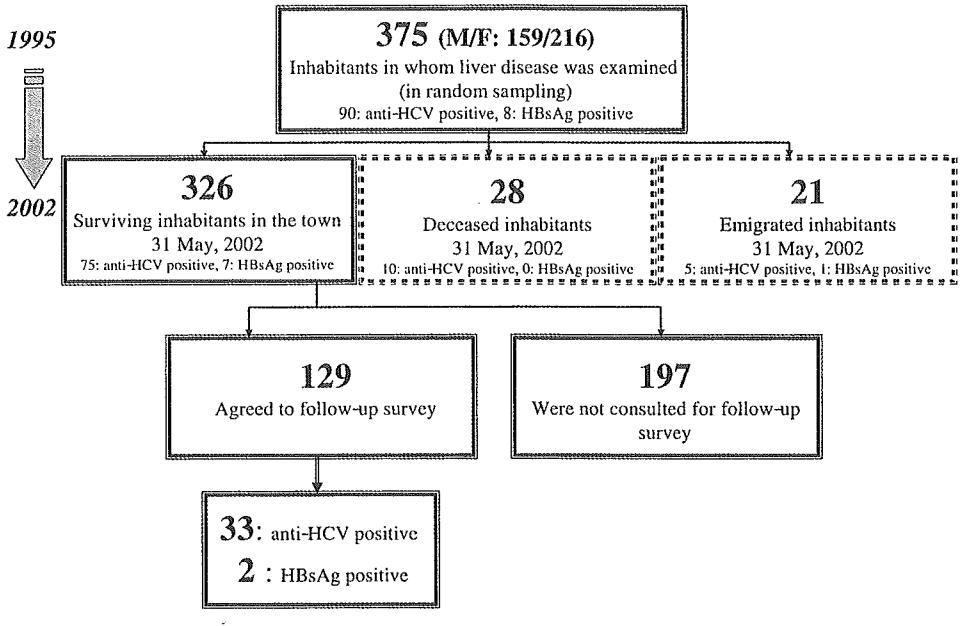

Figure 1. Diagram for pursuing the prognostic investigation of 375 inhabitants.

Table I. Characteristics of the subjects.

	Anti-HCV		
	Negative	Positive	p-value
Number	44	31	
Age (years)	59.6±9.8	59.7±10.6	NS
Sex ratio (male/female)	14/30	8/23	NS
Body mass index (kg/m^2)	22.7±2.7	22.1±1.9	NS
Lifetime use of alcohol (kg/life)	111.3±254.7	77.3±228.4	NS
Aspartate aminotransferase (U/l)	20.1±5.3	32.0±21.2	<0.01
Alanine aminotransaminase (U/l)	11.4±6.5	24.9±20.7	<0.01
γ-glutamyltranspeptidase (U/l)	10.9±6.4	13.8±9.3	NS

Data are expressed as mean ± SD or number of subjects. A p-value <0.05 was considered significant. Anti-HCV, anti-HCV antibody; NS, not significant.

inhibits insulin signaling by down-regulation of insulin receptor substrate (IRS) 1 and IRS2 in hepatocytes (19).

In this study, we evaluated insulin resistance among inhabitants with HCV infection in an HCV hyperendemic area of Japan. An association between HCV core and insulin resistance was also examined. Since association does not prove causality, we performed a case-cohort analysis to examine if inhabitants with HCV infection were at increased risk for type 2 diabetes mellitus after 7 years of follow-up.

Materials and methods

Study population. Since 1990, we have conducted health screenings of the residents of an HCV hyperendemic area (5). In 1995, 375 adult residents (159 men and 216 women) participated in this study. In the present study, we evaluated the insulin resistance of residents by using plasma and serum samples taken in 1995 and performed a case-cohort analysis to examine the relationship between HCV infection and the development of type 2 diabetes mellitus after 7 years of follow-up. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in prior approval by the Institutional Review Board at Kurume University School of Medicine. All participants provided written informed consent.

By 2002, 28 of 375 residents had died and 21 people had moved to other regions (Fig. 1). Thus, 326 residents of the original 375 residents remained in the area, and 129 residents agreed to participate in the medical follow-up survey, while the remaining 197 inhabitants did not declare their intention

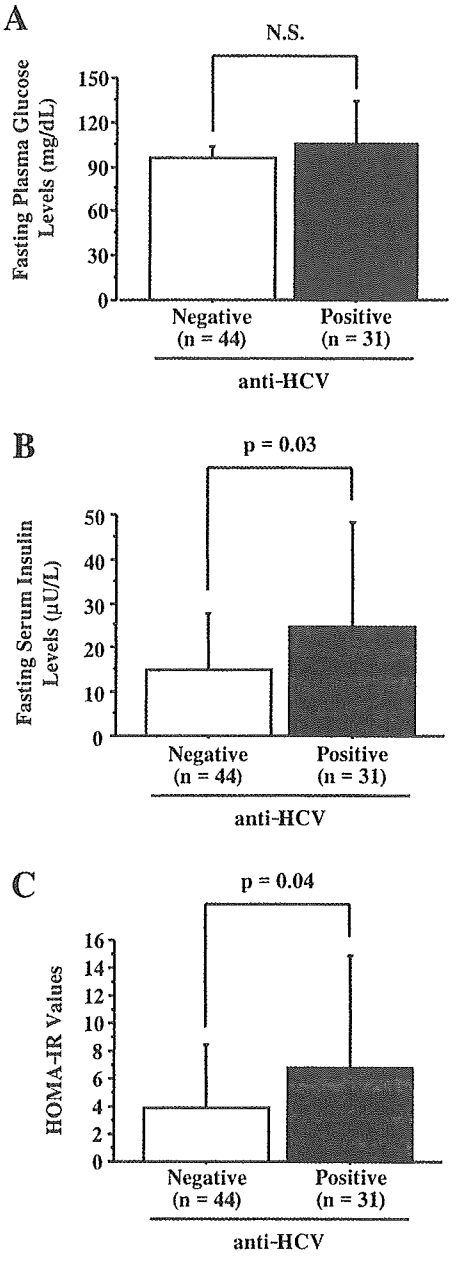

Figure 2. Glucose homeostasis in subjects with anti-HCV negative (n=44) and positive (n=31). (A) Fasting plasma glucose levels, (B) fasting serum insulin levels, and (C) insulin resistance determined by the homeostasis model assessment (HOMA-IR) values. Values are expressed as mean ± SD. NS, not significant.

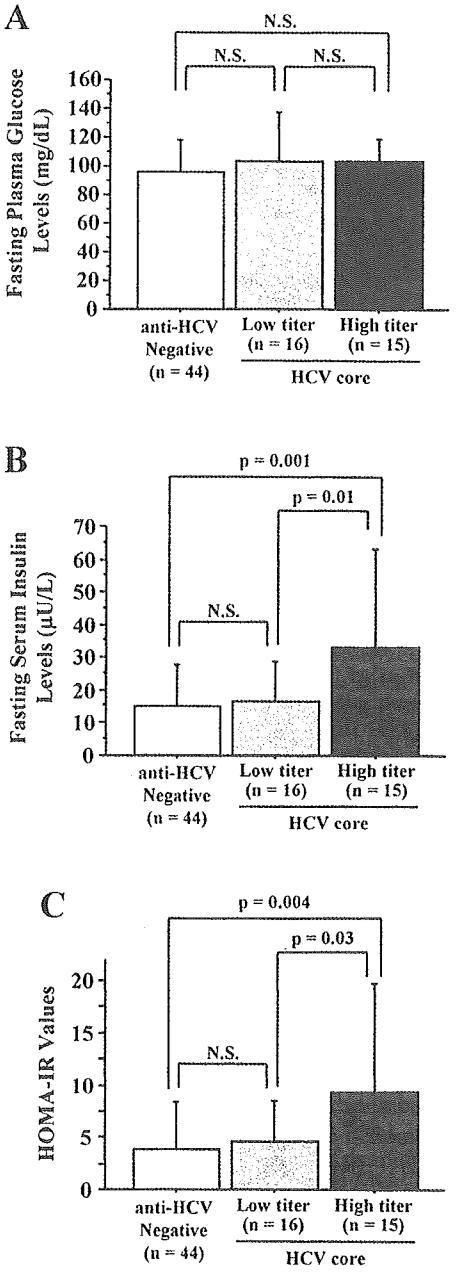

Figure 3. Glucose homeostasis in subjects with anti-HCV negative (n=44), low titer (<300 fmol/l; n=16), and high titer (>300 fmol/l; n=15) HCV core. (A) Fasting plasma glucose levels, (B) fasting serum insulin levels, and (C) insulin resistance determined by the homeostasis model assessment (HOMA-IR) values. Values are expressed as mean ± SD. NS, not significant.

either way in 2002. Among the surviving 129 residents who agreed to participate, 33 were anti-HCV positive. Of these 33, 2 residents were excluded from analysis because of indeterminate fasting glucose or insulin levels. The remaining 31 anti-HCV-positive residents and 44 randomly selected anti-HCV negative residents were enrolled in this study. All were hepatitis B virus surface antigen negative.

Clinical and serological assessment. Data including age, sex, and alcohol use were collected. Body mass index (BMI) was calculated as body weight in kilograms divided by the square of height in meters (kg/m^2). Venous blood samples were taken in the morning after a 12-h overnight fast. Sera were examined for the presence of anti-HCV by using a second-generation enzyme immunoassay test (Lumipulse II HCV, Fujirebio Inc., Tokyo, Japan). Sera were also assayed for HCV core by using a newly developed HCV Core Antigen ELISA Test system (Ortho-Clinical Diagnostics K.K., Tokyo, Japan), which has high stability and reproducibility under all conditions, and a detection limit of 44 fmol/l (22). The concentrations of serum HCV core antigen and serum HCV RNA correlated significantly. Based on the concentration of serum HCV RNA, <300 fmol/l of serum HCV core is classified as the low titer group and >300 fmol/l of

Table II. Relative risk of type 2 diabetes mellitus.

Anti-HCV	HCV core titer	Total number	No. of people with diabetes	p-value	Relative risk	95% Confidence interval
Negative		42	2		1	
Positive		29	5	0.08	3.62	0.83 to 20.89
	Low	14	1	0.73	1.50	7.66 to 18.11
	High	15	4	0.02	5.60	1.41 to 37.42

Subjects with anti-HCV positive were categorized according to HCV core titer into 2 groups: low titer, <300 fmol/l; and high titer, >300 fmol/l.

serum HCV core is classified as the high titer group. 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). Insulin resistance was calculated on the basis of fasting levels of plasma glucose and insulin, according to the homeostasis model assessment (HOMA-IR) method (23). The formula for the HOMA-IR is: HOMA-IR = fasting glucose (mg/dl) x fasting insulin (μU/ml)/405.

Diagnosis of type 2 diabetes mellitus. Type 2 diabetes mellitus was classified according to the 1997 American Diabetes Association criteria (24). Subjects who had a fasting plasma glucose level of ≥126 mg/dl were considered to have type 2 diabetes mellitus. Cases of type 2 diabetes mellitus were also defined by self-reported use of hypoglycemic medications. Subjects in whom diabetes was diagnosed at <30 years of age and who reported insulin use at the time of survey were categorized as having type 1 diabetes mellitus and excluded from this study.

Statistical analysis. All data are expressed as mean ± SD. Differences between 2 groups were analyzed using a Student's *t*-test. Statistical comparisons among multiple groups were performed by ANOVA followed by Scheffe's post-hoc test. Where indicated, simple correlation and linear regression analyses were done. A case-cohort analysis of the relationship of HCV infection and the development of type 2 diabetes mellitus was performed. The relative hazard for the development of type 2 diabetes mellitus, 95% confidence limit, and significance of the associations were evaluated based on 2 tests using Stat View® for Macintosh, version 5.0 (SAS Institute, Cary, NC). p-values <0.05 were considered significant.

Results

Characteristics of all subjects in an HCV hyperendemic area. In an HCV hyperendemic area, we enrolled 31 inhabitants who were anti-HCV-positive and 44 subjects who were anti-HCV-negative. Characteristics and laboratory data of these subjects are summarized in Table I. All subjects were Japanese. There was no significant difference in age, sex, BMI, lifetime alcohol use, and γ-glutamyltranspeptidase between the 2 groups. Although serum aspartate aminotransferase and alanine aminotransferase levels showed significant differences between the groups, both mean values were within normal range.

Insulin resistance in subjects with HCV infection. There was no significant difference in fasting glucose levels between anti-HCV-positive and anti-HCV-negative subjects (Fig. 2A). Anti-HCV-positive subjects showed increased fasting insulin levels compared to those subjects who were anti-HCV-negative (p=0.03; Fig. 2B). Values of HOMA-IR, an indicator of insulin resistance, were also increased in anti-HCV-positive subjects compared to those who were anti-HCV negative (p=0.04; Fig. 2C).

Association between HCV core and insulin resistance. Among 31 anti-HCV-positive subjects, 16 subjects showed low titer (<300 fmol/l) of serum HCV core and 15 subjects showed high titer (>300 fmol/l) of serum HCV core. Stratification of the anti-HCV-positive group according to serum HCV core titer revealed significant differences in fasting serum insulin levels, but not in fasting glucose levels between subjects with high titer of HCV core and subjects with anti-HCV negative (p=0.001; Fig. 3A and B). Similarly, HOMA-IR values were increased in subjects with high titer of HCV core compared to those in subjects with anti-HCV negative (p=0.004; Fig. 3C). A significant increase in fasting serum insulin levels and HOMA-IR values were also seen in subjects with a high titer of HCV core compared to those in subjects with a low titer of HCV core (Fig. 3B and C).

Correlations between fasting insulin levels of AST and ALT levels. Among anti-HCV-positive subjects, correlations between fasting insulin levels of AST and ALT levels were not statistically significant (r=0.33 and r=0.43, respectively). Similarly, correlations between HOMA-IR values of AST and ALT levels were not statistically significant (r=0.32 and r=0.45, respectively).

HCV infection and type 2 diabetes mellitus. To ascertain if HCV infection causes type 2 diabetes mellitus, we performed a case-cohort analysis in an HCV hyperendemic area. Of 75 subjects, 4 residents (2 anti-HCV-positive subjects and 2 anti-HCV-negative subjects) were excluded from the case-cohort analysis because the development of type 2 diabetes mellitus was at the baseline. Among 71 subjects free of diabetes mellitus at the baseline, 7 subjects developed type 2 diabetes mellitus during the 7-year follow-up evaluation.

Overall, anti-HCV-positive subjects were nearly 3-fold as likely as anti-HCV-negative subjects to develop diabetes mellitus, but this difference was not statistically significant (p=0.08; relative hazard, 3.62; 95% confidence interval, 0.83 to 20.89) (Table II). After stratification of the anti-HCV-positive group according to serum HCV core titer, a significant increase in the incidence of diabetes was seen in subjects with high titer of HCV core compared to anti-HCV-negative subjects (p=0.02; relative hazard, 5.60; 95% confidence interval, 1.41 to 37.42) (Table II).

Discussion

In this study, we described an association of HCV infection and the development of type 2 diabetes mellitus in an HCV hyperendemic area. Subjects with high titers of HCV core showed more severe insulin resistance than subjects with low titers of HCV core and anti-HCV negative subjects. Our pilot study showed a significant increase in the incidence of type 2 diabetes mellitus in subjects with high titers of HCV core compared to anti-HCV-negative subjects.

Although there was no significant difference in fasting glucose levels between subjects who were anti-HCV-positive and -negative, fasting insulin levels and HOMA-IR values (an indicator of insulin resistance) were significantly increased in anti-HCV-positive subjects compared to anti-HCV-negative subjects (Fig. 2A-C). These findings indicate that HCV infection-induced insulin resistance and fasting glucose levels were compensated by hyperinsulinemia.

Several studies and our previous reports proposed that HCV infection antedates insulin resistance (16-19,24). However, these studies were based in referral centers, and the association may therefore be restricted to subjects with more severe forms of the disease. In this study, we enrolled all anti-HCV-positive inhibitants, including asymptomatic HCV carriers, and showed increased serum fasting insulin levels and HOMA-IR values in subjects who were anti-HCV-positive compared to subjects who were anti-HCV-negative. The development of severe insulin resistance in the general population among subjects with HCV infection and less severe hepatitis may suggest an alternative mechanism to hepatitis-related insulin resistance. Our hypothesis is also supported by the fact that fasting insulin levels were not significantly correlated with AST and ALT levels.

HCV consists of envelope and core proteins. Although HCV envelope protein transgenic (Tg) mice develop no pathological changes in the liver (25), Shintani *et al* and our previous study showed that HCV core Tg mice developed hepatic insulin resistance (18,19). Thus, since HCV core is involved in the development of insulin resistance in Tg mice, we examined an association between HCV core and insulin resistance in an HCV hyperendemic area. By stratification of the anti-HCV-positive group according to serum HCV core titer, differences in fasting serum insulin levels and HOMA-IR values between subjects with high titer of HCV core (>300 fmol/l) and anti-HCV-negative subjects were made clear (Fig. 3B and C). Our previous study demonstrated one of the molecular mechanisms for HCV-induced insulin resistance; HCV core up-regulates SOCS3 and inhibits insulin signaling by the down-regulation of IRS1 and IRS2 in hepatocytes (19), and this molecular mechanism may account for an association of HCV core with insulin resistance. Thus, we provided additional evidence for an association between HCV core and insulin resistance.

Since it is also possible that subjects with type 2 diabetes mellitus are at increased risk for acquiring HCV infection, the above data were insufficient to conclude that HCV infection causes type 2 diabetes mellitus. We assessed whether subjects with HCV infection were at increased risk for type 2 diabetes mellitus. Although the difference in the development of type 2 diabetes mellitus between anti-HCV-positive and negative subjects was not statistically significant (p=0.08; Table II), stratification by serum HCV core titer revealed that the pre-existing high titer of HCV core increased the risk for type 2 diabetes mellitus (p=0.02; Table II). Mehta *et al* reported a preliminary causal relationship between HCV infection and type 2 diabetes mellitus (26); their data, however, were derived from a community with a low prevalence of HCV infection (0.8% residents were anti-HCV-positive). On the other hand, we performed this study in a community with a high prevalence of HCV infection (23.6% residents were anti-HCV-positive). In this study, we confirmed the preliminary causal relationship between HCV infection and type 2 diabetes mellitus and identified a new factor, 'HCV core,' responsible for the development of type 2 diabetes mellitus.

A limitation in this study was the absence of multivariate analysis. Since 197 subjects did not agree to the follow-up study, the small sample size limited our ability to perform multivariate analysis. However, this study had sufficient statistical power to detect an association between HCV core and type 2 diabetes mellitus because of high prevalence of anti-HCV and similar environmental factors, such as the eating habits and lifestyle of H-town residents.

In conclusion, HCV infection may cause the development of type 2 diabetes mellitus in an HCV hyperendemic area. The interaction between HCV infection and development of type 2 diabetes warrants further study.

Acknowledgements

The authors thank Syunichi Hattori and Hidetoshi Itoya from Ortho-Clinical Diagnostics, K.K. for technical assistance, and Kaoru Kobayashi (Health & Welfare Section, Haki Town Hall) for the collection of information. This study was supported in part by a Grant-in-Aid for Scientific Research (C) (no. 15590963 to T.K. and no. 16590648 to M.S.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan and a grant from the Ishibashi Foundation for the Promotion of Science.

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Extrahepatic manifestations and insulin resistance in an HCV hyperendemic area

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Received February 21, 2005; Accepted April 15, 2005

Abstract. Hepatitis C virus (HCV) causes extrahepatic manifestations as well as liver diseases, and contributes to insulin resistance and type 2 diabetes mellitus. The purpose of the present study was to evaluate the relationship of extrahepatic manifestations and insulin resistance in an HCV hyperendemic area. We investigated the incidence of extrahepatic manifestations among 139 inhabitants living in an HCV hyperendemic area in 2002 and compared it to 1999 data for the same inhabitants. Insulin resistance was tested for some non-HCV or HCV-infected inhabitants we had identified during mass screenings in 1999 and 2002. For some of the inhabitants in 2002, we examined records on the prevalence of insulin resistance seven years earlier. The prevalence of extrahepatic manifestations among individuals with positivity for anti-HCV antibodies was higher than among those without HCV in both 1999 and 2002. The prevalence of each extrahepatic manifestation which we identified in 2002 was higher than in 1999. Moreover, in some non-HCV or HCV-infected inhabitants, insulin resistance in 2002 was significantly higher than in 1999. Among inhabitants who had HCV infection with extrahepatic manifestations, fasting insulin levels or HOMA-IR findings seven years prior was significantly higher than for inhabitants who had neither HCV infection nor extrahepatic manifestations (p=0.03, p=0.01, respectively). Insulin resistance induces HCV infection, which causes an increase in the incidence of extrahepatic manifestations in HCV-infected individuals.

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Abbreviations: HCV, hepatitis C virus; HBV, hepatitis B virus; anti-HCV, antibodies to HCV; HBsAg, hepatitis B virus surface antigen; HCC, hepatocellular carcinoma; OLP, oral lichen planus; DM, diabetes mellitus

Key words: hepatitis C virus, extrahepatic manifestations, insulin resistance, lichen planus, diabetes mellitus

Introduction

It is common knowledge that hepatitis C virus (HCV) causes chronic hepatitis, cirrhosis, and even hepatocellular carcinoma (HCC). Of the HCC cases in Japan, approximately 16% are caused by hepatitis B virus (HBV) infection and approximately 80% by HCV infection. Therefore, the following are now implemented as emergency measures for hepatitis C in the Ministry of Health, Labour and Welfare in Japan. In our country, as part of a 5-year program from 2002 to 2007, candidates who reach certain turning points during every 5-year period from age 40-70, or those in whom abnormalities in liver function were pointed out during the basic health checkup according to the Health and Medical Service Law for the Aged will undergo examination for HCV and HBV infection.

On the other hand, HCV infection is known to be associated with extrahepatic manifestations including membranoproliferative glomerulonephritis, cryoglobulinemia, and oral lichen planus (OLP) (1,2). Many recent reports have described the relationship of LP to HCV, such as the incidence of HCV infection in LP patients (3-6) or the incidence of LP in subjects with HCV-related liver diseases (7-10). Diabetes mellitus (DM) is another extrahepatic manifestation attributed to HCV infection. Epidemiologic studies of liver disease patients (11-14) and of type 2 DM patients (13-15) have reported that HCV-related liver disease increases the risk of type 2 DM. One to two decades before type 2 DM is diagnosed, reduced glucose clearance is already present (16). Therefore, insulin resistance plays an important role in the development of type 2 DM. We recently showed molecular mechanisms for HCV core-induced insulin resistance (17). HCV core up-regulates the suppressor of cytokine signaling (SOCS) 3 and inhibits insulin signaling by down-regulation of insulin receptor substrate (IRS) 1 and IRS2 in hepatocytes.

We screened for HCC among inhabitants of 'H town' (adult population: 7,389), Fukuoka prefecture in northern Kyushu, Japan, which has been known for its high prevalence of liver diseases since 1990 (18-21). Anti-HCV positivity among H town residents in 1990 was 23.6% (18). We also screened for OLP in the same town in 1994 (7) and investigated the incidence of extrahepatic manifestations in 1999 (8) and 2002. Recently, we reported community-based evidence for an association between HCV core, insulin resistance, and the development of type 2 DM (22).

Table I. Characteristics of extrahepatic manifestation in 2002.

	Total	OLP[a]	Abnormal thyroid function	Rheumatoid arthritis	DM	Hypertension
Subjects (%)	139 (100)	15 (10.9)	3 (2.2)	3 (2.2)	13 (9.4)	33 (23.7)
Age (years) (mean ± SD)	60.6±13.1	62.7±12.5	51.3±6.6	66.3±11.8	66.5±8.8	66.9±10.7
Sex (M/F)	51/88	8/7	0/3	0/3	5/8	18/15
Anti-HCV (+) (%)	35 (25.2)	6/35 (17.1)	2/35 (5.7)	2/35 (5.7)	5/35 (14.3)	12/35 (34.3)
HCV RNA (+) (%)	21 (15.1)	5/21 (23.8)[b]	2/21 (9.5)[c]	1/21 (4.8)	4/21 (19.0)	7/21 (33.3)
Anti-HCV (-) and HCV RNA(-) (%)	104 (74.8)	9/104 (8.7)[b]	1/104 (1)[c]	1/104 (1)	8/104 (7.7)	21/104 (20.2)

[a]Examination of oral mucosa was performed for 138 subjects. [b]$p<0.05$, [c]$p=0.01$.

In the current study, we investigated the incidence of extrahepatic manifestations in 2002 in an HCV hyperendemic area of Japan and compared it with results for 1999. We also investigated insulin resistance and extrahepatic manifestations in some non-HCV and some HCV-infected inhabitants whom we had identified in both 1999 and 2002.

Finally, we examined insulin resistance for the prior seven years in some of the inhabitants in 2002.

Patients and methods

The 139 adult inhabitants we studied included 51 men and 88 women with a mean age of 60.6±13.1 years (mean ± SD). These subjects were inhabitants of H town, an area that was hyperendemic for HCV infection. These subjects participated in a liver disease examination in 2002, and 138 of 139 people (50 men and 88 women; mean age ± SD, 60.5±13.2) participated in an oral mucosa examination as well. These 139 subjects were interviewed in person by two trained interviewers. We inquired about the following information: present health condition, regular hospital visits, medical treatment received in the hospital, name of the family doctor, the kind of medicine taken, and the presence of extrahepatic manifestations of HCV infection such as thyroid dysfunction, articular rheumatism, DM, and hypertension. Informed consent was obtained from all subjects after the purpose and methods of the study were explained.

Examination for oral mucosa. Almost all (138 of 139) received oral mucosa medical examination by an oral surgeon. The diagnosis of OLP was made on the basis of clinical features. We used the headband fiber (50-100-10, Daiichi Medical Co., Ltd.) that had a brightness of 15,000 luxes for stomatic examination. When a lesion was found, we created a photograph as an intraoral record. A topographic map of the oral mucosa was precisely classified into 56 areas according to classification of Roed-Petersen *et al* (23) and examination of the oral mucosa was based on the 'Guide to epidemiology and diagnosis of oral mucosal diseases and conditions' published by the World Health Organization (WHO) (24). The OLP diagnosis was made on the basis of clinical features and WHO criteria.

Diagnosis of extrahepatic manifestations except for OLP. We asked the family doctors of the subjects about the presence of extrahepatic manifestations including thyroid dysfunction, articular rheumatism, type 2 DM, and hypertension. Moreover, for the subjects who did not have a family doctor, diagnosis of DM was based on the American Diabetic Association (ADA) criteria of 1997 (25). Persons in whom diabetes was diagnosed before 30 years of age and used insulin were categorized as type 1 DM and were excluded from this study.

Serological assay. Body mass index (BMI) was calculated as body weight in kilograms divided by the square of height in meters (kg/m^2). Sera from the 139 residents were evaluated for the following liver function tests: serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), gammaglutamyl transpeptidase (γ-GTP), lactate dehydrogenase (LDH), total bilirubin (T.Bil), total protein (TP), albumin (Alb) and gamma-globulin (γ-glob.). Sera were also examined for the presence or absence of HCV or HBV infection. Anti-HCV was measured by a chemiluminescent enzyme immunoassay (CLEIA) kit (Lumipulse II HCV, Fujirebio Inc., Tokyo, Japan). HCV RNA in the sera was detected using the Amplicore HCV test (Nippon Roche, Tokyo, Japan). Hepatitis B virus surface antigen (HBsAg) was assayed by a CLEIA kit (Architect™, HBsAg QT, Dainabot Co. Ltd., Tokyo, Japan). Ultrasonographic examination of subjects with abnormalities in the liver function tests and positive for anti-HCV or HBsAg was performed in order to investigate the shape of the liver and lesions occupying the hepatic space.

Plasma glucose levels were measured by a glucose oxidase method for all subjects. In 80 subjects (anti-HCV positive: 35 cases, anti-HCV negative: 45 cases), serum insulin levels were measured using a sandwich enzyme immuno assay kit (Eiken Chemical, Tokyo, Japan). Insulin resistance (IR) was calculated

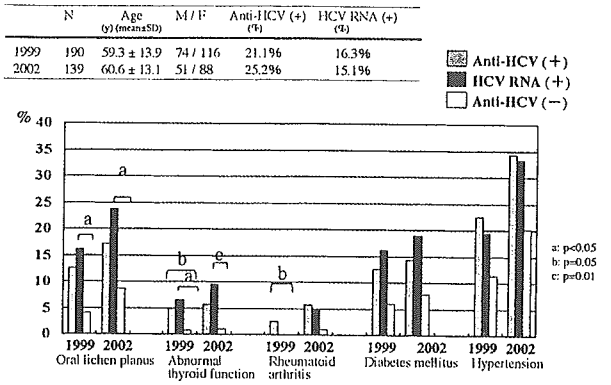

Figure 1. Prevalence of extrahepatic manifestations in HCV infection and in non-infected inhabitants. Note that the incidence of each extrahepatic manifestation was higher in those with HCV infection than in those without HCV. Note also that the incidence of each extrahepatic manifestation was higher in 2002 than in 1999. Y axix label should read: '% of total cases'.

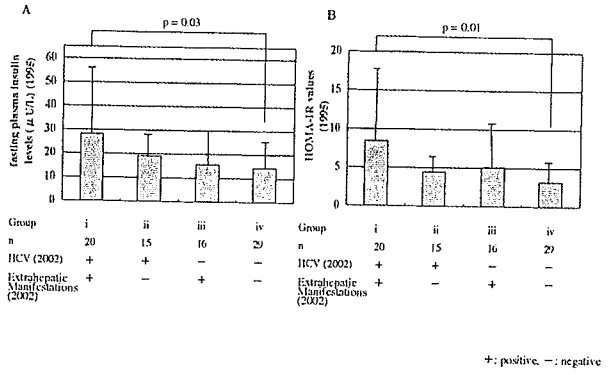

Figure 2. Association of insulin resistance in 1995 with the presence of HCV infection and extrahepatic manifestations in 2002. (A) Fasting serum insulin levels and (B) HOMA-IR values.

on the basis of fasting levels of plasma glucose and insulin, according to the homeostasis model assessment (HOMA-IR) method (26). The formula for the HOMA-IR is: HOMA-IR = fasting glucose (mg/dl) x fasting insulin (μU/ml)/405.

Comparison of prevalence of extrahepatic manifestation in 1999 and 2002. We reported the incidence of extrahepatic manifestations of HCV infection, including LP, for inhabitants of the same town in 1999 (8). We compared incidence of extrahepatic manifestations in 2002 with those in 1999.

Insulin resistance seven years prior in some inhabitants. The 80 of 139 people whom we followed-up in 2002, were divided into four groups: (i) extrahepatic manifestations with HCV infection, (ii) no extrahepatic manifestations with HCV infection, (iii) extrahepatic manifestations without HCV infection, (iv) no extrahepatic manifestations without HCV infection. We examined insulin resistance seven years prior in these four groups because we had surveyed HCC in the same town in 1995.

Statistical analysis. The chi-square test, the unpaired Student's t-test and Welch's t-test were used for statistical analyses. Differences were judged significant when $p<0.05$ (two-tailed). This study was approved by the Institutional Review Board/ Ethics Committee of our Institution.

Results

Extrahepatic manifestation in 2002. Anti-HCV antibodies and HCV RNA were detected in the sera from 35 (25.2%) and 21 (15.1%) of 139 inhabitants, respectively. HBsAg were detected in the sera from 4 subjects (4/139, 2.9%). The prevalence of OLP among all subjects was 10.9% (15/138), among HCV RNA positive subjects it was 23.8% (5/21), as shown in Table I. The incidence of OLP in those subjects who were serum HCV RNA-positive (23.8%, $p<0.05$ vs the OLP-HCV RNA negative group) was significantly higher than those without HCV RNA (Table I). The clinical appearances of 15 OLP subjects were: 5 with reticular, 5 with erosive, 4 with plaque-type, and 1 with atrophic OLP. Most (13/15 cases) of OLP had no pain. The most frequent site of OLP was the buccal mucosa. The diagnosis of all OLP subjects was made on the basis of clinical features. However, one OLP subject was biopsied and subsequently diagnosed as OLP histopathologically. There was no oral cancer. Besides OLP, the prevalences of extrahepatic manifestations among individuals with positivity for anti-HCV antibodies were higher than among those without HCV. The prevalence of thyroid dysfunction, articular rheumatism, DM, or hypertension in anti-HCV antibody positive subjects was 5.7, 5.7, 14.3, and 34.3%, respectively. Thyroid dysfunction, articular rheumatism, DM, or hypertension in HCV RNA positive subjects were 9.5, 4.8, 19.0, and 33.3, respectively (Table I). The prevalence of abnormal thyroid function among subjects with HCV infection was significantly higher than those without HCV infection.

Comparison with extrahepatic manifestations in 1999 and 2002. We had investigated the prevalence of extrahepatic manifestations for 190 people in the same town in 1999. Of the 190, 139 in 2002 participated in a second interview regarding extrahepatic manifestations. The prevalence of each extrahepatic manifestation in 2002 was higher than in 1999 (Fig. 1).

Characteristics of 35 subjects who were anti-HCV positive and 45 who were anti-HCV negative. We compared characteristics of 35 subjects who were anti-HCV positive (group 1) and 45 subjects who were anti-HCV negative (age and gender-matched controls, group 2). Table II indicates a comparison of characteristics of the 1999 and 2002 groups. For group 1 subjects, there were no significant differences between the two groups in age, sex, BMI, AST, ALT, gammaglutamyl transpeptidase or fasting plasma glucose between 1999 and 2002. Fasting serum insulin and HOMA-IR in 2002 levels were significantly increased over values in 1999. For group 2 subjects, fasting plasma glucose, fasting serum insulin, and HOMA-IR levels in 2002 were significantly increased over values in 1999, although there were no significant differences in age, sex, BMI, AST, ALT, and gammaglutamyl transpeptidase between 1999 and 2002.

Table II. Comparison of 35 HCV infected and 45 non-infected inhabitants in 1999 and 2002.

	Anti-HCV positive (35 cases)		
	1999	2002	p-value
Age (years) (mean ± SD)	62.4±10.0	67.1±10.6	ND
Sex (M/F)	9/26	9/26	ND
BMI	22.9±2.7	22.6±3.0	ND
AST	38.0±24.1	35.3±20.9	ND
ALT	37.9±27.7	31.7±26.9	ND
γGTP	36.8±25.9	34.3±25.4	ND
Fasting plasma glucose (mg/dl)	87.0±13.1	115.3±63.8	ND
Fasting serum insulin (μU/l)	12.6±7.3	29.3±28.1	0.04
HOMA-IR	2.8±1.9	8.8±9.0	0.02
	Anti-HCV negative (45 cases)		
	1999	2002	p-value
Age (years) (mean ± SD)	63.8±9.8	67.0±10.1	ND
Sex (M/F)	14/31	14/31	ND
BMI	22.9±3.5	22.9±3.5	ND
AST	22.4±5.6	21.6±4.9	ND
ALT	18.8±8.5	17.3±6.0	ND
γGTP	26.7±18.7	24.0±18.2	ND
Fasting plasma glucose (mg/dl)	92.6±26.6	109.6±46.8	0.04
Fasting serum insulin (μU/l)	14.0±12.3	22.3±17.1	0.01
HOMA-IR	3.5±4.5	6.4±5.7	0.01

ND, not statistically different.

Insulin resistance seven years prior for some of the inhabitants. Of the 139 people we examined on follow-up in 2002, 80 (35 anti-HCV positive and 45 anti-HCV negative; groups 1 and 2, respectively) were further studied, 20 had extrahepatic manifestations and HCV infection (group i), 15 had no extrahepatic manifestations and HCV infection (group ii), 16 showed extrahepatic manifestations without HCV infection (group iii), and 29 had no extrahepatic manifestations and no HCV infection (group iv). Fasting insulin levels seven years prior in these four groups were: 28.3±30.1, 19,2±9.6, 15.8±15.1, or 14.5±11.9, in groups (i), (ii), (iii), and (iv), respectively. Fasting insulin levels of group (i) was significantly higher than that of group (iv) (p=0.03, Fig. 2). HOMA-IR values seven years prior in groups (i), (ii), (iii), and (iv) were, respectively, 8.5±10.1, 4.5±2.3, 5.1±6.7, and 3.2±2.9. HOMA-IR values for group (i) was significantly higher than for group (iv) (p=0.01, Fig. 2).

Discussion

HCV is the cause of about 80% of HCC in Japan and contributes to various extrahepatic manifestations as well as chronic liver diseases. However, the mechanism through which various HCV-related extrahepatic manifestations develop has barely been elucidated. Detection of the HCV RNA negative strand has been reported not only in hepatocytes but also in many other cells, suggesting the extrahepatic replication of HCV (27). The fact that HCV replicates in tissue from OLP or oral cancer patients, both extrahepatic manifestations, has been demonstrated (28-31).

Diabetes mellitus has also been linked to HCV. Multiple studies have confirmed the suspicion that patients with HCV infection have a significantly higher prevalence of DM compared with patients with other liver diseases (11-14). An association of HCV and DM has also observed in cohorts of patients with type 2 DM (13,15), and in a large cross-sectional national survey (32). We recently showed molecular mechanisms for HCV core-induced insulin resistance. HCV core up-regulates suppressor of cytokine signaling (SOCS) 3 and inhibits insulin signaling by down-regulation of insulin receptor substrate (IRS) 1 and IRS2 in hepatocytes (17).

In the current study, we conducted a follow-up survey (in 2002) for the inhabitants who had been examined in 1999 for liver disease in an HCV hyperendemic area (18-20). We previously reported that medical treatment was considered to be a causative route of HCV transmission (18), that most HCV carriers died from HCC or liver cirrhosis (19), that the prevalence of OLP in HCV carriers was higher than in those without HCV (7,8), and that it is necessary to continuously provide medical treatment to recognized cases of HCV carriers (21). In the current study, we found: 1) a higher prevalence of extrahepatic manifestations in HCV positive subjects than in

those without HCV, 2) an increasing number of HCV positive subjects with advancing age, 3) a significantly increased incidence, with age, of fasting insulin levels and HOMA-IR levels, indicators of insulin resistance, in both anti-HCV positive and negative groups, and 4) significantly higher frequency of hyperinsulinemia among subjects who had HCV infection with extrahepatic manifestations than among subjects who had neither HCV infection nor extrahepatic manifestations. We think that the increased incidence of different extrahepatic manifestations with time may be due to insulin resistance. HCV infection induces insulin resistance and may cause extrahepatic manifestations. This supports our previous report of an association between HCV core, insulin resistance, and the development of type 2 DM (22). Anti-HCV-positive subjects were nearly 3-fold as likely as anti-HCV-negative subjects to develop DM (22).

In recent years, the importance of insulin resistance has come to the fore after its role in the pathogenesis of many metabolic disorders had been clarified. Among patients with essential hypertension who did not have clinically defined DM, insulin resistance was often found. Agata *et al* reported that 33.3% of essential hypertension cases were found to be insulin-resistant (33). They suggested that disturbances of glucose and lipid metabolism in essential hypertension may be related to both insulin resistance and compensatory hyperinsulinemia, and that insulin-resistant patients with essential hypertension may have more risk factors for arteriosclerotic complications than patients with essential hypertension who were not insulin-resistant. The insulin resistance syndrome includes hypertension, changes in atherogenic lipoproteins, diabetes, and hypercoagulability (34). The presence of insulin resistance in patients with white coat hypertension has also been reported in many studies (35). Thus, hyperinsulinism is an important background factor in the pathogenesis of essential hypertension.

On the other hand, an association between abnormal thyroid function and hyperinsulinemia can be explained as following: When thyroid hormone increases, absorption of glucose from the bowel is promoted, and blood glucose increases so that gluconeogenesis in liver is enhanced (36). Thus, increased thyroid hormone concentration causes hyperglycemia and subsequent hyperinsulinemia. Moreover, it has been reported that OLP is significantly associated with HCV and DM in southern Taiwan (37) and that the co-association of LP with HCV is significant (38). In our report, OLP patients with DM were found only in HCV-infected patients (5).

It is well known that HCV infection can result in increased development of OLP (3-10,28-31). In addition, an association between DM and HCV has been shown (11-14,32). Our study suggests a mechanism for the pathogenesis of HCV-related extrahepatic manifestations such as OLP, a precancerous condition. Knowing the mechanism should be beneficial in improving the prevention and treatment of extrahepatic manifestations of HCV infection. Improved treatments of various extrahepatic manifestations as well as chronic liver diseases and HCC will increase quality of life and reduce medical costs.

In summary, we investigated the incidence of HCV-related extrahepatic manifestations among inhabitants of an HCV hyperendemic area. The incidence of extrahepatic manifestations in subjects with HCV infection was significantly higher than in subjects without HCV infection. Regardless of the presence of HCV infection, insulin resistance increases with aging. Insulin resistance in inhabitants who have an extrahepatic manifestation with HCV infection increases more than the insulin resistance of inhabitants who have neither an extrahepatic manifestation nor HCV infection. HCV infection induces insulin resistance and may cause an extrahepatic manifestation. Insulin resistance appears to cause HCV-related extrahepatic manifestations.

Acknowledgements

As a project for establishing new high technology research centers this study was supported, in part by a Grant-in-Aid for Scientific Research (C) (No.10162398), by a Grant-in-Aid for Young Scientists (B) (No.14770256) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, by The 31st Daiwa Securities Health Foundation, and by Grant of the Clinical Research Foundation.

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J Gastroenterol 2005; 40:148–156
DOI 10.1007/s00535-004-1519-2

Journal of Gastroenterology

Risk factors for development of hepatocellular carcinoma in patients with chronic hepatitis C after sustained response to interferon

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Editorial on page 220

Background. Interferon (IFN) is expected to prevent the progression of hepatitis C virus infection to cirrhosis and the development of hepatocellular carcinoma (HCC), but there have been several reports of the development of HCC after a sustained response to IFN. Our aim was to elucidate the incidence and clinical features of, and risk factors for, HCC in sustained responders to IFN, taken for the treatment of chronic hepatitis C. ***Methods.*** We designed a retrospective cohort study conducted at 16 major Hospitals. The subjects were a total of 1056 patients showing sustained responses, 29 of whom developed HCC. ***Results.*** The incidence of HCC per 100 person-years was 0.56 (95% confidence interval, 0.35–0.76) in sustained responders. By the Cox proportional hazard model, we found that older age, higher serum aspartate aminotransferase level, and lower platelet count before IFN therapy were independent risk factors associated with the development of HCC. A risk index of HCC development, based on the coefficients of these risk factors, was used to classify patients into three groups, with low, intermediate, and high risk. The incidence rates of HCC for these three groups were 0.11, 0.44, and 1.98 per 100 person-years, respectively. The median period to the development of HCC was 4.6 years (range, 1.4–9.0 years), and there were no other specific clinical features of the HCC that developed in these patients. ***Conclusions.*** This study suggests that the risk of development of HCC is not completely eliminated in sustained responders to IFN. These findings may be useful in determining a follow-up strategy after a sustained response to IFN.

Received: May 24, 2004 / Accepted: August 18, 2004
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Key words: hepatitis C virus, hepatocellular carcinoma, interferon, sustained response

Introduction

Hepatitis C virus (HCV) infection is one of the most common causes of chronic hepatitis, and it is also a major risk factor for hepatocellular carcinoma (HCC).[1,2] Chronic hepatitis C is often asymptomatic and mild, but may slowly progress to liver cirrhosis and eventually to HCC.[3–5] Therefore, it has been assumed that eradication of HCV would provide the most effective means of preventing HCC.

Currently, interferon (IFN) represents the mainstay of treatment for chronic hepatitis C.[5–9] IFN therapy can lead to a decrease in serum transaminase activity, and to the disappearance of serum HCV RNA in patients with chronic hepatitis C. These patients appear to benefit by the prevention of progression to cirrhosis and HCC.[5,7,10–14] However, HCC can still occur in patients who are treated successfully with IFN, i.e., those showing a sustained response to the therapy.[5,10–25] The incidence and clinical features of HCC, and the risk factors for carcinogenesis, have not yet been investigated, although they have been documented in individuals and in small numbers of patients.[5,10–25] We investigated a large cohort of patients showing a sustained response to IFN therapy given for chronic hepatitis C. Our aims were to assess the incidence of HCC in these patients and to discover the clinical variables that may be associated with the development of HCC. Our study also focused on the clinical features of HCC. We designed a multicenter retrospective cohort study, because a single-institution study would have provided inadequate numbers of sustained responders who developed HCC.

Patients and methods

Patients

This study was conducted at 16 major hospitals belonging to the Japanese Society of Gastroenterology, Kyushu Division. A large cohort of sustained responders to IFN therapy given for chronic hepatitis C, in whom HCC had, or had not, been detected, was assembled consecutively by means of data collection instruments. All sustained responders included in the study were positive for HCV RNA before IFN therapy, and were followed up for more than 1 year after termination of IFN therapy, during the period July 1988 to August 2001. Sustained response was defined as the presence of HCV RNA negativity (determined by using qualitative HCV RNA assay) more than 6 months after the termination of IFN therapy. Diagnosis of HCC was based either on histological examination or on typical computed tomographic and/or angiographic findings at each institution. Patients were excluded if HCC was detected within 1 year after the termination of IFN therapy, because in such cases it was highly likely that the cancer had been present at the end of the IFN therapy. In Japan, at the time of the study, the standard schedule was 6–10 MU IFN-α every day for the first 2–4 weeks and then the same dose given three time a week for the following 20–22 weeks, or 6 MU IFN-β every day for 6–8 weeks.

During the study period at the 16 hospitals, a total of 3504 patients with chronic hepatitis C had received IFN therapy and had been followed up for more than 1 year thereafter, and a sustained response was obtained in 1091 (31.1%) of them. Among the sustained responders, 30 patients (2.7%) developed HCC. By means of the data collection instrument, we requested individual clinical data before IFN therapy for all sustained responders, as well as clinical data at the time of diagnosis of HCC for patients who had developed HCC. The clinical data for all 1091 sustained responders identified were obtained from the 16 hospitals (8 university hospitals and 8 regional hospitals) listed in the appendix. Of these patients, 35 were excluded from the analysis because of the development of HCC within 1 year after IFN therapy (1 patient) or insufficient clinical records before commencement of IFN therapy (34 patients). The final study population comprised a total of 1056 patients showing sustained response to IFN therapy given for chronic hepatitis C, 29 of whom had developed HCC.

Methods

To identify risk factors for the development of HCC in sustained responders to IFN therapy, we used univariate analysis and multivariate analysis to investigate 23 variables before IFN therapy for their relationship to the development of HCC. These variables were chosen by considering possible factors involved in the development of HCC, as indicated by previous investigations,[1–5,10–25] or suggested from our own clinical experience. Each variable, which was classified as host-related or treatment-related, was divided into one of two subgroups on the basis of clinically meaningful values. HCV RNA load was determined quantitatively by competitive reverse-transcription polymerase chain reaction (RT-PCR), branched-DNA probe assay, or Amplicor-HCV monitor assay.[26–28] When the serum HCV RNA level was more than 10^6 equivalents/ml by branched DNA assay, more than 10^6 copies/ml by competitive RT-PCR, or more than 10^5 copies/ml by Amplicor-HCV monitor assay, it was designated as a high viral load; an HCV RNA level of 10^5 copies/ml by the Amplicor-HCV monitor assay has already been demonstrated to correspond to approximately 10^6 equivalents/ml by the branched DNA probe assay or 10^6 copies/ml by competitive RT-PCR.[26–28] HCV subtype was classified by either the method of Okamoto et al.,[29] or Tanaka et al.'s method.[30] Genotypes 1a and 1b corresponded to serological group 1, and genotypes 2a and 2b corresponded to serological group 2, according to the Simmonds et al.[31] classification.[31] The data from liver biopsies that were done within 6 months before IFN therapy were included in this study. Assessments of the staging of liver fibrosis and the grade of inflammatory activity were based on the classification of Desmet and colleagues,[32] in which staging is defined as follows: F0 (no fibrosis), F1 (fibrous portal expansion), F2 (bridging fibrosis), F3 (bridging fibrosis with architectural distortion), and F4 (cirrhosis), and grading is defined as follows: A0 (no activity), A1 (mild activity), A2 (moderate activity), and A3 (severe activity).

To elucidate the clinical features of HCC that developed in sustained responders, 17 variables at the time of diagnosis of HCC were investigated. Number of tumors, maximum tumor size, portal vein invasion, hepatic vein invasion, and bile duct invasion were examined by ultrasonography, computed tomography, and/or angiography. The period to the development of HCC was measured from the day of termination of IFN therapy to the day when HCC was first diagnosed by imaging modalities, such as ultrasonography or computed tomography. The follow-up period for the detection of HCC after termination of IFN therapy was defined as the interval during which checks for HCC were done using tumor markers and/or imaging modalities.

Statistical analysis

Follow up ended with the last recorded visit before August 31, 2001. Incidences were calculated in person-

Table 1. Patient characteristics of 1056 sustained responders to interferon therapy given for chronic hepatitis C

		Number of patients
Host-related variables		
Age (years)	Median (range)	50 (11–76)
Sex	Male	711 (67%)
History of blood transfusion	Positive	266 (27%)
Alcohol abuse[a]	Positive	78 (8%)
Smoking habit[b]	Positive	248 (38%)
HCV viral load	High ($\geq 10^6$)	159 (21%)
HCV serologic group	Group 1	372
	Group 2	466
Hepatitis B surface antigen	Positive	17 (2%)
Treatment-related variables		
Interferon type	α	829 (79%)
	β	166 (16%)
	α + β	61 (6%)
Total amount of interferon (MU)	Median (range)	480 (42–1740)
Treatment period (weeks)	Median (range)	22 (2–56)
Prior interferon therapy	Positive	87

HCV, hepatitis C virus
[a] Alcohol intake, ≥80 g/day × 5 years
[b] Smoking habit, ≥20 cigarettes/day for ≥10 years

years; incidence curves of HCC were calculated by the Kaplan-Meier method; and differences in survival were evaluated by log rank tests. Hazard ratios and trend *P* values were calculated by treating the categories as ordinal variables. The Cox proportional hazard model was used to determine the most significant variables related to the development of HCC. All patients were then assigned a risk index value for the development of HCC, as follows: the value of each factor in the final model was multiplied by its corresponding regression coefficient, and these values were totaled to obtain the risk index for each patient. Stratification of the patients was conducted on the basis of this risk index. All *P* values were two-tailed and were considered significant when less than 0.05.

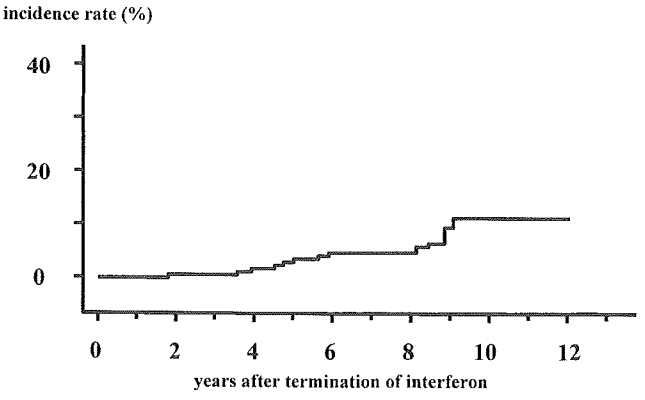

Fig. 1. Cumulative incidence of hepatocellular carcinoma in 1056 sustained responders to interferon therapy given for chronic hepatitis C

Results

Patient characteristics

Table 1 summarizes the patient characteristics of the 1056 sustained responders to IFN therapy given for chronic hepatitis C. The median age was 50 years (range, 11–76) years, and there were 711 men and 345 women (sex ratio, 2.1 : 1). Hepatitis B surface antigen was positive in 17 patients (2%). The HCV serological group was group 1 in 372 patients and group 2 in 466 patients, and thus a higher proportion of patients were in serological group 2. A total of 829 patients (79%) received IFN-α, 166 patients (16%) received IFN-β, and 61 patients (6%) received both. The median dose and duration of IFN administration were 480 MU and 22 weeks, respectively. No patients received peginterferon or combination therapy with ribavirin, and 87 patients (8%) received more than two cycles of IFN therapy.

Incidence of HCC

Twenty-nine of the 1056 sustained responders developed HCC, with a median follow-up period of 4.7 years. The incidence of HCC per 100 person-years was 0.56 (95% confidence interval, 0.35–0.76), and the incidences of HCC at 3, 5, 7, and 10 years after the termination of IFN therapy were 0.5%, 3.3%, 4.9%, and 11.1%, respectively (Fig. 1).

Univariate analyses

On univariate analysis (Table 2), age more than 60 years, positive smoking habit, platelet count less than $15 \times 10^4/mm^3$, aspartate aminotransferase (AST) more than 100 IU/l, prothrombin time less than 80%, and higher fibrosis stage (incidence of HCC per 100 person-years: F0, 0.00; F1, 0.27; F2, 0.47; F3, 0.62; F4, 1.31) were significant risk factors associated with the development of HCC. Alcohol abuse, total bilirubin, albumin, alanine aminotransferase, virological variables (viral load, serological group), tumor markers (alpha-fetoprotein, protein induced by vitamin K absence or antagonist-II), and treatment-related variables (treatment period, IFN type, total amount of IFN) were not significant risk factors.

Multivariate analyses

All variables whose P values were less than 0.20 on the univariate analyses were entered into the multivariate analyses (Table 3). However, history of blood transfusion, smoking habit, prothrombin time, and indocyanine green retention rate at 15 min (ICG R15) were not included in the model because inadequate data were available. Multivariate regression analysis, which assessed the independent predictive importance of each variable studied for the development of HCC, showed that older age, higher serum AST level, and lower platelet count were significantly related to the development of HCC.

Risk groups based on the regression model

For the clinical application of these findings, a risk index was calculated based on the regression coefficients derived from the three variables identified by multivariate analysis. The index equation was as follows: $1.14 \times$ (0, age $\leq$ 60 years; 1, age $>$ 60 years) + $1.13 \times$ (0, AST $\leq$ 100 IU/l; 1, AST $>$ 100 IU/l) + $1.02 \times$ (0, platelet count $\geq 15 \times 10^4/mm^3$; 1, platelet count $< 15 \times 10^4/mm^3$). The risk index was $\ln[hi(t)/h_0(t)]$, where $hi(t)/h_0(t)$ was the relative risk of the development of HCC for the i-th patient. The index values ranged from 0.00 to 3.29. The patients were then classified into three groups according to the risk index, as follows: low risk, risk index less than 1.00 (equivalent to patients with none of the three risk factors); intermediate risk, risk index from 1.00 to 2.00 (equivalent to patients with one of the three risk factors); and high risk, risk index greater than 2.00 (equivalent to patients with two or more of the three risk factors). The incidence curves for the three groups are shown in Fig. 2. The incidence rates of HCC per 100 person-years (95% confidence interval) in the low-, intermediate-, and high-risk groups were 0.11 (0.00–0.26), 0.44 (0.11–0.77), and 1.98 (1.09–2.87), respectively. There was a significant difference in survival time among the three groups ($P < 0.0001$).

Clinical features of HCC

The characteristics of the 29 patients in whom HCC developed after sustained response are shown in Table 4. All patients were HCV RNA-negative (determined by using qualitative HCV RNA assay), at the time of diagnosis of HCC. Twenty-five patients (86%) were aged 60 years or more, and 24 patients (83%) were men. Among the 13 patients in whom liver biopsy was done at the time of diagnosis of HCC, A0, A1, and A2 histological activity was observed in 5 (38%), 6 (46%), and 2 (15%) patients, respectively. F0, F1, F2, F3, and F4 histological stages were observed in 1 (8%), 1 (8%), 7 (54%), 2 (15%), and 2 (15%) patients, respectively. The median period from the termination of IFN therapy to the development of HCC was 4.6 years (range, 1.4–9.0 years), and there were 11 patients (38%) in whom HCC was detected more than 5 years after the termination of IFN therapy. The periods and methods of medical follow-up examination after the end of IFN therapy varied among the patients, and 8 patients did not receive a sufficient post-treatment medical examination. Among them, HCC of 5 cm or more in size was detected in 5 patients (63%).

Discussion

IFN is already widely used as a standard therapeutic modality for chronic hepatitis C.[5-9] It is generally assumed that eradication of HCV by IFN halts the progression of the disease and prevents clinical complications, including the development of HCC.[5,7,10-14] However, there have been reports of several patients in whom HCC developed after successful IFN therapy.[5,10-25] The incidence and clinical features of HCC, the risk factors for the disease, and the mechanism of carcinogenesis in these patients have not been fully elucidated, because the development of HCC is very rare in sustained responders to IFN therapy. This prompted us to perform a multicenter retrospective cohort study to gather clinical data on such patients.

Of all 1056 sustained responders to IFN therapy in the 16 hospitals in the study, 29 developed HCC, with a median period to development of 4.7 years, and the incidence of HCC was 0.56 (95% confidence interval, 0.35–0.76) per 100 person-years. This value was consistent with the results of previous studies of small numbers of sustained responders to IFN who developed HCC.[5,11-14,20,21-25] This rate was considerably lower than that in IFN-refractory patients or HCV-positive pa-

Table 2. Univariate analysis of 1056 sustained responders in relation to development of HCC

Variables		No. of patients	No. of patients developing HCC	Incidence (95% CI) (/100 person-years)	Hazard ratio (95% CI)	*P* value (log rank)
Host-related variables						
Age	≤60 years	840	13	0.32 (0.14–0.49)	—	
	>60 years	216	16	1.43 (0.73–2.13)	4.23 (2.04–8.80)	0.001
Sex	Male	711	24	0.67 (0.40–0.94)	—	
	Female	345	5	0.30 (0.04–0.57)	0.47 (0.18–1.23)	0.12
History of blood transfusion	Positive	266	11	0.80 (0.33–1.28)	—	
	Negative	723	16	0.45 (0.23–0.67)	0.60 (0.28–1.30)	0.19
Alcohol abuse[a]	Positive	78	2	0.53 (0.00–1.26)	—	
	Negative	946	26	0.56 (0.34–0.77)	1.05 (0.25–4.42)	0.95
Smoking habit[b]	Positive	248	14	1.16 (0.55–1.77)	—	
	Negative	405	7	0.36 (0.09–0.62)	0.30 (0.12–0.75)	0.009
HCV viral load	High ($\geq 10^6$)	159	1	0.15 (0.00–0.45)	—	
	Low ($<10^6$)	593	11	0.42 (0.17–0.66)	2.68 (0.35–20.77)	0.35
HCV serological group	Group 1	372	5	0.27 (0.03–0.52)	—	
	Group 2	466	10	0.47 (0.18–0.76)	1.78 (0.60–5.26)	0.30
Hepatitis B surface antigen	Positive	17	0	0.00	[c]	
	Negative	1008	27	0.54 (0.34–0.75)		0.56
Platelet count ($\times 10^4$/mm^3)	≥15	568	7	0.27 (0.07–0.46)	—	
	<15	358	21	1.15 (0.66–1.65)	3.95 (1.68–9.30)	0.002
Total bilirubin (mg/dl)	≥1.0	207	8	0.75 (0.23–1.27)	—	
	<1.0	824	21	0.52 (0.30–0.75)	0.37 (0.32–1.65)	0.45
Albumin (g/dl)	>4.0	564	17	0.59 (0.31–0.87)	—	
	≤4.0	396	8	0.42 (0.13–0.72)	0.78 (0.34–1.80)	0.56
Aspartate aminotransferase (IU/l)	>100	196	13	1.26 (0.57–1.94)	—	
	≤100	844	16	0.39 (0.20–0.58)	0.35 (0.17–0.73)	0.005
Alanine aminotransferase (IU/l)	>100	459	17	0.73 (0.38–1.07)	—	
	≤100	591	12	0.42 (0.18–0.66)	0.63 (0.30–1.32)	0.22
Prothrombin time (%)	≥80	493	9	0.39 (0.14–0.65)	—	
	<80	158	10	1.19 (0.45–1.93)	2.72 (1.10–6.74)	0.03
ICG R15 (%)	≥10	322	9	0.52 (0.18–0.86)	—	
	<10	274	1	0.08 (0.00–0.23)	0.18 (0.02–1.44)	0.11
Alpha-fetoprotein (ng/ml)	>20	66	2	0.58 (0.00–1.39)	—	
	≤20	554	16	0.58 (0.30–0.87)	1.10 (0.25–4.81)	0.78
PIVKA-II (AU/ml)	>0.063	42	0	0.00	[c]	
	≤0.063	235	8	0.66 (0.20–1.12)		0.63
Histological activity grade	A0 (No)	12	0	0.00		
	A1 (Mild)	309	6	0.40 (0.08–0.73)		
	A2 (Moderate)	359	11	0.64 (0.26–1.01)		
	A3 (Severe)	169	5	0.61 (0.07–1.14)	1.28 (0.74–2.21)	0.39
Histological fibrosis stage	F0 (No)	26	0	0.00		
	F1 (Mild)	405	5	0.27 (0.03–0.50)		
	F2 (Moderate)	301	7	0.47 (0.12–0.82)		
	F3 (Severe)	170	6	0.62 (0.12–1.11)		
	F4 (Cirrhosis)	97	4	1.31 (0.03–2.60)	1.56 (1.03–2.36)	0.03
Treatment-related variables						
Treatment period (weeks)	≥24	472	17	0.73 (0.38–1.08)		
	<24	584	12	0.41 (0.18–0.65)	0.56 (0.27–1.16)	0.11
Interferon type	α	829	25	0.61 (0.37–0.85)	—	
	β	166	4	0.55 (0.01–1.10)	0.99 (0.34–2.86)	0.98
	α + β	61	0	0.00	[c]	
Total amount of interferon (MU)	>500	491	10	0.42 (0.16–0.68)	—	
	≤500	534	16	0.60 (0.31–0.89)	1.34 (0.61–2.95)	0.47
Prior interferon therapy	Positive	87	2	0.46 (0.00–1.10)	—	
	Negative	955	27	0.57 (0.36–0.79)	1.17 (0.28–5.00)	0.82

HCC, hepatocellular carcinoma; CI, confidence interval; HCV, hepatitis C virus; ICG R15, indocyanine green retention rate at 15 min; PIVKA II, protein induced by vitamin K absence or antagonist-II; —; reference category

[a] Alcohol intake ≥80 g/day + 5 years

[b] Smoking habit, ≥20 cigarettes/day for ≥10 years

[c] not estimated

tients who did not receive IFN therapy, which has been reported to be 1.4%–7% yearly,[4–7,10–13,21–24] and it was obvious that IFN therapy decreased the risk of HCC in sustained responders. However, the incidence of HCC gradually increased over a period of at least 9 years after the termination of IFN therapy (Fig. 1). This suggests that the risk of HCC is not completely eliminated in patients who have a sustained response to IFN therapy,

at least for up to 9 years following cessation of the treatment.

Identification of the risk factors for the development of HCC in sustained responders is important, so that high-risk patients can be screened carefully for early detection of HCC and given potentially curative treatments such as hepatic resection; such patients generally have a good hepatic reserve after the elimination of HCV. Among the variables we investigated, multivariate analysis showed age to be an independent risk factor. As the patient ages, the period of HCV infection becomes longer, and the liver becomes more severely cirrhotic. Therefore, advanced age may simply represent the progression of associated liver disease. These findings are compatible with previous reports of the development of HCC in patients with chronic hepatitis C.[11–14,20–22]

Serum AST level and platelet counts were also independent risk factors in the present study. Some studies have reported that increased AST level and decreased platelet count are correlated with the progression of liver fibrosis,[33–34] which has been reported to be one of the most important risk factors for the development of HCC in patients with chronic hepatitis C.[5,11–13,21] Progression of liver fibrosis may reduce the clearance of AST,[35] leading to increased serum AST levels.[36] This progression is also associated with decreased production of thrombopoietin by hepatocytes[37] and progressive hypersplenism with worsening portal hypertension;[38] and, hence, reduced platelet production and increased platelet destruction. Moreover, in the present study, these factors were strongly associated with histological stage (Pearson's correlation coefficient; $P < 0.0001$). Therefore, increased AST level and decreased platelet count may reflect more progressive liver fibrosis.

For the clinical application of these findings, we proposed a risk index based on the independent risk factors. Patients were classified into three groups, with low, intermediate, and high risk ($P < 0.0001$ for difference in survival time among the three groups; Fig. 2). This index can be easily calculated, because it is based on variables obtained during routine laboratory examinations before IFN therapy is begun. This index, therefore, may be helpful in assessing the risk of development of HCC after sustained response to IFN therapy, although it is also important to validate this risk index by applying it to other populations of patients. Patients in the high-risk group (incidence rate, 1.98 per 100 person-years) may benefit from regular diagnostic imaging for the early detection of HCC.

In the analysis of the clinical features of HCC there were no specific findings. The period to the development of HCC after IFN therapy (median, 4.6 years; 1.4–9.0 range, years) was variable. HCC developed even in two patients whose liver showed improvement to mild fibrosis (stage F0 or F1) and in five patients whose liver improved to no activity (A0) after IFN therapy. The follow-up periods and methods for the detection of HCC after the termination of IFN therapy varied among the patients, and in some patients HCC was detected at far more advanced stages than in others, because of insufficient follow up after IFN therapy. This finding may suggest the need for regular follow up by diagnostic imaging, even after sustained response to IFN therapy for chronic hepatitis C, especially in the high-risk group.

Our study involved some uncertainties. First, because the study was retrospective, many data items were missing from the replies to the data collection instrument, and we had to ignore unmeasured or unrecorded data when conducting the statistical analyses. In the multivariate analysis, therefore, only variables whose P values were less than 0.20 on the univariate analysis were entered. Also, history of blood transfusion, smoking habit, prothrombin time, and ICG R15, whose P values were lower than 0.20, had to be excluded from the model because of missing data; these factors were potentially significant on multivariate analysis. Secondly, we sought information on serum hepatitis B virus DNA

Table 3. Significant risk factors identified in 1056 sustained responders, as determined by multivariate analysis with the Cox proportional hazard model

Variable	Hazard ratio (95% confidence interval)	P value
Age	3.13 (1.32–7.42)	0.01
Aspartate aminotransferase	3.10 (1.31–7.31)	0.01
Platelet count	2.78 (1.07–7.20)	0.04

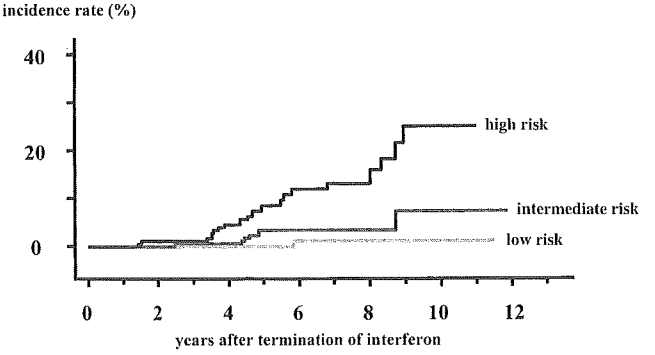

Fig. 2. Cumulative incidence of hepatocellular carcinoma for the three groups determined by a risk index based on the results of multivariate analysis. Low risk (risk index < 1.00); intermediate risk (risk index from 1.10 to 2.00); high risk (risk index, ≥2.00)

Table 4. Clinical features at the time of diagnosis of HCC in 29 patients who developed hepatocellular carcinoma after sustained response to interferon therapy given for chronic hepatitis C

Age (years)	Sex	HCV RNA	HBs Ag	Histological fibrosis stage	Histological activity grade	AFP (ng/ml)	PIVKA II (AU/ml)	Number of tumors
64	Male	Negative	Negative	NA	NA	2	0	4
60	Male	Negative	Negative	NA	NA	51	0.211	1
38	Male	Negative	Negative	NA	NA	4.3	NA	>5
67	Male	Negative	Negative	F4	A0	4.2	0.033	1
75	Male	Negative	Negative	F1	A1	5	0.054	1
65	Female	Negative	Negative	NA	NA	5	0.029	1
62	Male	Negative	Negative	NA	NA	3	NA	1
61	Male	Negative	Negative	F2	A0	3	0.001	1
64	Male	Negative	Negative	F2	A0	4	0.426	1
70	Male	Negative	Negative	NA	NA	46 000	NA	1
64	Male	Negative	Negative	NA	NA	146	0.049	1
54	Female	Negative	Negative	F3	A1	2165	6690	1
65	Male	Negative	Negative	F4	A2	25.9	0.015	>5
61	Male	Negative	Negative	F2	A1	4	1.79	1
64	Male	Negative	Negative	F2	A0	NA	NA	1
63	Male	Negative	Negative	NA	NA	135.3	0.06	1
67	Male	Negative	Negative	NA	NA	3.5	0.013	1
75	Male	Negative	Negative	NA	NA	2	NA	1
62	Male	Negative	Negative	F2	A1	1026	13.32	1
62	Male	Negative	Negative	F2	A1	2.3	1.79	1
68	Female	Negative	Negative	F3	A2	9.1	0.016	1
59	Male	Negative	Negative	F0	A0	29	0.029	1
70	Male	Negative	Negative	NA	NA	488.3	601 371	1
54	Male	Negative	Negative	NA	NA	258	2.1	1
68	Female	Negative	Negative	NA	NA	2.8	0.023	1
60	Male	Negative	Negative	F2	A1	3.2	0.023	1
70	Male	Negative	Negative	NA	NA	5463	6.566	2
70	Female	Negative	Negative	NA	NA	464.2	NA	1
77	Male	Negative	Negative	NA	NA	72	0.136	2

NA, not available; HBs Ag, hepatitis B surface antigen; AFP, alpha-fetoprotein; PIVKA II, protein induced by vitamin K absence or antagonist-II; Vp, portal vein invasion; Vv, hepatic vein invasion; B, bile duct invasion; US, ultrasonography; CT, computed tomography

in sustained responders in whom HCC developed after successful IFN therapy, but data could be obtained for only two patients, who were negative for hepatitis B virus DNA. We cannot rule out the presence of occult hepatitis B virus in the other patients, although all patients were negative for hepatitis B antigen. In spite of these uncertainties, this study represents a comprehensive analysis of HCC developing after sustained response to IFN therapy, because we were able to collect clinical data for a large number of sustained responders at 16 major hospitals.

In this study, we encountered 29 patients in whom HCC developed after successful IFN therapy, but the reason why HCC developed in these sustained responders is unclear. The existence of a small undetected HCC at the time of IFN therapy may have been responsible for the appearance of HCC after the sustained response to IFN therapy. However, in 11 patients (38%), HCC was detected more than 5 years after IFN therapy, and the incidence of HCC gradually increased for at least 9 years after IFN therapy. Considering the late onset of HCC in these patients, we cannot neglect the possibility of the de-novo development of HCC after the eradication of HCV. HCV is a single-stranded RNA virus without a DNA intermediate in its replicative cycle, so that the integration of HCV nucleic acid sequences into the host genome seems unlikely. Therefore, it is difficult to believe that HCV itself is a causative factor of HCC in the absence of chronic inflammation, liver cell necrosis and regeneration, and extensive fibrosis. It is probable that carcinogenesis is not a single-step event, but a complex multistep process. Future studies should aim to define the basic oncogenic mechanisms by which sustained responders to IFN develop HCC. Exploration of these mechanisms may point the way toward new strategies for the prevention of HCC.

In conclusion, some patients showing a sustained response to IFN therapy given for chronic hepatitis C demonstrated potential for the development of HCC for up to 9 years following cessation of the treatment. This suggests that the risk of HCC in sustained responders is not completely eliminated. The establishment of risk factors and an index for the development of HCC may be useful in determining follow-up strategy in patients after a sustained response to IFN therapy given for chronic hepatitis.

Table 4. *Continued*

Maximum tumor size (mm)	Vp	Vv	B	Differentiation of HCC	Period to development Of HCC (years)	Medical follow-up period (months)	Diagnostic modality
18	0	0	0	Moderately	1.43	3	US
16	0	0	0	NA	1.51	1	US
>20	3	0	0	NA	1.79	None	US
15	0	0	0	Moderately	2.52	1	US
25	0	0	0	Moderately	3.32	2	CT
20	0	0	0	Well	3.39	3	US
34	2	1	2	Well	3.54	2	US
20	0	0	0	Well	3.59	3	Laparoscopy
40	0	0	0	NA	3.70	None	US
50	2	2	2	NA	3.89	None	US
30	0	0	0	Well	4.35	1	US
110	0	0	0	Poorly	4.38	6	US
15	0	0	0	Well	4.48	6	US
50	1	0	1	Moderately	4.58	12	US
80	0	0	0	Moderately	4.60	None	CT
NA	0	0	0	NA	4.70	6	US
44	0	0	0	NA	4.88	6	US
28	0	0	0	NA	4.97	3	US
60	1	1	1	Moderately	5.52	None	US
50	1	0	1	Moderately	5.58	6	US
51	0	0	0	Combined type	5.80	3	US
40	0	0	0	Moderately	5.86	None	US
>20	2	0	0	NA	6.61	3	US
150	3	0	0	Poorly	6.86	None	US
15	0	0	0	NA	8.05	3	US
15	0	0	0	Well	8.39	6	US
60	0	0	0	Well	8.78	None	US
16	0	0	0	NA	8.79	3	US
42	0	0	0	NA	8.98	1	CT

Appendix

In addition to the study authors' hospitals (the four institutions listed on the title page), data were supplied by the following hospitals and clinics in the Kyushu Division of the Japanese Society of Gastroenterology: Shinnittetsu Yahata Memorial Hospital; Yame General Hospital; First Department of Internal Medicine, Ryukyu University School of Medicine; Second Department of Internal Medicine, Kagoshima University School of Medicine; Hayato Town Medical Association Medical Center; Department of Internal Medicine, Saga Medical School; Department of Medicine and Biosystemic Science, Kyushu University School of Medicine; Nishinihon Hospital; Kagoshima Kouseiren Hospital; Miyata Memorial Hospital; Second Department of Internal Medicine, Nagasaki University School of Medicine; and Yonabaru Central Hospital.

Acknowledgments. The authors thank Dr. Michitami Yano for suggestions and support of this study; Dr. Seiichiro Yamamoto for providing statistical suggestions; Dr. Takuji Okusaka for carefully reviewing the manuscript and providing valuable suggestions; and Ms. Mayuko Ikeda and Ms. Yuriko Kawaguchi for database development and manuscript preparation. This study was supported in part by the Japanese Society of Gastroenterology, Kyushu Division, as a new project for 2001.

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