J Gastroenterol (2009) 44:952–963
DOI 10.1007/s00535-009-0087-x

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Predictive values of amino acid sequences of the core and NS5A regions in antiviral therapy for hepatitis C: a Japanese multi-center study

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Received: 31 March 2009 / Accepted: 20 April 2009 / Published online: 11 June 2009

Abstract
Background Chronic hepatitis C (CHC) genotype 1b patients with high viral load are resistant to peginterferon (PEG-IFN) and ribavirin (RBV) combination therapy, especially older and female patients.
Methods To elucidate the factors affecting early and sustained viral responses (EVR and SVR), 409 genotype 1b patients CHC with high viral loads who had received 48 weeks of PEG-IFN/RBV therapy were enrolled. The amino acid (aa) sequences of the HCV core at positions 70 and 91 and of the interferon sensitivity determining region (ISDR) were analyzed. Host factors, viral factors, and treatment-related factors were subjected to multivariate analysis.
Results Male gender, low HCV RNA load, high platelet count, two or more aa mutations of ISDR, and wild type of core aa 70 were independent predictive factors for SVR. In patients with over 80% adherences to both PEG-IFN and RBV, male gender, mild fibrosis stage, and wild type of core aa 70 were independent predictors for SVR.
Conclusions Independent predictive factors for SVR were: no aa substitution at core aa 70, two or more aa mutations in the ISDR, low viral load, high values of platelet count, mild liver fibrosis and male gender.

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Keywords Chronic hepatitis C · Peginterferon and ribavirin · Core amino acid · Interferon sensitivity determining region

Abbreviations

CHC	Chronic hepatitis C
PEG-IFN	Peginterferon
RBV	Ribavirin
RVR	Rapid viral response
cEVR	Complete early viral response
LVR	Late viral response
ETR	End of treatment response
NR	Non response
SVR	Sustained viral response
ISDR	Interferon sensitivity determining region
Aa	Amino acid
ALT	Alanine aminotransferase
PLT	Platelet
HCC	Hepatocellular carcinoma

Introduction

A combination of pegylated interferon (PEG-IFN) and ribavirin (RBV) therapy for 48 weeks achieves a sustained viral response (SVR) rate of 40–50% in chronic hepatitis C (CHC) patients with a high viral load of genotype 1 [1–4]. The dose-reduction rate and the frequency of discontinuation of this treatment are high in aged patients [5]. The SVR rate of the therapy is lower in females than males, especially in older patients in Japan [6].

Around 30% of HCV carriers have serum alanine aminotransferase (ALT) levels within the upper limit of normal ranges [7, 8] and HCV carriers with persistently normal serum ALT (PNALT) and serum platelet (PLT) counts of over 15 × 10^4/mm^3 show low grade hepatic fibrosis and good prognosis [9]. Before treating HCV carriers, it is very important to predict non-response to PEG-IFN plus RBV therapy because of its medical cost, adverse effects, and its impact on the long term quality of life.

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There are many factors affecting response to IFN monotherapy and PEG-IFN/RBV therapy, including body mass index (BMI) [10, 11], steatosis [12, 13], insulin resistance [14], stage of liver fibrosis [15, 16], total cholesterol (T. Chol), triglyceride (TG), adherence to both PEG-IFN and RBV [17], race [18, 19], age [1, 2, 20], and viral factors including serum quantity of HCV RNA, HCV genotype and substitution of amino acids (aa) in the interferon sensitivity determining region (ISDR, 2209–2248) of the nonstructural protein 5A (NS5A) [21] and in the core protein [22, 23]. Early viral response is an important predictive factor in PEG-IFN/RBV therapy for CHC patients with genotype 1 and high viral loads [24–27].

The aim of this study was to elucidate the valuable predictive factors of SVR in Japanese patients with HCV genotype 1b high viral loads following 48 weeks of PEG-IFN/RBV therapy, focusing on the relationship between aa substitutions in the ISDR and at core aa 70 and 91 and early viral kinetics.

Patients and methods

Selection of patients

This retrospective study was conducted at 15 clinical sites in Japan which are part of the Study Group of Optimal Treatment of Viral Hepatitis supported by the Ministry of Health, Labor and Welfare, Japan. Eligible subjects were CHC patients, who (1) had received liver biopsy; (2) were genotype 1b with high viral load (≥100 KIU/ml by Cobas Amplicor Hepatitis C Virus Test, version 2.0) at the start of PEG-IFN/RBV therapy; (3) received weekly injections of PEG-IFN-α-2b (PEG-INTRON; Shering-Plough, Kenilworth, NJ) of 1.5 μg/kg bw and oral administration of RBV (Rebetol; Shering-Plough) for 48 weeks. The amount of RBV was adjusted based on the subject's body weight; (600 mg for ≤60 kg bw, 800 mg for 60–80 kg bw, 1,000 mg for > 80 kg bw); (4) were examined serially for quantitative and qualitative HCV RNA; and (5) the aa sequences at positions 70 and 91 in the core region and of the ISDR in the NS5A had been determined in pretreatment sera.

Hepatitis B virus (HBV) infection, human immunodeficiency virus (HIV) infection, autoimmune hepatitis, primary biliary cirrhosis, hemochromatosis, and Wilson's disease were excluded. Histopathological diagnosis was based on the scoring system of Desmet et al. [28]. The definition of alcohol abuse included patients having a history of more than 100 kg of total ethanol intake. Complete blood counts, liver function tests, serum lipids, serum ferritin, serum fibrosis markers, fasting plasma glucose (FPG), and immune reactive insulin (IRI) were examined in most cases. Written informed consent was obtained from all

patients before treatment, and the protocol was approved by the ethics committees in each site.

Study design

Four hundred and nine patients who completed 48 weeks of treatment and were followed for more than 24 weeks after treatment were enrolled in the first study (*Study design 1*).

To elucidate the effect of aa substitution of HCV core and in the ISDR on HCV dynamics, including a rapid viral response (RVR), complete early viral response (cEVR), a late viral response (LVR) and SVR, according to gender and age (<60 years $\geq$ 60 years), 201 of the 409 patients maintaining over 80% adherences to both PEG-IFN and RBV were enrolled in the second study (*Study design 2*).

Nucleotide sequencing of the core and NA5A gene

The nucleotide sequences encoding aa 1–191 (HCV core) and aa 2209–2248 (ISDR) were analyzed by direct sequencing as described by Akuta et al. [22, 27] and Enomoto et al. [21]. In brief, RNA was extracted from the sera and converted to cDNA and two nested rounds of polymerase chain reaction (PCR) were performed. Primers used in the PCR were as follows; (a) Nucleotide sequences of the core region: the first-round PCR was performed with CC11 (sense) and e14 (antisense) primers [22, 27], and the second-round PCR with CC9 (sense) and e14 (antisense) primers [22, 27]. (b) Nucleotide sequences of the ISDR in NS5A: the first-round PCR was performed with ISDR1 (sense) and ISDR2 (antisense) primers [21], and the second-round PCR with ISDR3 (sense) and ISDR4 (antisense) primers [21]. These sequences were compared with the consensus sequence of genotype 1b (HCV-J) [29]. Wild types virus encoded arginine and leucine at aa 70 and 91, respectively, and the aa substitutions were glutamine or histidine at aa 70 and methionine at aa 91.

Viral kinetic study

Serum HCV RNA levels were measured by PCR (Amplicor HCV RNA kit, version 2.0, Roche Diagnostics) using samples taken before treatment and at 4, 12, 24, and 48 weeks after the therapy. SVR was defined as HCV RNA negativity by qualitative analysis by PCR at 24 weeks after the treatment. RVR was defined as HCV RNA negativity at 4 weeks, cEVR as HCV RNA negativity at 12 weeks, LVR as HCV RNA negativity during 13–24 weeks and an end of treatment response (ETR) as HCV RNA negativity at the end of treatment. Patients who remained positive for HCV RNA at the end of the treatment and at 24 weeks after the therapy were defined as non-responders (NR).

Adherences to PEG-IFN and RBV

Adherences to PEG-IFN and RBV were assessed by separately calculating the actual doses of PEG-IFN and RBV received as percentages of the intended dosages. Adherences to PEG-IFN and RBV were divided into two groups; 80%$\leq$ and <80%.

Statistical analysis

All data analyses were conducted using the SAS version 9.1.3 statistical analysis packages (SAS Institute, Cary, NC, USA). Individual characteristics between groups were evaluated by Mann–Whitney U test for numerical variables or Fisher's exact test for categorical variables. Variables exhibiting values of $p < 0.1$ in the univariate analysis were subjected to stepwise multivariate logistic regression analysis. The grade of steatosis and iron deposition in liver tissue, BMI, albumin (Alb), low density lipoprotein-cholesterol (LDL-C), homeostasis model assesment-insulin resistance (HOMA-IR), ferritin, and hyaluronic acid were excluded from multivariate logistic regression analysis because of the absence of those data in more than 10% of the patients. All p values of $p < 0.05$ by the two-tailed test were considered statistically significant.

Results

Study design 1

Baseline backgrounds, characteristics and adherences of peginterferon and ribavirin in males and females

The treatment outcome of PEG-IFN and RBV combination therapy depends on gender in Japanese patients, so in addition to aa substitutions in the ISDR in NS5A [21] or at HCV core 70 and 91 [22, 27], we compared the baseline characteristics according to gender (Table 1). Males were younger and the grade of hepatic inflammation was milder in males. The serum levels of LDL-C, PLT count, and aa substitutions of ISDR and at core 70 and 91 did not differ significantly different between males and females. The frequency of no alcohol abuse was significantly ($p < 0.0001$) higher in females than males (Some of them are not described in Table 1).

The rates of over 80% adherences to PEG-IFN and RBV were significantly lower ($p = 0.0066$, $p < 0.00001$, respectively) in females than males. Only in those above 60 years did the rate of over 80% adherence to PEG-IFN not differ significantly between males and females, but the rate of over 80% adherence to RBV was significantly lower ($p = 0.035$) in females than males (Table 1).

Table 1 Backgrounds and characteristics of male and female patients

Factors	Gender		*p* value
	Male	Female	
No. of patients	256 (62.6%)	153 (37.4%)	
Age			
Median (range)	53 (18–73)	59 (23–75)	0.00001
F stage			
F0–2	206 (80.5%)	119 (77.8%)	0.592
F3–4	50 (19.5%)	34 (22.2%)	
Grade (A factor)			
A0–1	163 (63. 7%)	79 (51.6%)	0.026
A2–3	93 (36.3%)	74 (48.4%)	
HCV RNA load 0 week (KIU/mL)			
Median (range)	1500 (100–5000 <)	1280 (100–5000<)	0.384
ALT 0 week (IU/L)			
Median (range)	74.5 (16–504)	59 (19–391)	0.001
BMI			
Median (range)	23.6 (17.5–31.2)	22.1 (16.1–33.9)	0.00033
Alb (g/dL)			
Median (range)	4.0 (3.0–5.2)	3.8 (3.0–4.8)	0.011
LDL-C (mg/dL)			
Median (range)	97 (30–185)	90 (34–174)	0.612
T-Chol (mg/dL)			
Median (range)	167 (85–273)	176 (114–261)	0.0016
PLT count ($\times 10^4$/mm^3)			
Median (range)	17.0 (8.0–31.9)	16.4 (8.1–39.9)	0.350
Amino acid mutation of ISDR			
0–1	200 (78.1%)	121 (79.1%)	0.608
2≤	56 (21.9%)	32 (20.9%)	
Amino acid substitution of core 70			
Wild	177 (69.1%)	114 (74.5%)	0.261
Mutant	79 (30.9%)	39 (25.5%)	
Amino acid substitution of core 91			
Wild	153 (59.8%)	98 (64.1%)	0.403
Mutant	103 (40.2%)	55 (35.9%)	
PEG-IFN adherence			
<80%	41 (17.7%)	42 (30.4%)	0.0066
80%≤	190 (82.3%)	96 (69.6%)	
Ribavirin adherence			
<80%	54 (23.6%)	73 (52.1%)	<0.00001
80%≤	175 (76.4%)	67 (47.9%)	
Age: <60 years			
PEG adherence			
<80%	30 (17.8%)	23 (31.5%)	0.027
80%≤	139 (82.2%)	50 (68.5%)	
Ribavirin adherence			
<80%	27 (16.2%)	31 (42.5%)	0.000029
80%≤	140 (83.8%)	42 (57.5%)	
Age: 60 years≤			
PEG adherence			
<80%	11 (17.7%)	19 (29.2%)	0.147
80%≤	51 (82.3%)	46 (70.8%)	
Ribavirin adherence			
<80%	27 (43.5%)	42 (62.7%)	0.035
80%≤	35 (56.5%)	25 (37.3%)	

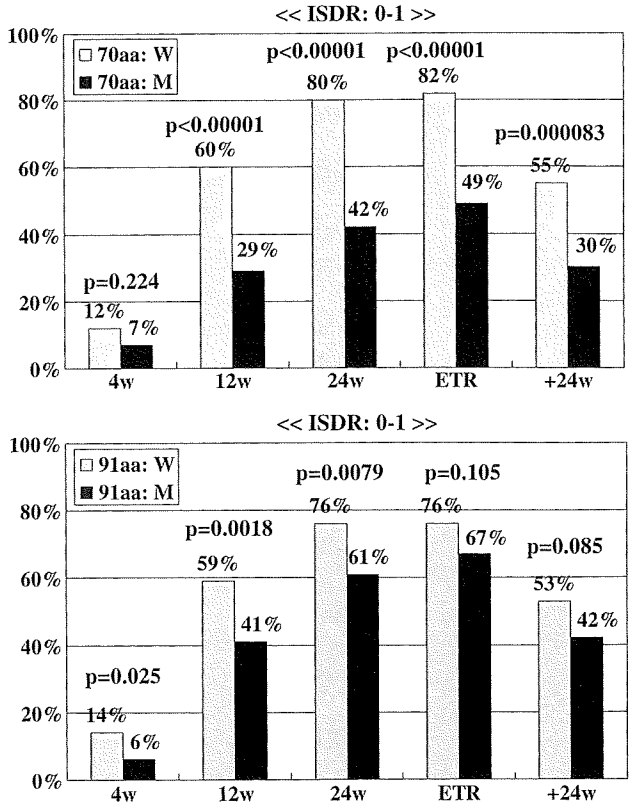

Fig. 1 Relationship between time course of serum HCV RNA negativity and amino acid substitutions in the ISDR and core amino acids 70 and 91. For cases with no or only one amino acid (aa) change in the ISDR, the rates of cEVR, LVR, ETR and SVR were significantly higher in patients with wild type core aa 70 but only the rates of RVR, cEVR, and LVR were significantly higher in patients with wild type core aa 91

Amino acid substitutions

There were no significant differences in the frequency of aa substitutions in the ISDR between males and females. Core aa substitutions at positions 70 and 91 were as follows; 291 (71.1%) were wild type and 118 (28.9%) were mutant at core aa 70, and 251 (61.4%) were wild type and 158 (38.6%) were mutant at core aa 91. There were no significant differences between males and females and between patients below and above 60 years of age.

Virological responses and aa substitutions

The rate of RVR did not differ significantly between males and females. However, more male patients showed HCV RNA negativity at 12 weeks (males vs. females; 60.7 vs. 48.4%, $p = 0.018$), 24 weeks (76.8 vs. 64.2%, $p = 0.0078$) and 48 weeks (78.2 vs. 68.6%, $p = 0.049$), and the proportion of male patients in SVR was significantly higher than that of females (61.3 vs. 37.3%, $p < 0.00001$).

RVR, cEVR and SVR rates were significantly higher in patients with two or more aa mutations in the ISDR compared to patients having no or one aa substitution in that region (20 vs. 11%, $p = 0.044$; 71 vs. 52%, $p = 0.0021$; 66 vs. 49%, $p = 0.0054$, respectively). AA substitution at core position 70 resulted in significantly lower rate of cEVR, LVR, ETR, and SVR (40 vs. 63%, $p = 0.000037$; 51 vs. 81%, $p < 0.00001$; 56 vs. 83%, 41 vs. 57%; $p < 0.00001$, $p = 0.0031$, respectively). Although the patients with the wild type aa at core 91 showed significantly higher rates of RVR and cEVR, the rate of SVR was not significantly higher in those patients ($p = 0.054$).

SVR rates were 30% for patients with no or one aa substitution in the ISDR and the core aa 70 substitution, and were significantly lower compared to those with the wild type aa core 70 (Fig. 1). These findings were not confirmed in patients with no or one aa substitution in the ISDR and the core aa 91 substitution (Fig. 1).

Factors affecting SVR by univariate analysis

Univariate analysis identified nine parameters that influenced non-SVR significantly: female gender, older age, advanced staged liver fibrosis, high viral load, low serum Alb level, low PLT count, no or one aa substitution in the ISDR, aa substitution at core aa 70, and low adherence to RBV (Table 2). The frequency of steatosis and HOMA-IR were significantly ($p = 0.0057$, $p < 0.00001$, respectively) lower in patients with SVR compared with non-SVR (data not shown). However, these factors were not entered in the multivariate analysis because of the absence of the data in many cases.

Factors affecting RVR, cEVR, and SVR by multivariate logistic regression analysis

Multivariate analysis identified four parameters that influenced RVR independently: low HCV RNA load, low serum ALT level, two or more aa mutations in the ISDR and the wild type aa at core position 91 (Table 3).

Concerning cEVR, male gender, mild fibrosis stage, low HCV RNA load, two or more aa mutations in the ISDR, and the wild type aa at core positions 70 and 91 were independent predictors (Table 3).

Concerning SVR, male gender ($p < 0.0001$), low HCV RNA load ($p = 0.013$), high PLT count ($p = 0.0019$), two or more aa mutations in the ISDR ($p = 0.024$), and wild type core aa 70 ($p = 0.0045$) were found to be independent predictors (Table 3).

The predictive values of the combination of gender, PLT count, ISDR and core aa 70 are shown in Fig. 2a. In male patients having PLT of $<15 \times 10^4/mm^3$, and, no or one aa substitution in the ISDR, the SVR rate was 68% when core 70

Table 2 Univariate analysis to identify the factors of SVR

Factors	Negative of HCV RNA after 24 weeks		*p* value
	(−)	(+)	
No. of patients	214 (52.3%)	195	
Gender			
Male	157 (61.3%)	99	<0.00001
Female	57 (37.3%)	96	
Age			
Median (range)	52.5 (18–75)	58 (20–74)	<0.00001
<60 years	155 (58.1%)	112	0.0018
60 years≤	59 (41.5%)	83	
Age: <60 years			
Male	118 (63.4%)	68	0.010
Female	37 (45.7%)	44	
Age: 60 years≤			
Male	39 (55.7%)	31	0.0011
Female	20 (27.8%)	52	
F stage			
F0–2	190 (58.5%)	135	0.000013
F3–4	25 (29.8%)	59	
Grade (A factor)			
A0–1	138 (56.8%)	104	0.130
A2–3	81 (48.5%)	86	
HCV RNA load 0 week (KIU/mL)			
Median (range)	1300 (100–5000<)	1700 (130–5000<)	0.016
ALT 0 week (IU/L)			
Median (range)	66 (16–391)	67 (19–504)	0.892
BMI			
Median (range)	23.0 (17.3–32.4)	23.25 (16.1–33.9)	0.714
Alb (g/dL)			
Median (range)	4.0 (3.2–5.2)	3.8 (3.0–4.8)	0.0088
LDL-C (mg/dL)			
Median (range)	94.5 (31–185)	97.5 (30–182)	0.611
T-Chol (mg/dL)			
Median (range)	169.5 (85–257)	170 (103–273)	0.511
PLT count ($\times 10^4/mm^3$)			
Median (range)	18.2 (8.7–39.9)	15.1 (8.0–31.9)	<0.00001
<15	54 (36.5%)	94	<0.00001
15≤	160 (61.3%)	101	
Amino acid mutation of ISDR			
0–1	156 (48.6%)	165	0.0054
2≤	58 (65.9%)	30	
Amino acid substitution of core 70			
Wild	166 (57.0%)	125	0.0031
Mutant	48 (40.7%)	70	
Amino acid substitution of core 91			
Wild	141 (56.2%)	110	0.054
Mutant	73 (46.2%)	85	
PEG-IFN adherence			
<80%	35 (42.2%)	48	0.063
80%≤	154 (53.8%)	132	
Ribavirin adherence			
<80%	55 (43.3%)	72	0.048
80%≤	132 (54.5%)	110	

Table 3 Multivariate logistic regression analysis to identify independent predictive factors of RVR, cEVR, and SVR

	Odds ratio	95% CI	*p* value
RVR factors selected by stepwise method			
F stage			
F0–2/F3–4	2.924	0.988–8.696	0.053
HCV RNA load 0 week (KIU/mL)			
<1000/1000≤	2.151	1.130–4.082	0.020
ALT 0 week (IU/L)			
<60/60≤	2.165	1.127–4.149	0.020
Amino acid mutation of ISDR			
2≤/0–1	2.371	1.187–4.735	0.014
Amino acid substitution of core 91			
W/M	2.137	1.021–4.464	0.044
cEVR factors selected by stepwise method			
Gender			
Male/female	1.912	1.209–3.021	0.0055
F stage			
F0–2/F3–4	2.079	1.133–3.817	0.018
HCV RNA load 0 week (KIU/mL)			
<1000/1000≤	1.608	1.002–2.577	0.049
PLT count ($\times 10^4$/mm^3)			
15≤/<15	1.427	0.882–2.309	0.148
Amino acid mutation of ISDR			
2≤/0–1	2.512	1.407–4.485	0.0018
Amino acid substitution of core 70			
W/M	2.513	1.508–4.184	0.0004
Amino acid substitution of core 91			
W/M	1.965	1.241–3.115	0.004
SVR factors selected by stepwise method			
Gender			
Male/female	3.704	2.132–6.410	<0.0001
F stage			
F0–2/F3–4	1.812	0.888–3.690	0.103
HCV RNA load0 week (KIU/mL)			
<1000/1000≤	2.024	1.163–3.534	0.013
PLT count ($\times 10^4$/mm^3)			
15≤/<15	2.469	1.394–4.372	0.0019
Amino acid mutation of ISDR			
2≤/0–1	2.148	1.107–4.170	0.024
Amino acid substitution of core 70			
W/M	2.415	1.316–4.444	0.0045
Amino acid substitution of core 91			
W/M	1.433	0.828–2.481	0.199
PEG adherence (%)			
80≤/<80	1.562	0.834–2.926	0.164

W Wild, *M* Mutant

was a wild type but only 16% in patients with mutant at core 70. In female patients, no or one aa substitution in ISDR and $<15 \times 10^4$/mm^3 of PLT count, the SVR rates were as low as 10 or 8%, irrespective of aa substitution at core 70. SVR was only 24% in patients with substitution of core aa 70 even when the PLT count was $\geq 15 \times 10^4$/mm^3. In this study, the combination analysis of PLT count, ISDR, and core aa substitution was useful for predicting non-SVR.

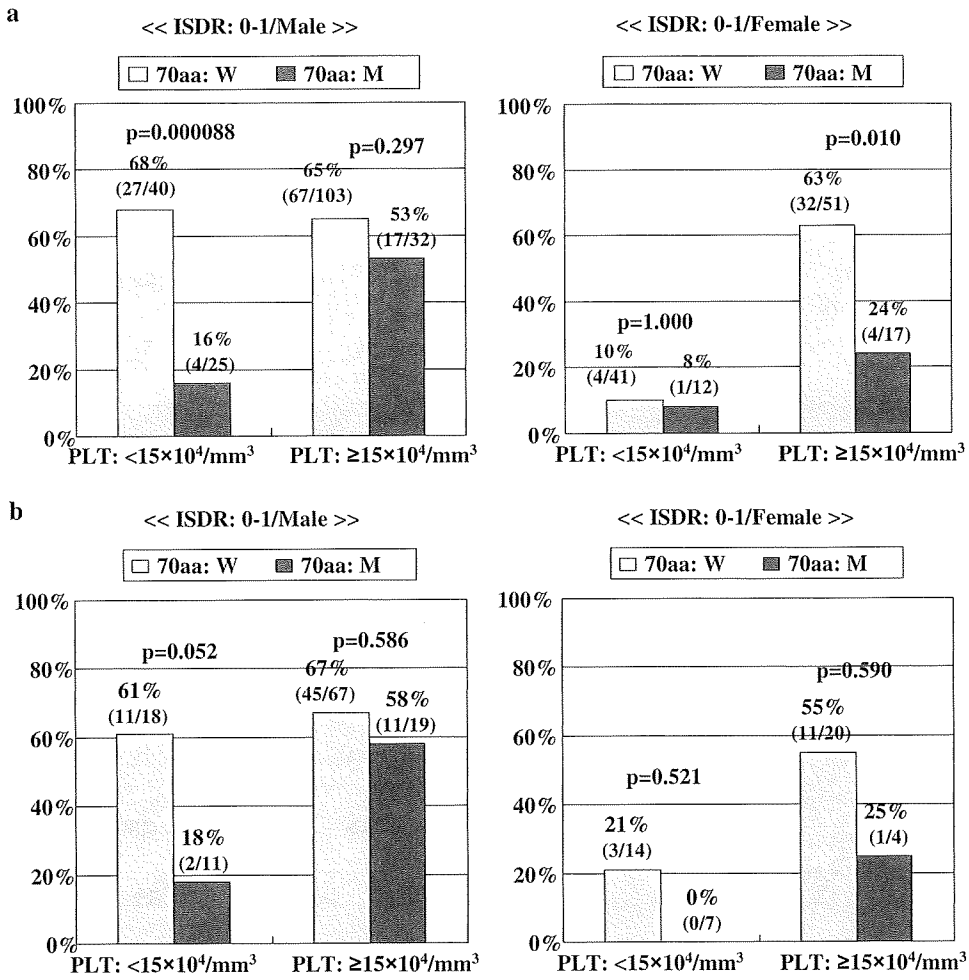

Fig. 2 Relationship between SVR rate and amino acid substitutions in the ISDR and core amino acids 70 and 91, PLT counts and gender difference. The two figures of **a** show the results of *Study 1* and the two figures of **b** show the results of *Study 2*. In male patients with no or only one amino acid (aa) substitution in the ISDR and PLT count of less than $15 \times 10^4/mm^3$, the SVR rate was 68% in those with wild type core aa 70, but only 16% in patients with mutant type of core aa 70, which is significantly different ($p = 0.000088$). There were no significant differences between wild type and mutant type of core aa 70 in the patients with no or one aa substitution in the ISDR and PLT count of over $15 \times 10^4/mm^3$. By contrast, in female patients with no or one aa substitution in the ISDR, there were no significant differences between wild type and mutant type of core aa 70 with PLT count of less than $15 \times 10^4/mm^3$, but there were significant differences between wild type and mutant type of core aa 70 with PLT counts of less than $15 \times 10^4/mm^3$ (**a**). For the patients maintaining over 80% adherences to both PEG-IFN and RBV, in males having no or one aa substitution in the ISDR and PLT counts of less than $15 \times 10^4/mm^3$, a wild type of core aa 70 could predict SVR with a positive predictive value (PPV) of 61% and negative predictive value (NPV) of 82% ($p = 0.052$). However, in male patients with PLT counts of over $15 \times 10^4/mm^3$, core aa 70 was not a useful marker for predicting SVR and non-SVR. The number of female patients with no or one aa substitution in ISDR was too small to reach a definite conclusion (**b**)

Study design 2

The basic features of 201 patients achieving 80% adherences to both PEG-IFN and RBV are as follows: the females were significantly ($p = 0.00006$) older than the males. Iron deposition in liver tissue, alcohol abuse, BMI, serum albumin level, serum ferritin level, and PLT count were significantly higher in males than females. Inflammatory activity was significantly ($p = 0.046$) higher in females than males (data not shown).

AA substitutions in the ISDR were as follows; in males 33 (22.3%) had two or more aa substitutions, in females 8 (15.1%) had two or more aa substitutions. The analysis of core aa position 70 and 91 sequences showed no significant differences in aa substitutions of either core aa 70 or 91 between males and females (data not shown).

In patients less than 60 years of age, SVR rate was significantly higher ($p = 0.0042$) in males than females, but no significant difference was noted between males and females over 60 years old. However, the number of patients over 60 years was small (Table 4).

Table 4 Univariate analysis to identify the significantly different factors between SVR and non-SVR (201 patients received over 80% adherences of both PEG-IFN and RBV)

Factors	Negative of HCV RNA after 24 weeks		*p* value
	(−)	(+)	
No. of patients	111 (55.2%)	90	
Gender			
Male	93 (62.8%)	55	0.00037
Female	18 (34.0%)	35	
Age			
Median (range)	51 (18–70)	56 (23–74)	0.00025
<60 years	91 (60.3%)	60	0.014
60 years≤	20 (40.0%)	30	
Age: <60 years			
Male	79 (66.4%)	40	0.0042
Female	12 (37.5%)	20	
Age: 60 years≤			
Male	14 (48.3%)	15	0.243
Female	6 (28.6%)	15	
F stage			
F0–2	103 (60.9%)	67	0.0012
F3–4	8 (25.8%)	23	
Grade (A factor)			
A0–1	80 (59.3%)	55	0.189
A2–3	31 (47.0%)	35	
HCV RNA load 0 week (KIU/mL)			
Median (range)	1300 (110–5000<)	1280 (130–5000<)	0.351
ALT 0 week (IU/L)			
Median (range)	74 (16–268)	67.5 (19–504)	0.752
BMI			
Median (range)	23.1 (17.3–31.0)	23.6 (16.1–33.9)	0.626
Alb (g/dL)			
Median (range)	3.95 (3.3–5.2)	3.9 (3.0–4.8)	0.079
LDL-C (mg/dL)			
Median (range)	96 (31–185)	97.5 (30–182)	0.865
T-Chol (mg/dL)			
Median (range)	170 (85–248)	170 (105–273)	0.624
PLT count ($\times 10^4/mm^3$)			
Median (range)	18.9 (8.7–30.9)	15.55 (7.2–28.4)	0.00003
<15	23 (35.9%)	41	0.00024
15≤	88 (64.2%)	49	
Amino acid mutation of ISDR			
0–1	84 (52.5%)	76	0.159
2≤	27 (65.9%)	14	
Amino acid substitution of core 70			
Wild	91 (61.5%)	57	0.0037
Mutant	20 (37.7%)	33	
Amino acid substitution of core 91			
Wild	73 (60.3%)	48	0.083
Mutant	38 (47.5%)	42	

Virological responses and aa substitution

The rates of RVR, cEVR, LVR, ETR and SVR in males and females were 12.5 versus 11.3% ($p = 1.000$), 59.6 versus 43.4% ($p = 0.053$), 74.3 versus 50.0% ($p = 0.0018$), 76.2 versus 66.7% ($p = 0.198$), and 62.8 versus 34.0% ($p = 0.00037$), respectively (data not shown). The backgrounds and characteristics of SVR and non SVR patients are shown in Table 4. There were significant differences in gender (male vs. female; $p = 0.00037$), age (<60 years vs. ≥60 years; $p = 0.014$), F stage (F0-2 vs. F3,4; $p = 0.0012$), PLT count ($<15 \times 10^4/mm^3$ vs. $15 \times 10^4/mm^3 \leq$; $p = 0.00024$), and substitution of core aa 70 (wild type vs. mutant, p = 0.0037) between SVR and non-SVR patients. The distribution of fatty change in liver tissue (≤10% vs. 11–33% vs. 34%≤; $p = 0.046$) and the grade of HOMA-IR (1.7 vs. 3.9, $p = 0.0018$) were significantly different between SVR and non-SVR (data not described in Table 4).

Factors affecting SVR by multivariate logistic regression analysis

Male gender ($p = 0.0006$), mild fibrosis stage ($p = 0.027$), and wild type of core aa 70 ($p = 0.043$) were independent predictors of SVR.

Valuable markers for predictions of sustained virological response to peginterferon and ribavirin therapy

Two or more aa mutations in the ISDR, wild type core aa 70, $\geq 15 \times 10^4/mm^3$ of PLT count, and male gender were selected statistically as independent predictors of SVR. We show here SVR rates of the patients having over 80% adherences to both PEG-IFN and RBV (Fig. 2b). In males having no or one aa substitution in the ISRD and PLT count of $<15 \times 10^4/mm^3$, wild type core aa 70 could predict SVR with a positive predictive value (PPV) of 61% and negative predictive value (NPV) of 82% ($p = 0.052$). In females, the SVR rate was very low in those who had substitution of core aa 70, but there was no significant difference between patients with wild type and substitution of core aa 70. The number of female patients was too small to provide a definite conclusion.

Discussion

The present multivariate logistic regression analysis revealed that male gender, low HCV RNA load, high PLT count, and two or more aa mutations in the ISDR and wild type core aa 70 were independent predictors for SVR. PLT count significantly decreased corresponding to the progression to the stage of liver fibrosis in CHC [9, 30, 31].

It has been considered that the low adherence level to PEG-IFN/RBV is a major cause of a significantly lower SVR rate in females and older patients [32]. The percentage of patients having over 80% adherences to both PEG-IFN and RBV was significantly lower in females than males, however, differences in the adherence to PEG-IFN/RBV between males and females were not independent predictive factors of non-SVR.

A recent report from Japan showed six or more mutations in the variable region 3 (V3) of nonstructural protein 5A (NS5A) plus upstream flanking region NS5A (aa 2334–2379), referred to as the IFN/RBV resistance determining region (IRRDR), was a useful marker for predicting SVR, but the ISDR sequence was not valuable for predicting SVR [33]. However, the number of subjects in that study was too small ($n = 45$) to reach an acceptable conclusion.

To elucidate the factors affecting low SVR rate in older female patients, we performed a multivariate logistic regression analysis using patients who achieved ≥80% adherence to both PEG-IFN and RBV. Male gender, stage of mild liver fibrosis, and wild type core aa 70 were independent predictors of SVR. In this study, blood concentration of RBV was determined in fewer than 50% of cases during treatment. Thus we cannot exclude the possibility of the effect of the blood concentration of RBV during treatment on the low SVR rate in females and older patients.

From the present analysis, it was clear that ALT, BMI, Alb, T. Chol, and adherence to RBV differed significantly between males and females, however, these factors were not independent predictors of SVR. There is a report that steatosis is an important cofactor that reduces the SVR rate in genotype 1 infected patients [34], however, such an effect was not seen in this study. Thus we could not identify the factors associated with a significantly lower SVR rate in females than males.

In the present multivariate logistic regression analyses, patients having wild type core aa 91 had significantly higher rates of RVR and cEVR, but not SVR, and patients with wild type core aa 70 had significantly higher rates of cEVR and SVR, but not RVR. Patients having two or more aa substitutions in the ISDR had significantly higher rates of RVR, cEVR, and SVR. Although several possibilities have been considered concerning the effects of aa substitutions of core protein on SVR in PEG-IFN/RBV therapy for CHC patients, the exact mechanisms have not yet been elucidated.

Recent reports have indicated that low serum IP-10 (interferon-γ inducible protein 10 kDa) [35], a higher HCV-specific CD8 cell proliferation potential [36], and a high ratio of Th1/Th2 [37] are good predictors of SVR to

PEG-IFN/RBV therapy. These results indicate the importance of immunological status and immunological response to treatment in patients difficult to treat with PEG-IFN/RBV therapy for CHC.

The present univariate analyses revealed that there were many factors relating to RVR, cEVR, and SVR including LDL-C, HOMA-IR, fatty change in liver tissue, and hyaluronic acid, however some of these factors had not been examined in some participating institutes. We consider that we must perform a prospective mass study using many factors including immunological aspects, viral factors, disease status, and therapeutic aspects to elucidate the reason that older female patients are resistant to a combination of PEG-IFN and RBV therapy in CHC with a high viral load genotype 1b.

In conclusion, our results demonstrated that wild type core aa 70, two or more aa mutations in the ISDR, low viral load, high PLT counts, and male gender are useful markers for predicting SVR.

Acknowledgments We express our thanks to other members of the Study Group of Optimal Treatment of Viral Hepatitis; Hideyuki Nomura, Shin-Kokura Hospital; Yoshiyuki Ueno, University of Tohoku; Hisataka Moriwaki, Gifu University; Makoto Oketani, Kagoshima University Graduate School of Medical and Dental Sciences; Masataka Seike, Oita University; Hiroshi Yotsuyanagi, The University of Tokyo. This study was supported in part by a Grant-in-Aid from the Ministry of Health, Labor and Welfare, Japan.

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J Gastroenterol (2010) 45:86–94
DOI 10.1007/s00535-009-0128-5

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Etiology of liver cirrhosis in Japan: a nationwide survey

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Received: 17 July 2009 / Accepted: 19 August 2009 / Published online: 30 September 2009

Abstract
Background Little is understood about worldwide changes in the epidemiological distribution of the etiology of liver cirrhosis (LC). The present study examines the etiology of liver cirrhosis in Japan using a nationwide survey.
Methods We analyzed data from 33,379 patients with LC at 58 hospitals and presented the findings in a poster symposium regarding the etiology and clinical features of LC in Japan that was included in the program of the 44th Annual Meeting of the Japan Society of Hepatology. We identified the distribution of the etiology of LC and compared the present with previous Japanese findings to estimate the future of etiological changes in LC.
Results The etiological agents were as follows: hepatitis B virus (HBV) 13.9%, hepatitis C virus (HCV) 60.9%, alcohol 13.6%, primary biliary cirrhosis (PBC) 2.4% and autoimmune hepatitis (AIH) 1.9%. Cirrhosis was considered to be related to nonalcoholic steatohepatitis (NASH) in 2.1% of the patients. The ratio of HCV-related LC was significantly higher among patients with hepatocellular carcinoma (HCC) ($P < 0.0001$) compared to those without, whereas the ratios of alcohol, PBC, AIH were lower. HCC was evident in 31.5% of NASH-related LC.
Conclusions The major etiology of liver cirrhosis in Japan remains HCV. Our survey revealed the prevalence of NASH-related LC in Japan and the frequency of HCC. Future changes in etiology must be considered in establishing preventive or educational strategies, as well as in developing new treatment strategies.

Keywords Carcinogenesis · Hepatitis B virus · Hepatitis C virus · Hepatocellular carcinoma · Nonalcoholic steatohepatitis

Abbreviations

AIH	Autoimmune hepatitis
ANA	Anti-nuclear antibody
BMI	Body mass index
DM	Diabetes mellitus
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
LC	Liver cirrhosis
MetS	Metabolic syndrome

Participating investigators of The Japan Etiology of Liver Cirrhosis Study Group are listed in the Appendix.

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NAFLD Nonalcoholic fatty liver disease
NASH Nonalcoholic steatohepatitis
PBC Primary biliary cirrhosis
PSC Primary sclerosing cholangitis

Introduction

Liver cirrhosis (LC) is a life-threatening, major worldwide health problem that is defined as regenerative nodule development after chronic liver diseases. A considerable ratio of patients with LC can progress to liver failure, hepatocellular carcinoma (HCC) and portal hypertension. Chronic infection with hepatitis B virus (HBV), hepatitis C virus (HCV), and alcohol consumption are the major global causes of liver cirrhosis, but the epidemiology and etiology are not well described. Alcohol and HCV are common causes of LC in European, North American and other developed countries, whereas HBV is the major cause in many Asian and African countries [1, 2]. Information about the exact ratios of LC etiology in individual areas or countries is minimal, and few Japanese reports describe analyses of large patient cohorts or nationwide surveys. Globally, about 57% of cirrhosis was attributable in 2007 to either HBV (30%) or HCV (27%) [2]. Reports indicate that alcohol is the leading cause of LC, followed by HCV in the United States of America (USA) and the United Kingdom (UK), whereas HCV is the major cause in Italy [1–5]. On the other hand, HBV is the major cause of LC in endemic areas. Liver cirrhosis of unknown causes has been referred to as cryptogenic cirrhosis, and nonalcoholic steatohepatitis (NASH) is recognized as an important cause of cryptogenic LC and/or HCC [6–9]. However, the exact prevalence of NASH-related LC is unknown.

Based on this background we analyzed the etiology and clinical features of 33,379 patients with LC from 58 hospitals nationwide and then determined accurate etiological ratios for liver cirrhosis in Japan in July 2008.

Patients and methods

Patients

A group of 58 hospitals throughout Japan responded to mailed questionnaires regarding the etiology of liver cirrhosis. The data from 33,379 patients with liver cirrhosis were presented in a poster symposium regarding the etiology and clinical features of LC at the 44th Annual Meeting of the Japan Society of Hepatology during June 2008. We included all university hospitals and other major hospitals in Japan that contribute to the care of liver diseases. The appendix lists the cooperating institutions. The ethics committees of the appropriate institutional review boards approved this study in accordance with the Declaration of Helsinki 2000.

Criteria and questionnaire

Table 1 lists the criteria for LC and the definition of etiology applied in this study. We enrolled patients who were

Table 1 Criteria for diagnosis of liver cirrhosis and classification of etiology

I. Criteria for diagnosis of liver cirrhosis

Autopsy, laparoscopy or abdominal imaging (left lobe hypertrophy with splenomegaly, nodular changes in liver surface) and laboratory findings (low platelet count, albumin, and/or prolonged prothrombin time) compatible with liver cirrhosis. Also clinically diagnosed in patients with clinical findings of esophageal varices, ascites, or hepatic encephalopathy. Patients diagnosed solely based on histological liver biopsy findings are excluded.

II. Criteria for classification of etiology

1. Hepatitis B virus (HBV): positive for HBsAg and/or anti-HBc with high titer
2. Hepatitis C virus (HCV): positive for anti-HCV and HCV-RNA
3. HBV + HCV
4. Alcohol: criteria proposed by the Japanese Study Group of Alcoholic Liver Disease
5. Primary biliary cirrhosis
6. Other biliary cirrhosis (primary sclerosing cholangitis, etc.)
7. Autoimmune hepatitis
8. Metabolic diseases (Wilson disease, hemochromatosis, etc.)
9. Congestive disease
10. Parasites
11. Other known etiology
12. Nonalcoholic steatohepatitis (NASH): fulfillment of criteria described below and not meeting any criteria for above known etiologies.
13. Unknown etiology

Table 2 Criteria for NASH-related cirrhosis

I. Clinically supposed NASH-related cirrhosis

Fulfillment of the following criteria but without liver biopsy.

1. Alcohol consumption: less than 20 g/day
2. No other etiology for liver disease
3. Combined with diseases or states that could cause fatty liver diseases such as obesity (body mass index >25), diabetes mellitus and metabolic syndrome.

II. Histologically diagnosed NASH-related cirrhosis

Fulfillment of Criteria I, and histological liver biopsy findings are suitable with NASH (micronodular cirrhosis, perisinusoidal fibrosis, fatty change).

histologically and clinically diagnosed with LC, and with LC complicated by HCC. Since consensus has not been reached regarding criteria for liver cirrhosis caused by NASH, we tentatively established the criteria shown in Table 2. Final diagnosis of LC and diagnosis of etiology was determined in each institution. We also collected clinical information (age, gender, body mass index, complicating diseases and laboratory data) about patients with LC related to NASH or of unknown etiology. Alcoholic LC was diagnosed according to the criteria proposed by Takada et al. [10]. The time of diagnosis was not restricted in this retrospective study.

Statistical analyses

Data were statistically analyzed by the χ^2 test and by Student's t test using SPSS version 13.0J software (SPSS, Inc., Tokyo, Japan). All statistical tests were two-sided. P values below 0.05 were considered significant.

Results

Etiology of liver cirrhosis

Of the 33,379 patients with LC included in this study, 20,817 (62.4%) were male, 12,562 (37.6%) were female and 16,117 overall (48.3%) had HCC at the time of diagnosis with LC.

Figure 1 compares the etiology with previous Japanese data. The present study found the following causes of LC: HCV 60.9%, HBV 13.9%, alcohol 13.6%, PBC 2.4%, NASH-related 2.1% and AIH 1.9%. Among the remaining 4.0% of patients, other known and unknown etiologies were identified in 1.0 and 3.0%, respectively. Other known etiologies comprised: other biliary cirrhosis 0.3%, congestive cirrhosis 0.3% and parasites 0.1%. Other biliary cirrhosis ($n = 104$ patients) comprised primary sclerosing cholangitis (PSC; $n = 66$), congenital biliary atresia ($n = 16$), and others ($n = 22$). Metabolic diseases ($n = 91$) comprised Wilson's disease ($n = 55$), hemochromatosis ($n = 24$), glycogen storage disease ($n = 5$), citrunemia ($n = 3$), porphyria ($n = 3$) and amyloidosis ($n = 1$).

The results show that hepatitis virus, particularly HCV, remains a major cause of liver cirrhosis in Japan. On the other hand, the incidences of HBV and of alcohol-induced LC are decreasing. We focused on NASH-related LC for this analysis, and speculated that NASH represents a major unrecognized etiology. Data from 1998 show total values for LC excluding HBV, HCV and alcohol, of 8.8%, compared with the current value of 9.9%. Thus, NASH seemed to have historically been categorized as liver cirrhosis of unknown etiology.

Geographic differences in Japan

Figure 2 shows the geographic distribution of the etiology of liver cirrhosis in Japan. The most prevalent source in almost all areas in Japan except Okinawa was HCV. Alcohol was the most prevalent in Okinawa, followed by HCV and HBV. The prevalence of HBV was relatively higher in Hokkaido, Kyushu and in some western areas, and NASH was also more frequent (10%; fivefold higher than in other areas) in Japan.

Differences between males and females

Figure 3a shows differences in the etiology of LC between males and females. The ratio of alcohol was higher among males (19.2 vs. 4.3%; $P < 0.0001$, χ^2 test), whereas the ratios of PBC (0.6 vs. 5.3%), AIH (0.4 vs. 4.3%) and NASH (1.4 vs. 3.4%) were higher among females ($P < 0.0001$ for all). More female patients had LC of unknown etiology (2.3 vs. 4.0%, $P < 0.0001$). The numbers of males and females in the group with other known etiology were PSC, 44 and 22, Wilson's disease, 31 and 24 and hemochromatosis, 18 and 6, respectively.

Difference in etiology with or without HCC

The patients were categorized based on the presence of HCC, and then the etiology was analyzed (Fig. 3b). The

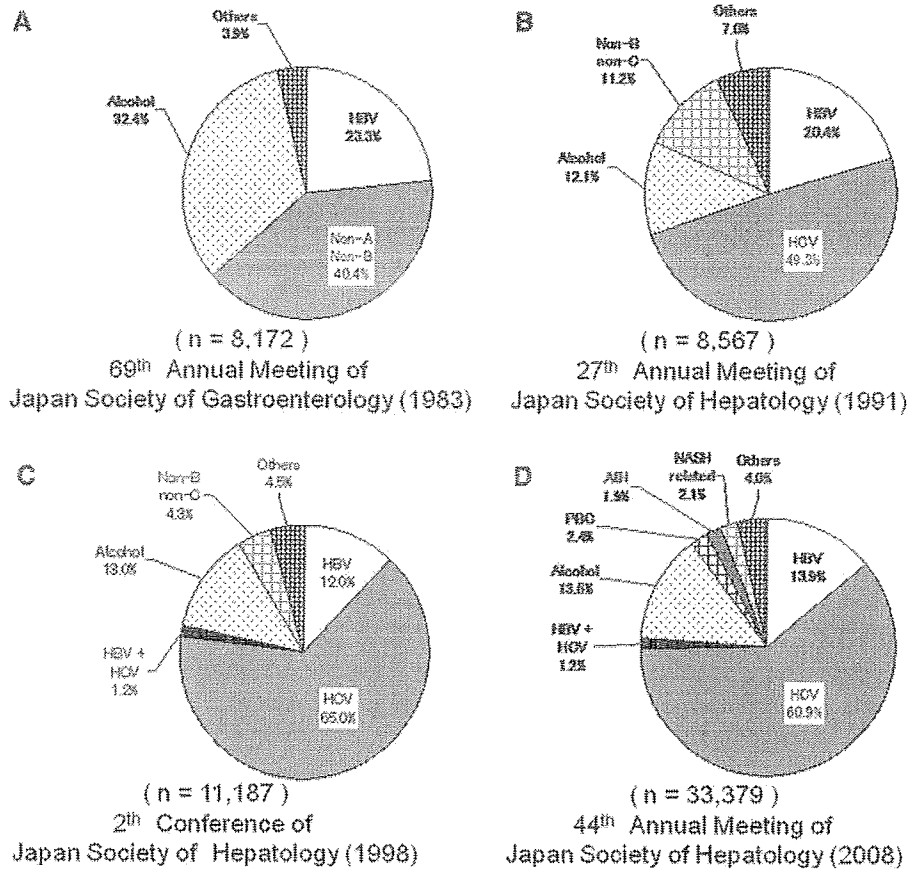

Fig. 1 Etiology of liver cirrhosis in Japan. Data presented at the 69th Annual Meeting of Japan Society of Gastroenterology in 1983 (edited by Dr. Sukeo Yamamoto) (**a**), 27th Annual Meeting of Japan Society of Hepatology in 1991 (edited by Dr. Yasuyuki Ohta) (**b**), 2nd Conference of Japan Society of Hepatology in 1998 (edited by Dr. Kennichi Kobayashi) (**c**), and 44th Annual Meeting of Japan Society of Hepatology (present study) (**d**)

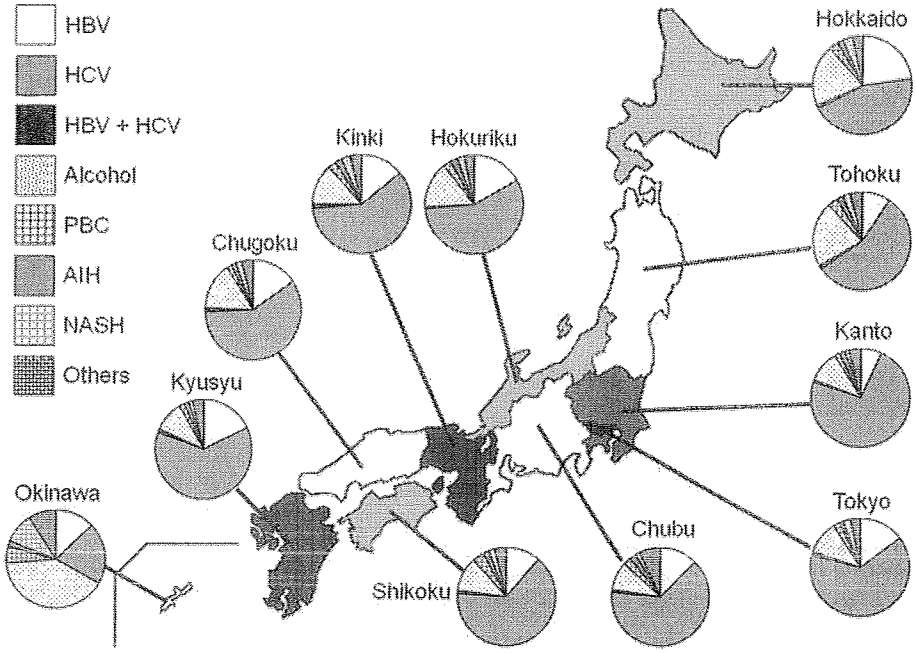

Fig. 2 Geographic distribution of etiology of liver cirrhosis in Japan

ratios among patients without HCC were HCV 49.4%, alcohol 20.5%, HBV 13.7%, PBC 4.0% and AIH 3.1%. On the other hand, the ratio of HCV was significantly higher (73.1%; $P < 0.0001$; χ^2 test), whereas those of alcohol, PBC, AIH were significantly lower (6.3, 0.6, 0.6%, $P < 0.0001$, respectively), and the findings were similar for HBV (14.1%). Among the metabolic diseases, HCC was complicated with Wilson's disease, hemochromatosis and

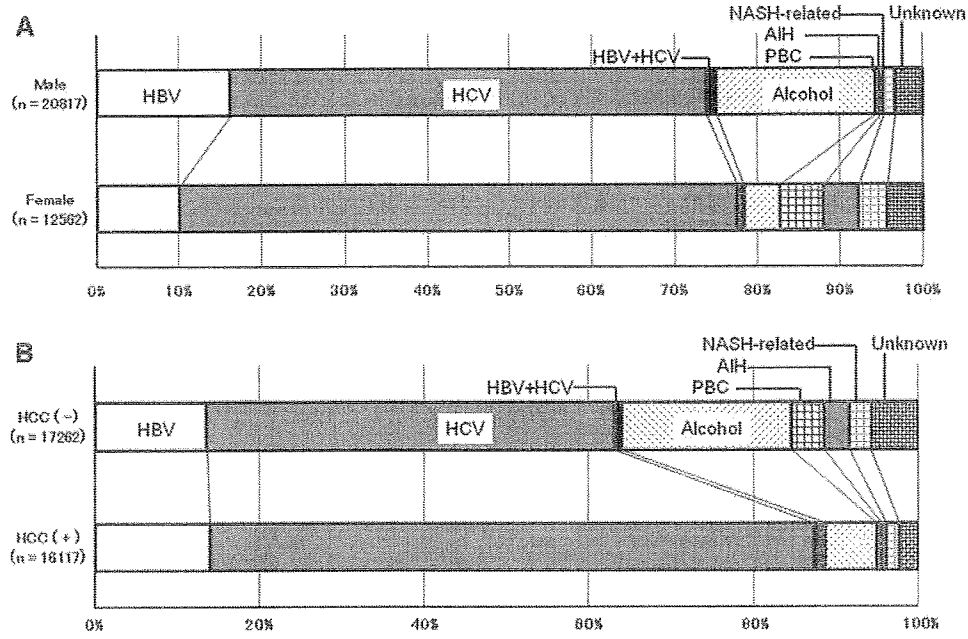

Fig. 3 Etiology of liver cirrhosis classified by gender and hepatocellular carcinoma. Data groups are separated based on gender (**a**) and hepatocellular carcinoma (**b**)

Table 3 Clinical features of NASH-related liver cirrhosis

	Total	Male (M)	Female (F)	*P* value (M vs. F)
Patients (%)	647	261 (40.3)	386 (59.7)	
Age (years)	66.6 ± 10.9	64.2 ± 12.2	68.2 ± 9.5	<0.001*
BMI (kg/m^2)	27.6 ± 4.5	26.9 ± 4.1	28.0 ± 4.8	<0.005*
Total cholesterol (mg/dl)	168.7 ± 49.1	170.1 ± 49.0	167.7 ± 49.2	NS
Triglyceride (mg/dl)	114.6 ± 114.6	124.5 ± 93.7	107.9 ± 68.0	<0.05*
Fasting plasma glucose (mg/dl)	138.6 ± 56.8	140.6 ± 58.5	137.2 ± 55.6	NS
Fasting insulin (μU/ml)	22.5 ± 25.2	20.1 ± 14.8	24.1 ± 30.3	NS
HOMA-IR	7.74 ± 9.24	7.51 ± 9.05	7.90 ± 9.41	NS
Hypertension (%)	316 (50.2)	110 (43.1)	206 (54.9)	<0.001*
Diabetes Mellitus (%)	424 (66.6)	175 (67.8)	249 (65.7)	NS
Hepatocellular carcinoma (%)	199 (31.5)	109 (42.2)	90 (24.1)	<0.05*

NS not significant

* *P* value determined by Student's *t* test

glycogen storage disease in 2 of 55, 4 of 24 and 2 of 5 patients, respectively.

NASH-related cirrhosis

Nonalcoholic steatohepatitis was associated with 2.1% of all LC; that is, in 2.7 and 1.6% of the groups without and with HCC complications, respectively. Table 3 shows the clinical background including laboratory data, complications and features of NASH-related LC in 647 patients. Mean age and body mass index (BMI) were 66.6 ± 10.9 and 27.6 ± 4.5, respectively. The women were older and had a higher BMI ($P < 0.001$ and <0.005, respectively) than the men. Hypertension and diabetes mellitus were complications in 50.2 and 66.6%, respectively, of those with NASH-related LC. HCC was frequently complicated with NASH-related LC (31.5%), especially among males (males vs. females: 42.2 vs. 24.1%, $P < 0.005$). Moreover, 10% of NASH-related LC was complicated with HCC during our 10-year study period. However, precise data about the study period of each followed-up patient was unavailable, so the accurate occurrence rate of HCC among patients with NASH could not be determined from this study. The prevalence of hypertension was higher in women, whereas that of HCC was higher among men. Anti-nuclear antibody (ANA) was found in 36.7% of all patients (males vs. females: 31.5 vs. 37.2%). One-third of patients with NASH were also positive for ANA, and the

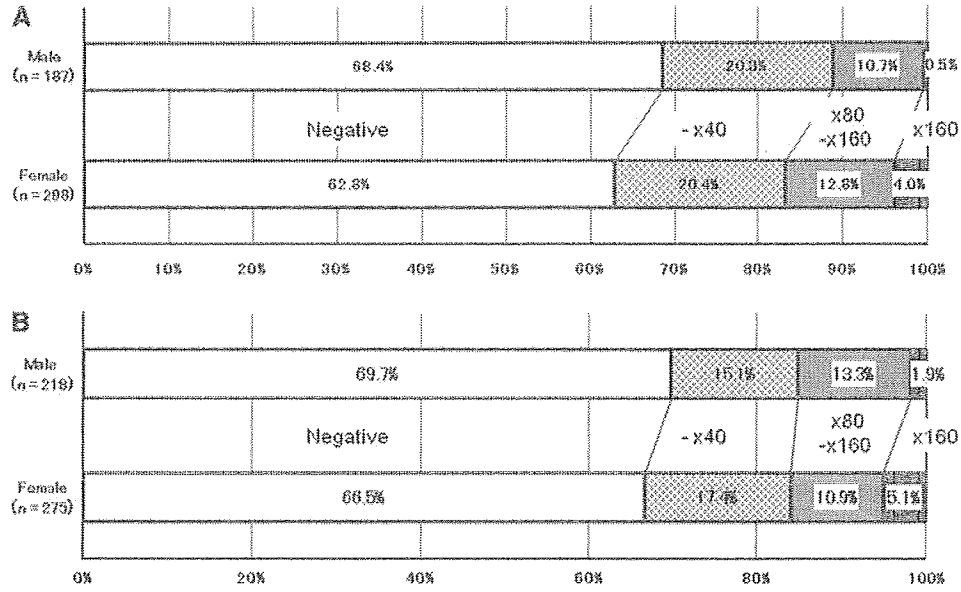

Fig. 4 Prevalence of anti-nucleic antibody (ANA) in liver cirrhosis that is NASH-related and of unknown etiology. Data show prevalence of ANA in LC related to NASH (**a**), and in LC of unknown etiology classified according to gender (**b**)

Table 4 Clinical features of liver cirrhosis of unknown etiology

	Total	Male (M)	Female (F)	*P* value (M vs. F)
Case (%)	801	385 (48.1)	416 (51.9)	
Age (years)	68.6 ± 11.1	66.8 ± 11.4	70.3 ± 10.5	<0.001*
BMI (kg/m^2)	23.9 ± 4.3	23.6 ± 3.8	24.2 ± 4.7	<0.001*
Total cholesterol (mg/dl)	152.4 ± 46.8	155.3 ± 51.1	149.7 ± 42.5	NS
Triglyceride (mg/dl)	88.3 ± 53.0	94.7 ± 58.6	82.4 ± 46.6	<0.01*
Fasting plasma glucose (mg/dl)	118.1 ± 46.6	118.5 ± 42.2	117.8 ± 50.3	NS
Fasting insulin (μU/ml)	14.0 ± 12.2	14.4 ± 14.4	13.6 ± 10.2	NS
HOMA-IR	4.0 ± 3.8	3.9 ± 2.8	4.2 ± 4.5	NS
Hypertension (%)	230 (30.7)	97 (27.6)	133 (33.5)	NS
Diabetes mellitus (%)	229 (30.3)	120 (33.8)	109 (27.3)	<0.001*
Hepatocellular carcinoma (%)	248 (32.6)	157 (43.0)	91 (23.0)	<0.001*

NS not significant

* *P* value determined by Student's *t* test

prevalence of ANA positivity was higher among females than males ($P < 0.005$; Fig. 4a).

Table 2 shows that we diagnosed NASH-related LC in one group that had been histologically diagnosed with NASH (39.4%; 255/647), and in another with clinically supposed NASH (60.6%; 392/647) without a histological examination. The frequency of HCC did not differ between histologically proven and supposed NASH (28.2 vs. 33.6%). Moreover, the positive rates of HBc antibodies did not differ between NASH-related LC with and without HCC (22.2 vs. 23.0%). We could not identify any other significant differences in the backgrounds of the patients between these groups. The positive ratio for ANA also did not differ between histologically diagnosed and supposed NASH (33.0 vs. 36.8%, respectively).

Cirrhosis of unknown etiology

We found LC of unknown etiology in 3.0% of the patients (3.8 and 1.8% with and without HCC complications, respectively). Table 4 shows the clinical data of 801 patients (mean age 68.6 ± 11.1 years). Age, BMI, triglyceride levels and HCC occurrence were significantly higher among females than males, findings similar to those of NASH-related LC (Table 3). Complication with diabetes mellitus was more prevalent among males than females among the patients with LC of unknown etiology. On the other hand, hypertension was more evident in females than in males among the patients with NASH-related LC. ANA was found in 33.9% of all patients (males vs. females: 30.3 vs. 33.5%; Fig. 4b). The positivity rate in females was

higher than that in males ($P < 0.005$), but that of ANA did not differ between total LC of unknown etiology and NASH-related LC (33.9 vs. 36.7%, respectively).

Discussion

The present study confirmed that HCV infection is the most prevalent cause of LC in Japan, accounting for about 60% of all LC. The induction of LC by HBV and alcohol was similarly prevalent, and these comprised the second and third most prevalent etiological factors. Causes related to NASH accounted for only 2.1% of LC in Japan. Among other types of metabolic cirrhosis, Wilson's disease was the leading cause, followed by hemochromatosis. A comparison with previous Japanese data showed that HCV remains the major etiology of liver cirrhosis in Japan, while the ratio of HBV has decreased over the past 17 years (Fig. 1b). Patients in Japan become infected with HCV at peak ages that are about 10–20 years older than those in the USA and European countries [11]. The present etiological features of HCV-induced LC in Japan are thus likely to be reflected in the USA and in European countries 10–20 years later.

The distribution of the etiology of liver cirrhosis in each area was similar in all geographic areas of Japan except Okinawa (Fig. 2), which was under American occupation for 27 years after World War II. Compared with other parts of Japan, Okinawa has more American cultural influences including food (such as fast-foods that have higher fat content and more calories). By the year 2000 (60 years after World War II), the mean lifespan in Okinawa had decreased from being the longest in Japan, while the prevalence of obesity had increased to being the highest in Japan [12, 13]. Such influences will be related to the increased ratio of NASH compared with other areas of Japan. Moreover, the prevalence of alcohol-related cirrhosis is higher and the ratio of HCV is lower in Okinawa than in other areas of Japan. These reasons will be clarified in future studies.

We analyzed the data based on gender and HCC (Fig. 3). The results indicated that alcohol-induced LC is more predominant among males, whereas HCV, autoimmune liver diseases (AIH or PBC) and NASH are more predominant among females. AIH occurs more frequently among females, and males consume more alcohol than females in Japan. Annual health screening has revealed that more males are HBs-antigen positive [14], whereas the frequency of HCV-antibody positivity is similar in both males and females [15]. More females than males had LC of unknown etiology. We suspect that this group included some patients with autoimmune or NASH-related cirrhosis that was not classified by the present criteria.

The ratios of etiologies among patients with HCC in the present study were comparable with those of other national surveys of HCC in Japan [16]. Additionally, our analyses clarified differences in etiological ratios between LC with and without HCC (Fig. 3). The ratio of HCV was higher among LC patients with HCC (67.2 vs. 57.5%, $P < 0.0001$), indicating that HCV itself has the potential to evoke hepatocarcinogenesis in patients with LC. On the other hand, HBV, alcohol, AIH and NASH would have less potential to evoke HCC than HCV. Although a prospective study is required to confirm this notion, these data are nevertheless sufficient to suggest that HCV infection contributes to hepatocarcinogenesis. Notably, many patients with HCC also had LC caused by alcohol, PBC, AIH and that related to NASH. Therefore, cirrhotic patients with not only viral hepatitis but also with these non-viral etiologies should be screened for HCC.

Recently, NASH has become recognized as an important cause of LC. Diagnostic criteria for nonalcoholic fatty liver disease (NAFLD) or NASH have been discussed elsewhere [17, 18]. Although the gold standard for a diagnosis of NASH is a liver biopsy, a risk of sampling error persists [17]. Furthermore, to obtain liver biopsies from patients with advanced cirrhosis is hazardous. Therefore, the actual prevalence of NASH-related LC has been difficult to define. Cryptogenic cirrhosis is thought to include NASH-related LC, and metabolic syndrome (MetS) is often a complication of NASH [18]. However, the importance of NASH in the etiology of liver cirrhosis remains ambiguous.

We considered that clinical etiologic criteria without a liver biopsy are needed to determine the accurate ratios of NASH-related cirrhosis due to the above reasons. The etiological criteria for clinically supposed NASH-related cirrhosis satisfied all of the factors listed in Table 2. We included obesity, diabetes mellitus (DM) and MetS among these criteria. The backgrounds of the patients diagnosed histologically and non-histologically with NASH-related LC did not differ. Under this classification, 0.9 and 1.2% of the 2.1% of patients with NASH-related LC were diagnosed histologically and non-histologically, respectively. Bell et al. reported that NAFLD accounted for 14.7% of LC in USA [4]. Thus, the frequency of NASH (NAFLD)-related LC is lower in Japan than in the USA, as is the frequency of MetS [19, 20]. The data show that the present frequency of NASH-related LC in Japan is quite low. However, the frequency of NASH-related LC will increase in Japan due to alterations in lifestyles (such as increased food consumption, type of food, stress and sedentary lifestyles), whereas that of HCV or HBV-related LC will decrease [21], as shown in Okinawa.

The frequency of HCC combined with NASH-related LC was high, especially among males (Table 3). The total

frequency of HCC in NASH-related LC herein was higher than in previous reports [22]. One reason for this is that our criteria for NASH-related LC included patients with higher-risk HCC compared with other studies. However, the frequencies of HCC in histologically defined and non-histologically supposed NASH did not differ. Another explanation is that occult HBV could be related to hepatocarcinogenesis in NASH-related HCC. However, the positivity rates of HBc antibody did not differ between NASH-related LC with and without HCC. Thus, the above factors probably did not influence our results. The reported incidence of HCC is higher in males with type 2 DM than in females [23, 24]. This evidence might be related to our findings, but further studies are required to reach a conclusion.

According to our criteria, cryptogenic LC (or LC of unknown etiology) of patients who do not consume alcohol but who were obese or complicated with DM or MetS were classified as having NASH-related LC. On the other hand, some patients with NASH-related cirrhosis without obesity, DM and MetS might have been included in the group with LC of unknown etiology. This group accounted for 3% of the studied patients, which was lower than that previously reported [1, 3, 4]. This group included LC due to viruses other than HBV and HCV, undiagnosed congenital diseases, undiagnosed AIH and patients with NASH who did not fulfill the study criteria. Patients who had been HBV carriers but who had become negative for HBsAg, or patients with occult HBV might have also been included [25]. However, the backgrounds of the male and female patients with NASH-related LC and LC of unknown etiology were quite similar (Tables 3, 4), as were the positivity and distribution of the ANA titers in both groups (Fig. 4). Further studies should clarify the real cause of LC with unknown etiology. Nevertheless, the present results suggest that some patients with NASH-related LC were included in the group with unknown etiology, rather than patients with undiagnosed AIH who are positive for ANA. If so, the estimated frequency of NASH-related LC will be 5–6% of all LC.

Our nationwide survey determined the etiology of liver cirrhosis in Japan. Infection with HCV remains a major cause of LC, and the ratio has persisted at around 60% for 10 years. Liver cirrhosis associated with HCV accounted for significantly more patients with LC with HCC than without, suggesting that HCV has carcinogenic potential. NASH-related LC accounted for 2.1% of the total LC in Japan and this might increase in the future. The present epidemic status in Japan might reflect the status of LC in the USA and European countries 10–20 years later.

Acknowledgments The authors thank the collaborators at the 58 institutions who participated in the survey and provided the data.

Appendix

The Japan Etiology of Liver Cirrhosis Study Group consists of the following members: Takashi Goto (Akita University), Mamiko Takeuchi (Anjo Kosei Hospital), Shigeki Miyoshi (Asahikawa Medical College), Yutaka Yonemitsu (Chiba University), Ikuta Tanaka (Date Red Cross Hospital), Toshimitsu Murohisa (Dokkyo Medical University School), Yoshio Tokumoto (Ehime University Graduate School of Medicine), Yoshinori Horie (Eiju Sogo Hospital), Atsushi Takahashi (Fukushima Medical University School of Medicine), Makoto Shiraki (Gifu University Graduate School of Medicine), Hiroko Yamada (Gunma University Graduate School of Medicine), Ryoichi Okamoto (Hiroshima City Hospital), Takahiro Asakami (Hiroshima University), Shuhei Hige (Hokkaido University Hospital), Yoshiaki Inui (Hyogo Prefectural Nishinomiya Hospital), Kazuto Fukuda (Ikeda Municipal Hospital), Yuko Nagaoki (International Medical Center of Japan), Hidekatsu Kuroda (Iwate Medical University), Takuya Nagano (Kagawa Prefectural Central Hospital), Akihiro Deguchi (Kagawa University School of Medicine), Masayoshi Yamada (Kanazawa Medical University), Akito Sakai (Kanazawa University Graduate School of Medical Science), Nobuyuki Toshikuni (Kawasaki Hospital, Kawasaki Medical School), Keisuke Ojiro (Keio University School of Medicine), Chitomi Hasebe (Keiyukai Yoshida Hospital), Yoko Kudo (Kumamoto University of Medicine), Kazuhisa Nakamura (Kyorin University School of Medicine), Kanji Yamaguchi (Kyoto Prefectural University of Medicine), Eiji Takeshita (Matsuyama Red Cross Hospital), Satoshi Nakayama (Mishuku Hospital), Yuka Takahashi (Musashino Red-Cross Hospital), Shunsuke Nojiri (Nagoya City University Graduate School of Medical Sciences), Masao Fujimoto (Nara Medical University), Naota Taura (NHO Nagasaki Medical Center), Hiroshi Matsumura (Nihon University School of Medicine), Minoru Nomoto (Niigata University), Shinichi Fujioka (Okayama Saiseikai General Hospital), Bon Shoji (Okayama University Graduate School of Medicine), Hiroyasu Morikawa (Osaka City University School of Medicine), Ryoichi Ebara (Osaka Police Hospital), Mie Inao (Saitama Medical University), Ayana Endo (Sapporo City General Hospital), Hideyuki Nomura (Shin-Kokura Hospital), Satoru Jyoshita (Shinshu University School of Medicine), Yoshihiko Morisawa (Teikyo University School of Medicine), Takeshi Matsui (Teine Keijinkai Hospital), Masanori Ito (Tokyo Medical University Kasumigaura Hospital), Naoaki Hashimoto (Tokyo Teishin Hospital), Maki Tobari (Tokyo Women's Medical University), Miharu Hirakawa (Toranomon Hospital), Kenji Oyama (Tottori University), Shingo Arakaki (University of Ryukyus), Makoto Kadokura (University of Yamanashi), Masanori Matsuda