Table 2. Time from onset of coma to death and rate of usage of narcotic analgesics in each group

	Cirrhosis ($n = 48$)	HCC ($n = 35$)	Lung cancer ($n = 33$)	$p^{\dagger}$	$p^{\dagger\dagger}$	$p^{\S}$	$p^{\P}$
Duration from coma to death (hours)	10.5 (0.5–192)	5.0 (0.5–168)	44.0 (1.0–528)	0.045	0.06	0.09	0.31
Using rate of narcotic drugs (yes/no)	4/44	13/22	20/13	<0.01	0.053	<0.01	<0.01
Serum total bilirubin just before death (mg/dL)	4.15 (0.6–31.7)	11.1 (0.4–32.1)	—	—	—	—	<0.01
Ascites (yes/no)	41/6	29/6	—	—	—	—	ns

Data are the median (range). †Lung cancer compared with liver cirrhosis and HCC, ††lung cancer compared with HCC, §lung cancer compared with liver cirrhosis, ¶liver cirrhosis compared with HCC. HCC, hepatocellular carcinoma; ns, not significant.

shorter in liver disease patients (cirrhosis and/or HCC: 7.0 h) compared with lung cancer patients (44.0 h).

Metastasis from HCC might not be common compared with cancers originating in other organs.[6] Recently, survival with HCC has been prolonged by advances in therapeutic approaches that have delayed metastasis to the late phase of the disease.[7,8] A recent report from Japan observed metastasis from HCC to lung, bone, lymph nodes, adrenal gland, brain and peritoneum; the most common site was the lung.[9] Lung metastases did not commonly lead to serious clinical symptoms; by contrast, metastasis to bone was not common (2–15%) but caused severe pain, walking difficulties and paralysis of the lower half of the body.[10] The low incidence of serious complications from metastasis of HCC might be a major reason for the low frequency of use of narcotic analgesics compared with lung cancer, in which severe clinical symptoms in the early stage lead to early introduction of palliative care.[11]

Highly impaired liver function might explain why the time between onset of coma and death was significantly shorter in the liver disease patients compared with the lung cancer patients. Surgical resection of up to two-thirds of the liver is possible even in cirrhosis patients if their total bilirubin is within the normal range,[12] so coma does not occur in liver cirrhosis and/or HCC until the terminal stage of the disease. In contrast, lung cancer is complicated by dysfunctions of ventilation, pulmonary function and gas exchange,[13] which lead to coma in the early stage of the disease.

Few studies have focused on the terminal stage of liver cirrhosis and/or HCC. The present study suggests that these patients do not always require palliative care and that the survival time from onset of coma is not prolonged. The limitations of this study, such as the small number of patients, the retrospective chart review and the comparison with lung cancer only, warrant further investigations.

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Original Paper

Survival Advantage of Radiofrequency Ablation for Hepatocellular Carcinoma: Comparison with Ethanol Injection

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ABSTRACT

Background/Aims: The aims of this study were to compare long-term prognosis of patients with hepatocellular carcinoma (HCC) treated with radiofrequency ablation (RFA) and percutaneous ethanol injection (PEI). **Methodology:** Two hundred and thirteen patients with HCC were initially treated with PEI or RFA at Saga University Hospital between 1990 and 2004. The present study included 190 patients: 98 treated with PEI from 1990 to 1999, and 92 with RFA from 2000 to 2004. The association of treatment method with survival prognosis was evaluated by multivariate analysis. **Results:** There were no significant differences in gender, etiology, and tumor stage between the two groups. Five-year survival rate in the PEI group was 40% and 51% in the RFA group. According to tumor stage, there were no differences in 5-year survival rate between the two groups for tumor stage I and III. For stage II patients, RFA had better survival than PEI (48% *vs.* 28%, $p = 0.03$). Multivariate analysis indicated that RFA was more effective for long-term survival than PEI in patients with tumor stage II ($p = 0.04$). **Conclusions:** Compared to PEI, RFA improved survival in patients with stage II HCC, indicating a therapeutic advantage of RFA.

Key Words: Hepatocellular carcinoma; Radiofrequency ablation; Percutaneous ethanol injection; Cause of death; Survival prognosis.

Abbreviations: Hepatocellular carcinoma (HCC); Percutaneous Ethanol Injection (PEI); Radiofrequency Ablation (RFA); Randomized Controlled Trials (RCTs); Ultrasonography (US); Computed Tomography (CT); Magnetic Resonance Imaging (MRI); Hepatitis B Surface Antigen (HBsAg); Hepatitis C Virus (HCV); Aspartate Aminotransferase (AST); Alanine Aminotransferase (ALT); γ-glutamyl Transpeptidase (GGT); α–Fetoprotein (AFP).

INTRODUCTION

In Japan, hepatocellular carcinoma (HCC) has been treated according to 2009 clinical practice guidelines (1). According to these guidelines, resection or local therapy is adopted for patients with Child-Pugh class A or B, ≤3 tumors, and ≤30 mm tumor diameter. Currently, percutaneous ethanol injection (PEI) or radiofrequency ablation (RFA) is generally selected for local therapy (2-5). Several randomized controlled trials (RCTs) or quasi-RCTs that have investigated the differences in safety and efficacy between PEI and RFA have been reported (6-11). Although RFA sometimes has severe complications such as obstructive jaundice caused by bile duct injury or hepatic infarction due to vascular ablation, most studies have indicated that there are no significant differences in complications between the two therapeutic approaches (8,10,11). Ablation-site recurrence in patients treated with RFA is less frequent than in patients treated with PEI (7-11), which might lead to superior overall survival for RFA compared to PEI (8-10). However, some studies have indicated that there are no significant differences in survival prognosis between the two treatment methods (7,11).

One problem with these previous RCTs is that the observation period was short-term (22.4–37.2 mo), and there have been few studies that have evaluated the long-term prognosis of RFA and PEI. The aim of this historical comparison was to compare PEI with RFA from the point of view of long-term survival rate.

METHODOLOGY

Patients

Two hundred and thirteen patients with HCC were initially treated with PEI or RFA at Saga University Hospital between 1990 and 2004. The present study enrolled 190 patients who were followed up for >3 years: 98 patients treated with PEI from 1990 to 1999, and 92 patients treated with RFA from 2000 to 2004. Median observation period in the PEI group was 54.4 (range: 2.5–157.4) mo, and 54.9 (range: 5.2–119.2) mo in the RFA group. Local therapy for HCC was indicated for patients with ≤3 nodules, each with a maximum diameter of 30 mm.

Diagnosis and staging of HCC

HCC was diagnosed by using at least two imaging tests including ultrasonography (US), dynamic computed tomography (CT), dynamic magnetic resonance imaging (MRI) and/or angiographic CT. Tumor stages were classified according to the article published by the Liver Cancer Study Group of Japan (12). This method includes the following three conditions: i) tumor diameter <20 mm; ii) single tumor; and iii) no vascular invasion. When all the three conditions were met, the tumor was classified as stage I. When two conditions were met, the tumor was classified as stage II. When one condition was met, the tumor was classified as stage III. When none of the conditions were met, the tumor was classified as stage IV.

Hepato-Gastroenterology 2013; 60:00-00 doi 10.5754/hge121270

TABLE 1. Patient characteristics of PEI and RFA groups.

Factors	PEI (n = 98)	RFA (n = 92)	*p* value
Gender			
Female / Male	41 / 57	34 / 58	0.492
HCC occurrence age	64.4 ± 7.6 (43-82)	67.0 ± 7.9 (47-80)	0.022
Etiology			
HCV / HBV / B+C / NBNC	83/ 8 / 2 / 5	80 / 5 / 3 / 4	0.833
Child-Pugh class			
A / B / C	55 / 39 / 4	66 / 26 / 0	0.025
Tumor stage			
I / II / III	41 / 39 / 18	37 / 46 / 9	0.166
Tumor diameter (mm)	21 ± 7 (8-30)	18 ± 6 (8-30)	0.003
Platelet count ($\times 10^4/\mu L$)	8.4 ± 3.9 (3-22.3)	9.1 ± 4.6 (2.5-28.3)	0.293
Prothrombin activity (%)	70.3 ± 21.1 (26-149)	74.6 ± 11.9 (36.5-101.1)	0.107
Albumin (g/dL)	3.5 ± 0.5 (2.4-4.5)	3.7 ± 0.5 (2.5-4.6)	0.060
Total bilirubin (mg/dL)	1.3 ± 0.7 (0.2-4.2)	1.1 ± 0.6 (0.4-3.1)	0.020
AST (IU/L)	79 ± 42 (18-246)	69 ± 41 (23-253)	0.129
ALT (IU/L)	69 ± 47 (12-237)	64 ± 42 (14-241)	0.414
GGT (IU/L)	78 ± 65 (15-397)	92 ± 92 (15-564)	0.255
AFP (ng/mL)	296 ± 1432 (2-12454)	212 ± 765 (3-5740)	0.638

Data are expressed as means ± SD or as the number of patients. Figures in parentheses indicate range.

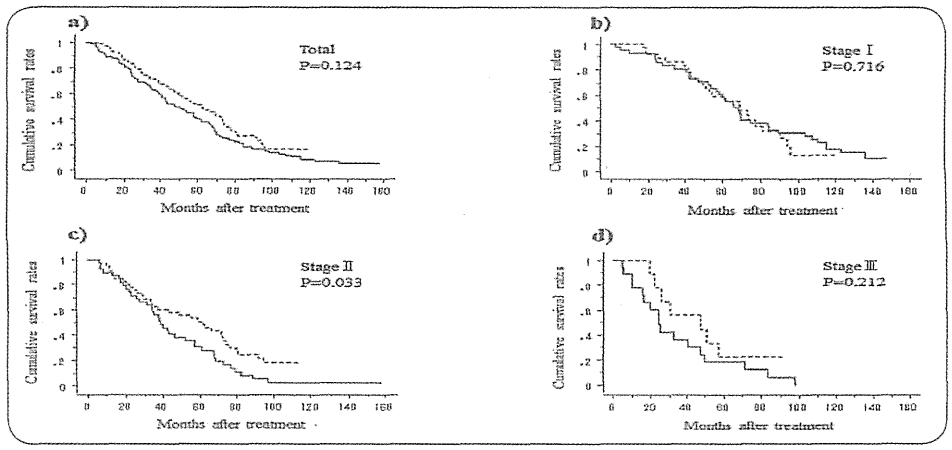

FIGURE 1. Cumulative survival curves in RFA group (---) and PEI group (—). (a) Total; (b) stage I; (c) stage II; (d) stage III.

Procedure of PEI and RFA

PEI was performed using real-time US under intramuscular administration of pentazocine and regional anesthesia of the puncture site. A 21-gauge needle was used for purpose of liver puncture and ethanol injection. According to the distribution of ethanol, tumor size, and the patient's condition, ethanol was injected at a dose of 1–4 mL per session once or twice weekly. The number of sessions was decided on the basis of the required dose of ethanol calculated by tumor volume on CT or MRI.

RFA was performed using the following three RF systems under general, sometimes local, anesthesia. From January to December 2000, 12 patients underwent RFA using an RF 2000 generator system (Radio Therapeutics, Mountain View, CA, USA) with 10 expandable hook-shaped electrode tines, and 17 patients underwent RFA using a model 500PA generator system (Rita Medical Systems, Mountain View, CA, USA) with four expandable hook-shaped electrode tines (Model 30). From January 2001 to December 2004, 63 patients underwent RFA with a cool-tip RF system (Radionics, Burlington, MA, USA) with a 17-gauge cooled-tip electrode with a 2- or 3-cm metallic tip. Ablation procedures were performed according to a globally standardized regimen (13).

TABLE 2. Patient characteristics of PEI and RFA groups (stage I HCC).

Factors	PEI (n = 41)	RFA (n = 37)	*p* value
Gender			
Female / Male	14 / 27	15 / 22	0.560
HCC occurrence age	63.2 ± 8.5 (43-82)	66.4 ± 8.2 (48-79)	0.099
Etiology			
HCV / HBV / B+C / NBNC	34 / 4 / 1 / 2	31 / 2 / 1 / 3	0.849
Child-Pugh class			
A / B / C	27 / 12 / 2	32 / 5 / 0	0.078
Tumor diameter (mm)	16 ± 4 (8-20)	14 ± 4 (8-20)	0.076
Platelet count ($\times 10^4/\mu L$)	8.7 ± 3.9 (3-22.3)	9.3 ± 4.9 (2.2-3.6)	0.602
Prothrombin activity (%)	75.3 ± 20.4(37-149)	77.7 ± 12.4 (36.5-101)	0.548
Albumin (g/dL)	3.7 ± 0.4 (2.5-4.5)	3.8 ± 0.5 (2.9-4.6)	0.109
Total bilirubin (mg/dL)	1.2 ± 0.5 (0.5-2.6)	1.0 ± 0.4 (0.4-1.9)	0.033
AST (IU/L)	77 ± 47 (18-246)	70 ± 43 (23-219)	0.482
ALT (IU/L)	70 ± 43 (12-201)	63 ± 40 (14-147)	0.527
GGT (IU/L)	65 ± 41 (16-189)	86 ± 76 (15-408)	0.148
AFP (ng/mL)	66 ± 140 (2-730)	125 ± 261 (4-1400)	0.244

Data are expressed as means ± SD or as the number of patients. Figures in parentheses indicate range.

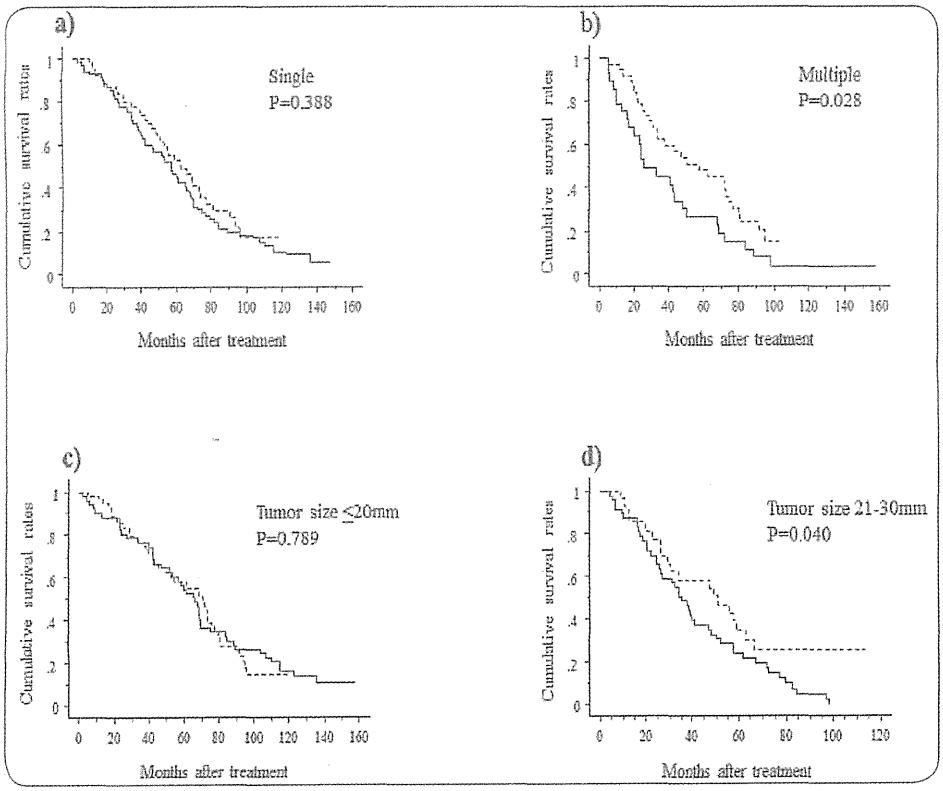

FIGURE 2. Cumulative survival curves in RFA group (---) and PEI group (—). (a) Single tumor; (b) two or three tumors; (c) tumor diameter ≤20 mm; (d) tumor diameter 21–30 mm.

TABLE 3. Patient characteristics of PEI and RFA groups (stage II HCC).

Factors	PEI (n = 39)	RFA (n = 46)	*p* value
Gender			
Female / Male	20 / 19	17 / 29	0.184
HCC occurrence age	64.7 ± 7.2 (53-82)	67.6 ± 7.8 (48-80)	0.081
Etiology			
HCV / HBV / B+C / NBNC	32 / 3 / 1 / 3	40 / 3 / 2 / 1	0.647
Child-Pugh class			
A / B / C	17 / 21 / 1	27 / 19 / 0	0.245
Tumor diameter (mm)	24 ± 6 (10-30)	21 ± 5 (11-30)	0.009
Platelet count ($\times 10^4/\mu L$)	7.8 ± 4.0 (3.3-18.9)	9.1 ± 4.7 (3.2-28.3)	0.185
Prothrombin activity (%)	66 ± 20.6 (26-103)	71.7 ± 11.3 (41.1-98)	0.127
Albumin (g/dL)	3.3 ± 0.5 (2.7-4.2)	3.5 ± 0.5 (2.5-4.3)	0.092
Total bilirubin (mg/dL)	1.4 ± 0.7 (0.2-3.1)	1.2 ± 0.6 (0.4-3.1)	0.350
AST (IU/L)	72 ± 40 (18-175)	70 ± 43 (26-253)	0.866
ALT (IU/L)	58 ± 38 (15-188)	65 ± 47 (17-241)	0.486
GGT (IU/L)	73 ± 62 (15-304)	101 ± 107 (18-564)	0.165
AFP (ng/mL)	77 ± 164 (2-792)	291 ± 1045 (3-5740)	0.241

Data are expressed as means ± SD or as the number of patients. Figures in parentheses indicate range.

TABLE 4. Patient characteristics of PEI and RFA groups (stage III HCC).

Factors	PEI (n = 18)	RFA (n = 9)	*p* value
Gender			
Female / Male	7 / 11	2 / 7	0.387
HCC occurrence age	66.4 ± 5.9 (58-82)	66.3 ± 7.3 (55-77)	0.976
Etiology			
HCV / HBV / B+C / NBNC	17 / 1 / 0 / 0	9 / 0 /0 / 0	0.471
Child-Pugh class			
A / B / C	11 / 6 / 1	7 / 2 / 0	0.607
Tumor diameter (mm)	28 ± 3 (22-30)	24 ± 4 (21-30)	0.013
Platelet count ($\times 10^4/\mu L$)	9.1 ± 3.7 (3.4-17.2)	8.2 ± 2.6 (5.4-13.3)	0.541
Prothrombin activity (%)	68.7 ± 22.6 (38-111)	76.9 ± 9.7 (56.4-87)	0.335
Albumin (g/dL)	3.6 ± 0.5 (2.4-4.3)	3.6 ± 0.6 (2.7-4.4)	0.877
Total bilirubin (mg/dL)	1.5 ± 0.9 (0.5-4.2)	1.0 ± 0.5 (0.5-1.8)	0.188
AST (IU/L)	102 ± 56 (20-198)	63 ± 21 (39-101)	0.073
ALT (IU/L)	93 ± 63 (15-237)	58 ± 26 (33-104)	0.146
GGT (IU/L)	120 ± 96 (29-397)	65 ± 62 (25-206)	0.162
AFP (ng/mL)	1278 ± 3202 (4-12454)	160 ± 263 (4-682)	0.340

Data are expressed as means ± SD or as the number of patients. Figures in parentheses indicate range.

Laboratory data

We collected laboratory data just before initial treatment including hepatitis B surface antigen (HBsAg), hepatitis C virus (HCV) antibody, platelet count, prothrombin activity, albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin and γ–glutamyl transpeptidase (GGT) and α–fetoprotein (AFP). Etiology of liver disease was decided as follows: patients with positive HBsAg were HBV; patients with positive HCV antibody were HCV; patients with both HBsAg and HCV antibody were HBV+HCV (B+C); and patients with negative for HBsAg and HCV antibody were non-HBV non-HCV (NBNC).

Statistical analysis

Comparisons of clinical characteristics between the two treatment groups were made using the unpaired Student's *t*-test for continuous variables and the χ^2 test for categorical data. The survival periods were estimated using the Kaplan-Meier method, and the survival curves

were compared using the log-rank test. Factors related to survival prognosis were analyzed by using multivariate Cox proportional hazards model. Two-tailed p values <0.05 were considered significant. Statistical analyses were performed with SPSS Statistics ver. 19 (SPSS Japan, Tokyo, Japan).

RESULTS

Baseline characteristics

Clinical characteristics of the patients are shown in **Table 1**. Although there were no significant differences in gender, etiology of liver disease, platelet count, prothrombin activity, albumin, AST, ALT, GGT and AFP between the PEI and RFA groups, the RFA group was older, and Child-Pugh score and total bilirubin level of the RFA group were lower than those of the PEI group. Although tumor diameter in the PEI group was longer than that in the RFA group, there was no difference in stage classification of HCC.

Cumulative survival rates overall and according to tumor stage

Regarding overall cumulative survival rates, 5-year survival rates were 40% and 51% in the PEI and RFA group, respectively (**Figure 1a**), and there was no difference between the two groups. **Tables 2-4** show the clinical characteristics of the patients categorized by tumor stage. There were no significant differences in gender, age, etiology, Child-Pugh class, platelet count, prothrombin activity, albumin, AST, ALT, GGT and AFP between the RFA and PEI group for each tumor stage. Total bilirubin level in tumor stage I was higher in the PEI than RFA group. Regarding the cumulative survival rate at each stage, the RFA group had significantly better survival compared to the PEI group for tumor stage II (**Figure 1c**, $P = 0.033$). This better survival in the RFA group was not detected for tumor stage I and stage III (**Figure 1b** and **1d**). Five-year survival rates in the two groups were as follows: PEI, 60% and RFA, 59% for stage I; PEI, 28% and RFA, 48% for stage II; and PEI, 18% and RFA, 22% for stage III.

Comparison of causes of death

During the observation period, 156 of 190 patients died (PEI: 93, RFA: 63). Causes of death were identified in 112 patients. Fifty-nine patients died from growth of HCC (PEI: 38, RFA: 21); 29 from hepatic failure (PEI: 16, RFA: 13); seven from extrahepatic rupture of HCC (PEI: 5, RFA: 2); and six from rupture of esophageal or gastric varices (PEI: 3, RFA: 3). Eleven patients died from causes other than liver-related diseases: six from infectious disease; two from other carcinomas; two from perforation of the gastrointestinal tract; and one from rupture of aortic aneurysm. Distribution in causes of death did not differ between the PEI and PFA groups, and it was similar according to stage of tumor (data not shown).

Cumulative survival rates according to tumor size and number

Figure 2 is the Kaplan-Meier curve with regard to the number of tumors and tumor size. There was no significant difference in cumulative survival rate between the two groups in patients with a single tumor or tumor diameter <20 mm (**Figure 2a** and **2c**). The RFA group had better survival than the PEI group in patients with multiple tumors or tumor diameter 21-30 mm (**Figure 2b** and **2d**).

TABLE 5. Univariate analysis of factors for survival periods in stage II HCC patients.

Variables	Odds ratio	95%CI		p value
Gemder: Female	1.19	0.742	1.893	0.476
HCC occurrence age: ≥66	1.24	0.777	1.984	0.365
Child-Pugh class: B, C	1.62	1.016	2.591	0.043
Etiology: non HBV	2.30	0.718	2.296	0.161
Treatment: PEI	1.66	1.037	2.658	0.035
Tumor diameter: ≥20 mm	1.00	0.973	1.034	0.851
Platelet: <7.7×10⁴ /μL	1.46	0.893	2.371	0.132
AST: ≥70 IU/L	1.28	0.784	2.091	0.323
ALT: ≥80 IU/L	0.81	0.463	1.432	0.476
GGT: ≥100 IU/L	0.91	0.516	1.597	0.738
AFP: ≥100 ng/mL	1.32	0.734	2.355	0.358

AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: gamma-glutamyl transpeptidase; AFP: α-fetoprotein.

TABLE 6. Multivariate analysis of factors for survival periods in stage II HCC patients.

Variables	Odds ratio	95%CI		p value
Gender: Female	1.24	0.742	- 2.064	0.414
HCC occurrence age: ≥66	1.32	0.774	- 2.235	0.311
Child-Pugh class: B, C	1.55	0.907	- 2.642	0.109
Etiology: non HBV	3.88	0.887	- 16.988	0.072
Treatment: PEI	1.84	1.023	- 3.295	0.042
Tumor diameter: ≥20 mm	0.98	0.947	- 1.013	0.231

Analysis for factors associated with survival in tumor stage II patients

There was a significant difference in survival prognosis of tumor stage II patients between the two groups; therefore, we analyzed factors associated with survival in stage II patients. As a result of the univariate analysis for factors including gender, age, Child-Pugh class, tumor diameter, etiology of liver disease, initial treatment method (PEI or RFA), platelet count, AST, ALT, GGT and AFP, the initial treatment method and Child-Pugh class were significant factors associated with survival prognosis (**Table 5**). Multivariate analysis adjusted by gender, age, Child-Pugh class, tumor diameter, and etiology of liver disease showed that RFA for initial treatment was a significant factor for good survival prognosis in patients with stage II HCC ($p = 0.042$, **Table 6**).

DISCUSSION

Our comparative study demonstrated that long-term survival in patients treated with RFA was superior to that with PEI in patients with stage II HCC, although this advantage was not observed when the outcome was evaluated in the overall or other stage patients.

An important feature of RFA is that a larger ablated area is obtained from one treatment session compared to PEI (6,8). It is speculated that the difference in sur-

vival of stage II HCC between the two treatment methods might have originated from the extent of the ablated margin. Previous pathological studies have shown that one fifth to one third of small HCCs, ≤3.0 cm in diameter, had satellite lesions that were not detected during pretreatment evaluation (14,15). Although several studies have indicated that ablation-site recurrence in patients treated with RFA was less than that in those treated with PEI (7-11), there are some reports that RFA was not significantly better than PEI for tumors <20 mm in diameter (8,9,16). Regarding survival prognosis, although some studies have indicated no significant difference between these two treatment methods (7,11), many more studies have indicated that RFA treatment results in less local recurrence than does PEI, which results in a longer survival period (8-10). These studies suggest that RFA might be more effective for HCC >20 mm in diameter compared with PEI with regard to local regulation and subsequent improved survival. This agrees with our result that RFA has a better prognosis than PEI for HCC of 21–30 mm diameter.

HCC stage II contains multiple tumors ≤20 mm in size, as well as single tumors >20 mm in size. Our result indicated that RFA was more effective against multiple (2 or 3 tumors) HCC than PEI was. We cannot explain the reason for this result from our data, although it is speculated that it resulted from the completeness of local ablation of each tumor.

The superiority of RFA was not observed in tumor stage I and stage III. We speculate that stage I HCC could be cured by PEI, similar to RFA, because the tumor size was small (<20 mm), and stage III HCC could not be regulated by not only PEI but also RFA, because these tumors were multiple and >20 mm in diameter. From the point of view of cost benefit, PEI might be a better selection for local treatment of stage I HCC, because the cost of PEI is much lower than that of RFA. For stage III HCC, we should consider combination therapy with other treatment methods including transarterial chemoembolization.

It is noteworthy that the observation period (median: 54.6 mo) of the present study was longer than that of previous studies indicating the advantage of RFA over PEI, and causes of death were examined. It was revealed that there was no difference in cause of death between the two treatment methods.

The limitation of the present study was that it was not prospective and not an RCT. The data were selected from a retrospective chart review for historical comparison, and the local regulatory rate could not be investigated. Moreover, because the follow-up period was different, the survival of the patients might have been influenced by several other factors, including progress in supportive care and innovation of another treatment for HCC. The PEI group included some cases treated with RFA for second and subsequent treatment after initial treatment with PEI. However, many previous studies have indicated that the initial therapy strongly affects the survival prognosis of HCC. Moreover, because PEI has rarely been selected for initial therapy for HCC in our country, it is almost impossible to compare long-term survival between PEI and RFA at present.

In conclusion, this historical comparative study suggests that RFA is appropriate compared to PEI for stage II HCC patients evaluated for long-term survival.

ACKNOWLEDGEMENTS

The authors would like to thank Ms Yukie Watanabe, Ms Chieko Ogawa, and all the medical staff at Saga Medical School Hospital for their assistance and excellent advice.

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JOURNAL OF VIRAL HEPATITIS

Journal of Viral Hepatitis, 2013, **20**, e124–e126 doi:10.1111/jvh.12026

An automated rapid detection system using the quenching probe method for detecting interleukin 28B and inosine triphosphatase single nucleotide polymorphisms in chronic hepatitis C

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Received July 2012; accepted for publication October 2012

SUMMARY. Single nucleotide polymorphisms (SNPs) in the interleukin 28B gene (*IL28B*) are good pretreatment predictors of anti-hepatitis C virus (HCV) therapy with interferon. SNPs of the inosine triphosphatase (*ITPA*) gene are associated with reduced haemoglobin levels during treatment with ribavirin. The i-densy (Arkray, Inc.), which is based on the quenching probe (QP) method, automatically detects target genes in blood samples by fluorescence quenching within 100 min. Using a QP and primer set, a gene amplification response is generated that can quickly and easily detect a specific gene's arrangement by fluorometry. The present study was conducted to compare the utility of i-densy (QP method) with that of conventional direct sequencing (DS) for detecting SNPs in the *IL28B* and *ITPA* genes in chronic hepatitis C patients. Between June 2011 and January 2012, 73 consecutive patients underwent genotyping of IL28B, and 54 patients underwent genotyping of ITPA. All of the patients were seropositive for HCV-RNA. The IL28B and ITPA genotypes were tested for bi-allelic polymorphisms in rs8099917 (T/T, T/G and G/G; minor allele, G) and rs1127354 (C/C, C/A and A/A; minor allele, A), respectively. The results obtained with the QP method were identical to those obtained with the conventional DS method. The frequency of the *IL28B* genotypes TT, GT and GG were 74%, 24.7% and 1.4%, respectively, and those of the *ITPA* genotypes CC, AC and AA were 68.5%, 29.6% and 1.9%, respectively. These results indicate that the i-densy using the QP method can automatically, quickly and easily identify genotypes of *IL28B* and *ITPA*.

Keywords: fluorometry, IL28B, inosine triphosphatase, quenching probe.

BACKGROUND AND AIM

Recent reports have shown that single nucleotide polymorphisms (SNPs) in the interleukin 28B (*IL28B*) gene, which encodes IFN-lambda 3, are good pretreatment predictors for peginterferon α (Peg-IFNα) plus ribavirin (RBV) therapy and triple therapy [1–3]. Additionally, a recent genome-wide association study showed a strong association between reductions in haemoglobin levels during RBV treatment and the SNP rs6051702 in the inosine triphosphatase (*ITPA*) gene [4]. Therefore, easy and rapid detection of SNPs in *IL28B* or *ITPA* is important for routine clinical practice and to guide treatment decisions. As previously reported, the quenching probe (QP) method is extremely effective in detecting the *KRAS* mutations in lung adenocarcinoma [5]. Following the addition of a QP and a primer set, a gene amplification response is generated, and the gene's arrangement is quickly and easily detected by fluorometry. In the present study, we compared the utility of the QP method with that of the conventional DS method for detecting *IL28B* and *ITPA* SNPs in blood samples from chronic hepatitis C patients.

Abbreviations: DS, direct sequencing; HCV, hepatitis C virus; ITPA, inosine triphosphatase; QP, quenching probe; RBV, ribavirin.

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MATERIALS AND METHODS

Patients and blood samples

Between June 2011 and January 2012, 73 consecutive patients underwent *IL28B* genotyping, and 54 patients underwent ITPA genotyping. All patients were seropositive

for HCV-RNA. Venous blood samples were collected and stored at 4 °C.

Detection of IL28B and *ITPA* by direct sequencing

Human genomic DNA was extracted from peripheral blood using a blood DNA extraction kit (QIAGEN, Tokyo, Japan). Allelic typing was performed by real-time PCR with the Applied Biosystems 104 prIsM dye terminator cycle sequencing method on an ABI 105 PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using a fluorescein amidite-labelled SNP primer for the locus rs8099917 (Applied Biosystems). The *IL28B* polymorphism (rs8099917) and the *ITPA* polymorphism (rs1127354) were detected by PCR amplification with specific primers according to the manufacturer's instructions. The possible genotypes for each bi-allelic polymorphism in rs8099917 are T/T, T/G and G/G (minor allele, G). *ITPA* was genotyped at the polymorphic site for rs1127354 on chromosome 20 using the ABI TaqMan allelic discrimination kit and the same system as for *IL28B* genotyping. The possible genotypes in rs1127354 are C/C, C/A and A/A (minor allele, A).

Detection of IL28B and *ITPA* by the quenching probe method

The i-densy fully automated genotyping system (ARKRAY, Inc., Kyoto, Japan) was used. In this system, genomic DNA is purified from a blood sample using an FTA® matrix (Whatman, Middlesex, UK) followed by PCR amplification of the target SNPs. The SNP genotypes are determined by monitoring the fluorescence intensity of a QP. Each QP contains cytosine at its 5′ or 3′ end, which is labelled with a guanine quench fluorophore. When the QP hybridizes to its target DNA, its fluorescence is quenched by the guanine residue in the target that is complementary to the modified cytosine. The system performs these processes automatically and can genotype up to three SNP sites using four blood samples. For this study, the forward and reverse PCR primers and guanine QP (J-Bio21, Tokyo, Japan) were 5′-caacatggagagttaaagtaagtcttgtatttcacc-3′, 5′-cagctaccaaactgtatacagcatggttc-3′ and 5′-ctgtgagcaatgtcaccc-3′, respectively, for *IL28B*, and 5′-aagtgttctcttttctcttggaacag-3′, 5′-ag aa(or g)acatacggtcaattttctgtg-3′ and 5′-gcatgtaaacttatctcc-3′, respectively, for *ITPA*. For *IL28B*, PCR consisted of

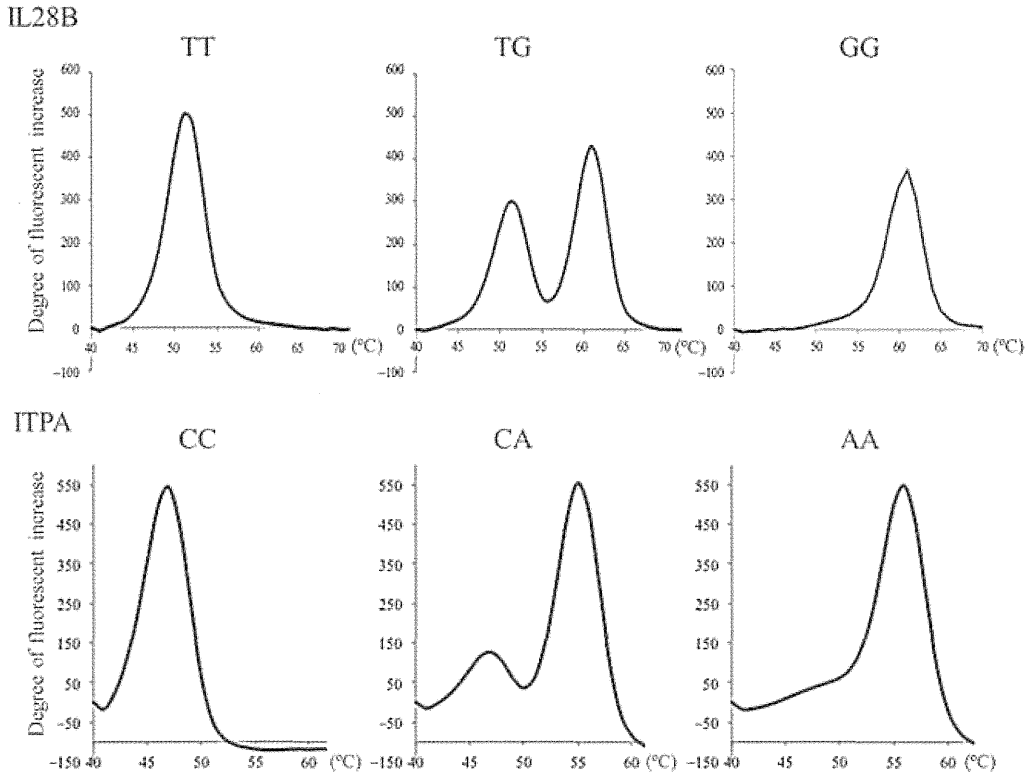

Fig. 1 Genotyping of the *IL28B* and *ITPA* SNPs by the quenching probe (QP) method. The temperature was gradually increased from 40 to 95 °C, and the increased fluorescence of the probe was measured. The excitation and emission wavelengths were 445–480 and 520–555 nm, respectively, for *IL28B*, and 587–700 and 520–555 nm, respectively, for *ITPA*. The TT and GG genotype of *IL28B* were detected as a single fluorescent peak at 53 and 62 °C. The heterozygous genotype TG was detected as a double peak at 53 and 62 °C. The CC and AA genotypes of *ITPA* were detected as single peak at 48 and 56 °C, respectively. The CA genotype was detected as a double fluorescent peak at 48 and 56 °C.

initial denaturation for 1 min at 95 °C, and 50 cycles of denaturation at 95 °C for 1 s and annealing at 60 °C for 30 s. For *ITPA*, PCR consisted of initial denaturation at 95 °C for 1 min, and 50 cycles of denaturation at 95 °C for 1 s and annealing at 64 °C for 30 s. After the PCR was complete, melting temperature (T_m) analyses were performed. The SNPs were identified based on differences in temperature and fluorescence.

RESULTS AND CONCLUSION

By differentiating the fluorescence intensities by the temperature, the T_m was obtained within 100 min (Fig. 1). All 73 patients were successfully genotyped for *IL28B*. The results obtained with the QP, and conventional DS methods were identical. Fifty-four patients were successfully genotyped for *ITPA*, and the results were identical with both methods. The frequencies of TT, GT and GG for *IL28B* were 74%, 24.7% and 1.4%, respectively. The frequencies of CC, AC and AA for *ITPA* were 68.5%, 29.6% and 1.9%, respectively. In conclusion, the QP method, with its high sensitivity, effectiveness and speed, allows for the determination of *IL28B* and *ITPA* genotypes in clinical settings.

ACKNOWLEDGEMENTS

This work was supported in part by Grants-in Aid from the Ministry of Health, Labour and Welfare of Japan.

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J Gastroenterol (2012) 47:586–595
DOI 10.1007/s00535-012-0533-z

Prevalence and associated metabolic factors of nonalcoholic fatty liver disease in the general population from 2009 to 2010 in Japan: a multicenter large retrospective study

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Received: 7 July 2011 / Accepted: 9 December 2011 / Published online: 11 February 2012

Abstract
Background The prevalence of nonalcoholic fatty liver disease (NAFLD) has been increasing. This study aimed to assess the recent prevalence of NAFLD and to predict the prevalence of nonalcoholic steatohepatitis (NASH) with liver fibrosis using established scoring systems in the general population.
Methods A cross-sectional study was conducted among 8352 subjects who received health checkups from 2009 to 2010 in three health centers in Japan. Subjects with an intake over 20 g of alcohol/day or with other chronic liver diseases were excluded. Fatty liver was detected by ultrasonography. The probability of NASH with advanced fibrosis was calculated according to the body mass index, age, ALT, and triglyceride (BAAT) and FIB-4 (based on age, aspartate aminotransferase and alanine aminotransferase levels, and platelet counts) indices.
Results A total of 5075 subjects were enrolled. The overall prevalence of NAFLD was 29.7%. There was a significant threefold difference in the mean prevalence between males (41.0%) and females (17.7%). This prevalence showed a linear increase with body mass index, triglycerides, and low-density lipoprotein cholesterol regardless of threshold values, even without obesity. The estimated prevalence of NASH according to the BAAT index ≥ 3 was 2.7%, and according to the FIB-4 index it was 1.9%.
Conclusions The prevalence of NAFLD has increased in the general population, especially in males. There is a linear relationship between the prevalence of NAFLD and various metabolic parameters, even in nonobese subjects. The prevalence of NASH with advanced fibrosis is estimated to be considerably high in subjects with NAFLD.

Keywords Abdominal obesity · Central obesity · Metabolic syndrome

Abbreviations

Ht	Body height
BW	Body weight
BMI	Body mass index
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
AAR	AST/ALT ratio
ALP	Alkaline phosphatase
GGT	Gamma-glutamyl transferase
ChE	Cholinesterase
FPG	Fasting plasma glucose

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Hb	Hemoglobin
PLT	Platelet
TC	Total cholesterol
TG	Triglyceride
HDL-C	High-density lipoprotein cholesterol
LDL-C	Low-density lipoprotein cholesterol

Introduction

Obesity and life-related diseases due to obesity are rising at an alarming rate in Japan, many Western countries, and worldwide. Nonalcoholic fatty liver disease (NAFLD), a hepatic manifestation of metabolic syndrome, is associated with an increased risk for development of life-related disease including type 2 diabetes, cardiovascular disease, and cerebral vessel disease. NAFLD covers a spectrum of liver diseases that range from benign simple steatosis to hepatic inflammation and fibrosis of nonalcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma [1, 2].

NAFLD is rapidly becoming the most common liver disorder worldwide [3–6]. Currently, NAFLD is present in 20–40% the general population of industrialized countries [7, 8]. Among all subjects with NAFLD, features of NASH are observed in 10–20%. Recent studies reported that the prevalence of NASH in Western countries is approximately 2–12% [7–9].

The degree of fatty infiltration in NAFLD is graded according to the percentage of hepatocytes with fat deposits: mild NAFLD involves less than 30% hepatocytes, moderate NAFLD up to 60%, and severe NAFLD more than 60%. The degree of liver fibrosis must be estimated to determine the surveillance, prognosis, and optimal treatment for NAFLD, similar to the situation for other liver diseases such as chronic hepatitis C [10, 11]. Liver biopsy is recommended as the gold standard method for the diagnosis and staging of NAFLD/NASH, but it is invasive and is associated with a high risk of complications [1, 12]. In fact, it is impossible to recommend a liver biopsy to all NAFLD patients, because the number of NAFLD patients has reached 80–100 million in the USA and an estimated 10 million in Japan. Previous studies proposed novel scoring systems to estimate NASH with advanced liver fibrosis, because it was just not realistic to conduct a liver biopsy in a large number of subjects with fatty liver. The scoring system consisting of body mass index (BMI), age, serum alanine aminotransferase (ALT), and triglyceride (BAAT score) and the novel index proposed by Sterling et al. based on age, serum aspartate aminotransferase (AST), ALT level, and platelets (FIB-4 index) are simple and useful to predict NASH with advanced liver fibrosis. It might therefore be possible to estimate the approximate prevalence of NASH with advanced liver fibrosis in the Japanese general population by using these predictive formulae [13–15].

It is well known that there are age and gender differences in both the prevalence and severity of NAFLD. These age and gender differences are caused by differences in the prevalence of obesity and lifestyle-related diseases. According to annual health check findings in Japan, the prevalence of NAFLD in men is approximately 27% for all ages above 30 years. In contrast, in women, it gradually increases from 7% in their 30s to 23% above 60 years of age [16, 17]. However, this information was reported from studies conducted at the end of 1990–2000. According to the worldwide systemic analysis of health examination surveys and epidemiological studies, the prevalence of obesity is increasing year-on-year and varies substantially between nations. It is predicted that the prevalence of NAFLD in the general population is increasing and there might be a difference between each country [18].

Because of the dramatic increase in obesity in Japan and many other industrialized countries, it is plausible that there also has been a dramatic increase in the prevalence of NAFLD and NASH. However, the most recent prevalence of NAFLD has not been well established in Japan. Therefore, the aim of this study was to investigate the prevalence of NAFLD/NASH using the latest database of a large proportion of the general population who underwent an annual health checkup from 2009 to 2010 in Japan and to estimate the prevalence of NASH with liver fibrosis using established scoring systems.

Patients and methods

Study population

We studied 8352 subjects (51.8% males) aged 21–86 years (mean 50.0 years), who received a health checkup from 2009 to 2010 in three health centers, namely Eguchi Hospital Health Center in Saga prefecture, Kawamura Clinic Heath Center in Hiroshima prefecture, and Kochi Medical School Hospital in Kochi prefecture in Japan. Subjects were included if they fulfilled the following criteria: (1) absence of markers of hepatitis B virus infection (hepatitis B surface antigen and anti-hepatitis B core antibody) and hepatitis C virus infection (anti-hepatitis C virus antibodies); (2) no alcoholic liver disease (more than 20 g of alcohol per day); and (3) no use of insulin-sensitizing medication. Finally, 5075 subjects who met the inclusion criteria were enrolled. All subjects provided written informed consent to the use of their data for an epidemiological study under anonymity. The study design

was approved by each institutional review board (Saga Medical School, "2011-06-04" as Eguchi Hospital; Hiroshima University, "Eki-241" as Kawamura Clinic Health Center; Kochi Medical School, "23-74"). The study was conducted in accordance with the Declaration of Helsinki.

Physical examination and serum biochemistry

Body weight and height were obtained for both sets of subjects, and BMI was calculated. Waist circumference was measured at the umbilical level. Venous blood samples were taken from all subjects at 0900 hours following a 12-h overnight fast and AST, ALT, gamma-glutamyl transpeptidase (GGT), total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, triglycerides, and fasting plasma glucose (FPG) levels were measured using standard techniques in the subjects who received a health checkup.

Abdominal ultrasound protocol and definition of fatty liver

All subjects received abdominal ultrasonography to determine fatty liver. First, examination of all visible liver parenchyma was performed with a conventional convex array transducer. Liver parenchyma was examined with sagittal as well as longitudinal guidance of a probe and completed by lateral and intercostals views. Use of tissue harmonic imaging with both transducers was encouraged. The presence of steatosis was recognized as a marked increase in hepatic echogenicity, poor penetration of the posterior segment of the right lobe of the liver, and poor or no visualization of the hepatic vessels and diaphragm. The severity of hepatic steatosis present by imaging was not graded with careful consideration of the error due to the difference of ultrasonography equipment and examiner. The liver was considered normal if the hepatic parenchyma was homogeneous with no acoustic attenuation, the portal veins were visible, the diaphragm was well visualized, and the echogenicity was similar or slightly higher than the echogenicity of the renal cortex.

Ultrasonography was performed with the following units: LOGIQ 7 with a 4-MHz convex array transducer (GE Health Care) at Eguchi Hospital; Pro Sound Alpha-10 with 3.5 MHz with a convex array transducer (Hitachi Aloka Medical) at Kawamura Clinic Health Center; and Xario with a 3.5-MHz convex array transducer (Toshiba Medical Systems) at Kochi Medical School. Experienced sonographers, who were trained by gastroenterologists with more than 5 years' experience, performed examinations over 5 years. Technical parameters were adjusted for each subject using the standard protocol for ultrasonography. Each certificated gastroenterologist independently reviewed the images and evaluated the liver for the presence of steatosis.

Algorithms for prediction of NASH

In this study, two representative algorithms based on the BAAT score and the recently proposed FIB-4 index [13, 14] were employed to predict the prevalence of subjects with NASH with advanced liver fibrosis. BAAT scores consist of the sum of the following categorical variables: BMI ($\geq$28 = 1, <28 = 0), age ($\geq$50 years = 1, <50 = 0), ALT [$\geq$2 UNL (males, ALT $\geq$60 IU/L; females, ALT $\geq$40 IU/L) = 1, <2 UNL = 0) and serum triglycerides [1.7 mmol/L (=150 mg/dL) = 1, <1.7 = 0], thus ranging from 0 to 4, and a cutoff value to predict NASH with advanced liver fibrosis was defined as BAAT score $\geq$3 in this study [13]. The FIB-4 index was calculated as [age (years) $\times$ AST (U/L)]/[platelets (10^9) $\times$ root ALT (U/L)]. The subjects were classified into three groups on the basis of the following values: FIB-4 index $\geq$2.67 and <1.30, because previous studies reported that a FIB-4 index $\geq$2.67 had an 80% positive predictive value and a FIB-4 index <1.30 had a 90% negative predictive value to predict NASH with advanced liver fibrosis [14, 15].

Statistical analysis

Descriptive statistics (means and standard deviations) were calculated for all continuous variables. Differences between the two groups were compared by the Mann–Whitney U test. Differences were considered significant at $p < 0.05$. All analyses were carried out using IBM SPSS Statistics Ver. 19.

Results

Clinical and biochemical characteristics and the prevalence of NAFLD in enrolled subjects

A total of 5075 subjects were enrolled from July 2009 to June 2010. The clinical and biochemical characteristics of these subjects are summarized in Tables 1 and 2. The subjects were predominantly middle-aged (50.0 $\pm$ 9.5 years; range 21–86 years) and 48.2% were female. The mean BMI of the whole cohort was 23.0 $\pm$ 3.3 kg/m^2 with 23.6% of the subjects meeting the criteria for obesity (BMI $\geq$25). The mean age was not significantly different between subjects with or without NAFLD (51.1 $\pm$ 8.9 vs. 49.5 $\pm$ 6.7 years). A total of 1509 subjects (29.7%) had evidence of NAFLD on ultrasonography. There was a significant threefold difference in the mean prevalence of NAFLD between males (41.0%) and females (17.7%). The

Table 1 Characteristics of all patients

	All ($n = 5075$)	Non-NAFLD ($n = 3566$)	NAFLD ($n = 1509$)	p value
Gender (M/F)	2627/2448	1551/2015	1076/433	<0.0001
Age (years)	50.0 ± 9.5	49.5 ± 6.7	51.1 ± 8.9	<0.0001
Ht (m)	1.631 ± 0.086	1.62 ± 0.08	1.65 ± 0.08	<0.0001
BW (kg)	61.4 ± 11.7	57.7 ± 9.6	70.2 ± 11.3	<0.0001
BMI (kg/m^2)	23.0 ± 3.3	21.8 ± 2.6	25.6 ± 3.3	<0.0001
AST (IU/L)	21.5 ± 9.1	20.1 ± 6.7	24.7 ± 12.4	<0.0001
ALT (IU/L)	22.6 ± 16.6	18.3 ± 9.9	32.7 ± 23.5	<0.0001
AAR	1.11 ± 0.37	1.21 ± 0.35	0.87 ± 0.29	<0.0001
ALP (IU/L)	210.6 ± 65.7	205.3 ± 65.3	223.3 ± 64.7	<0.0001
GGT (IU/L)	34.4 ± 36.1	28.4 ± 27.0	48.5 ± 48.6	<0.0001
ChE (IU/L)	293.6 ± 126.0	277.0 ± 122.0	33.4 ± 126.3	<0.0001
Albumin (g/dL)	4.5 ± 0.2	4.5 ± 0.2	4.6 ± 0.2	<0.0001
FPG (mg/dL)	99.6 ± 17.7	96.3 ± 13.3	107.3 ± 23.3	<0.0001
TC (mg/dL)	207.1 ± 34.1	205.6 ± 33.9	210.5 ± 34.3	<0.0001
TG (mg/dL)	111.8 ± 76.9	93.0 ± 52.6	155.6 ± 102.3	<0.0001
HDL-C (mg/dL)	60.6 ± 16.2	64.4 ± 16.2	51.5 ± 12.1	<0.0001
LDL-C (mg/dL)	121.6 ± 32.1	118.3 ± 32.2	129.4 ± 30.6	<0.0001

Values are expressed as mean ± SD. Statistical analysis was conducted using Mann–Whitney U test

Ht body height, *BW* body weight, *BMI* body mass index, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *AAR* AST/ALT ratio, *ALP* alkaline phosphatase, *GGT* gamma-glutamyl transferase, *ChE* cholinesterase, *FPG* fasting plasma glucose, *Hb* hemoglobin, *PLT* platelet, *TC* total cholesterol, *TG* triglyceride, *HDL-C* high-density lipoprotein cholesterol, *LDL-C* low-density lipoprotein cholesterol

prevalence of NAFLD in men was greater than 30% in all ages above the third decade. This prevalence was higher in males than that in females for all ages, and it gradually increased from only 3.3% in the second decade to 31.3% above the sixth decade in females (Fig. 1). Furthermore, there was a significant difference in most of the clinical factors except BMI and LDL cholesterol between males and females (Table 2). By multivariate logistic regression in each male and female with NAFLD, there was a difference in the independent variables significantly associated with NAFLD (Table 3).

Relationship between anthropometric and biochemical features and the presence of NAFLD

BMI in subjects with NAFLD was significantly higher than that in those without NAFLD ($p < 0.01$). The prevalence of NAFLD showed a linear increase with the increase of BMI (BMI <23 kg/m^2, 10.5%; BMI ≥23 kg/m^2 and <25 kg/m^2, 37.9%; BMI ≥25 kg/m^2 and <28 kg/m^2, 58.4%; BMI ≥28 kg/m^2, 84.2%; a 7.4–11.4% increase per 1 kg/m^2 between 20 and 30) (Fig. 2), and it was 18.4% in nonobese subjects (BMI <25 kg/m^2) with NAFLD, 63.4% in obese subjects (BMI >25 kg/m^2 but <30 kg/m^2) with NAFLD, and 89.1% in morbid obese (BMI >30 kg/m^2) subjects with NAFLD. Serum levels of LDL cholesterol, triglycerides (TG), FPG, and liver enzymes including AST and ALT were significantly higher in subjects with NAFLD than those in subjects without NAFLD ($p < 0.01$). Serum levels of HDL cholesterol were significantly lower in subjects with NAFLD than those without NAFLD ($p < 0.01$, Tables 1, 2). The prevalence of NAFLD showed a linear increase with the increase of serum triglycerides and LDL cholesterol levels (Fig. 3a, d), and a linear decrease with the increase of serum HDL cholesterol levels (Fig. 3c). The prevalence of NAFLD was 22.8% in subjects with normal triglyceride levels (triglycerides <150 mg/dL) and 59.5% in subjects with hypertriglyceridemia (triglycerides >150 mg/dL). The prevalence of NAFLD was 27.3% in subjects with normal HDL cholesterol levels (HDL cholesterol >40 mg/dL) and 61.7% in subjects with hypo-HDL cholesteremia (HDL cholesterol <40 mg/dL). The prevalence of NAFLD was 26.4% in subjects with normal LDL cholesterol levels (LDL cholesterol <140 mg/dL) and 38.5% in subjects with hyper-LDL cholesteremia (LDL cholesterol >140 mg/dL). The prevalence of NAFLD showed a linear increase with FPG levels (<120 mg/dL) and this prevalence was approximately 60% and reached a plateau with FPG ≥120 mg/dL, especially in males (Fig. 3e). The prevalence of NAFLD was 25.6% in subjects with a normal fasting glucose, 56.2% in subjects with impaired FPG (FPG >110 mg/dL

Table 2 Characteristics of the patients according to gender

	Male			Female			
	Non-NAFLD (n = 1551)	NAFLD (n = 1076)	p value*	Non-NAFLD (n = 2015)	NAFLD (n = 433)	p value*	p value**
Age (years)	49.8 ± 10.2	49.9 ± 8.8	0.651	49.2 ± 9.3	54.0 ± 8.3	<0.0001	<0.0001
Ht (m)	1.69 ± 0.58	1.694 ± 0.059	0.886	1.567 ± 0.055	1.552 ± 0.055	<0.0001	<0.0001
BW (kg)	64.8 ± 7.9	73.6 ± 10.3	<0.0001	52.2 ± 6.8	61.8 ± 9.2	<0.0001	<0.0001
BMI (kg/m^2)	22.6 ± 2.4	25.6 ± 3.1	<0.0001	21.2 ± 2.6	25.7 ± 3.7	<0.0001	0.5978
AST (IU/L)	20.9 ± 7.8	25.2 ± 11.7	<0.0001	19.5 ± 5.7	23.5 ± 14.0	<0.0001	<0.0001
ALT (IU/L)	21.1 ± 11.7	35.3 ± 24.0	<0.0001	16.1 ± 7.5	26.2 ± 20.6	<0.0001	<0.0001
AAR	1.09 ± 0.34	0.81 ± 0.26	<0.0001	1.30 ± 0.32	1.01 ± 0.30	<0.0001	<0.0001
ALP (IU/l)	214.3 ± 63.9	218.6 ± 58.5	<0.01	198.1 ± 65.6	235.4 ± 77.3	<0.0001	<0.01
GGT(IU/L)	36.5 ± 31.5	53.8 ± 48.7	<0.0001	22.1 ± 20.9	35.4 ± 45.9	<0.0001	<0.0001
ChE (IU/L)	289.2 ± 120.5	333.2 ± 130.0	<0.0001	267.2 ± 122.3	333.8 ± 116.9	<0.0001	<0.05
Albumin (g/dL)	4.5 ± 0.2	4.6 ± 0.2	<0.0001	4.4 ± 0.2	4.5 ± 0.2	<0.0001	<0.0001
FPG (mg/dL)	99.7 ± 15.2	108.7 ± 23.8	<0.0001	93.7 ± 11.0	103.9 ± 21.5	<0.0001	<0.0001
TC (mg/dL)	200.7 ± 32.1	208.0 ± 33.9	<0.0001	209.4 ± 34.7	216.6 ± 34.7	<0.0001	<0.0001
TG (mg/dL)	109.6 ± 59.1	167.2 ± 106.7	<0.0001	80.2 ± 42.9	127.0 ± 84.1	<0.0001	<0.0001
HDL-C (mg/dL)	57.5 ± 14.5	48.6 ± 10.4	<0.0001	69.8 ± 15.4	58.6 ± 13.0	<0.0001	<0.0001
LDL-C (mg/dL)	118.5 ± 31.8	128.6 ± 30.5	<0.0001	118.1 ± 32.5	131.3 ± 30.8	<0.0001	0.1288

Values are expressed as mean ± SD. Statistical analysis was conducted using Mann–Whitney U test. Abbreviations are the same as those in Table 1

*p value for comparison between non-NAFLD and NAFLD in each gender group

**p value for comparison between male and female with NAFLD

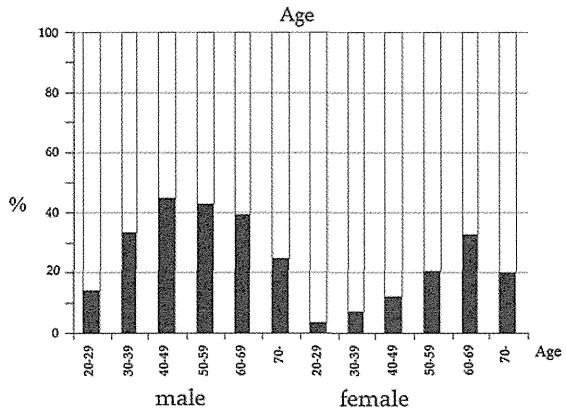

Fig. 1 Prevalence of NAFLD in patients according to age. The prevalence of NAFLD is higher in males than that in females at all ages, and it gradually increases with age in females

but <126 mg/dL), and 68.0% in subjects with FPG greater than 126 mg/dL, respectively. The prevalence of NAFLD gradually increased with an elevation of ALT. The prevalence of NAFLD was 70.6 and 35.8% in subjects with abnormal ALT levels in males (ALT ≥30) and females (ALT ≥20); the prevalence of NAFLD was 29.5 and 10.7% in subjects with normal ALT levels in males (ALT <30) and females (ALT <20), respectively (Fig. 3f).

Prevalence of NASH in the general population and subjects with fatty liver predicted by established scoring systems

In this study, the prevalence of NASH was estimated by BAAT score and FIB-4 index. Tables 4 and 5 show the distribution of subjects estimated by BAAT score and FIB-4 index, respectively. The estimated prevalence of NASH according to the BAAT index was 16.7% (BAAT score ≥2) and 2.7% (BAAT score ≥3) in the whole cohort, whereas it was 36.1% (BAAT score ≥2) and 8.3% (BAAT score ≥3) in subjects with NAFLD.

Mean FIB-4 indices in the whole cohort, in subjects without NAFLD, and in those with NAFLD were 1.15 ± 0.60, 1.17 ± 0.62, and 1.10 ± 0.55, respectively. The estimated prevalence of NASH according to the FIB-4 index was 1.9% (cutoff ≥2.67) in the whole cohort and it was 2.7% in subjects with NAFLD. In contrast, the estimated prevalence of NAFLD without advanced fibrosis was 74.0% (cutoff <1.30) in subjects with NAFLD.

Discussion

Using the latest large database in Japan, our study showed that the prevalence of NAFLD was high in the general

Table 3 Clinical factors associated with NAFLD in each male and female group using multivariate logistic regression analysis

	Male				Female			
	Coefficient	*p*	Odds ratio	95% confidence interval	Coefficient	*p*	Odds ratio	95% confidence interval
BMI >25	1.34	0.00	3.81	3.11–4.67	1.98	0.00	7.23	5.50–9.50
Age >50	–	–	–	–	0.59	0.00	1.80	1.37–2.37
ALT (M >30; F >20)	0.93	0.00	2.54	2.00–3.23	0.50	0.00	1.65	1.20–2.67
AAR <1	1.08	0.00	2.53	2.02–3.15	1.07	0.00	2.56	1.87–3.48
FPG >110	0.851	0.00	2.34	1.83–2.99	0.96	0.00	2.61	1.76–3.87
TG >150	0.792	0.00	2.21	1.78–2.74	0.95	0.00	2.58	1.83–3.63
GGT >35	0.360	0.00	1.43	1.17–1.75	–	–	–	–
HDL <40	0.306	<0.05	1.36	1.00–1.84	–	–	–	–
LDL >140	0.264	<0.05	1.30	1.05–1.60				

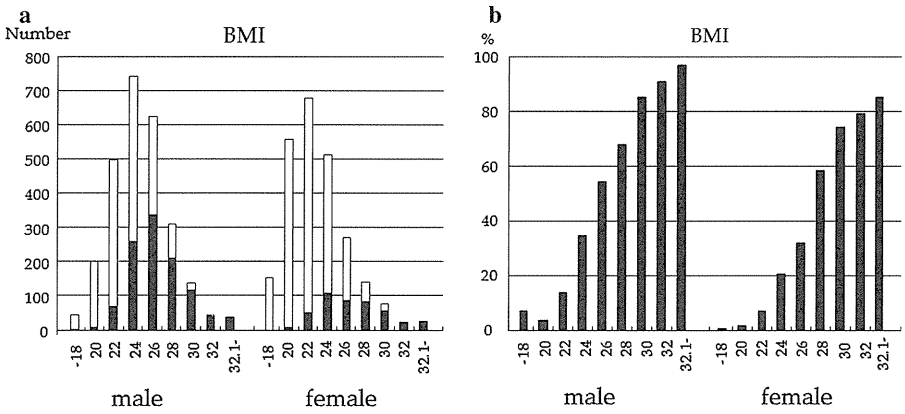

Fig. 2 **a** Distribution of subjects with NAFLD (*black columns*) and without NAFLD (*white columns*) according to body mass index (BMI). **b** Relative percentage of NAFLD according to BMI. The prevalence of NAFLD shows a tendency to increase linearly with BMI in males and females

population, especially in males, even though subjects were not obese. Our study suggested that the prevalence of NAFLD is still increasing in Japan. The present study showed the most recent frequency of NAFLD and a 10% increase from a previous Japanese study conducted in subjects who received a health checkup from 1989 to 2000 [17]. A recent study reported that mean BMI has globally increased in adults 20 years and older in 199 countries and territories between 1980 and 2008 [18]. In 2008, an estimated 1.46 billion adults worldwide had a BMI of 25 kg/m^2 or greater, and of these, 205 million men and 297 million women were obese.

Our study found that there was a linear relationship between the prevalence of NAFLD and an increase in BMI, serum triglycerides, and cholesterol, whereas the increase of prevalence showed a plateau at 120 mg/dL for FPG levels, especially in males. It is well known that NAFLD and NASH are strongly associated with the presence of obesity and lifestyle-related diseases, especially type 2 diabetes mellitus [6–8]. According to annual health check findings in Japan and Asian countries, the prevalence of NAFLD increases with BMI; it has been reported to be 10–20% in nonobese subjects, approximately 50% in those with a BMI ranging from more than 25 kg/m^2 to less than 30 kg/m^2, and approximately 80% in those with a BMI over 30 kg/m^2 [19].

A previous study reported that the crude prevalence of NAFLD increased with deterioration of glucose homeostasis, from 27% in patients with normal fasting glucose, 43% in patients with impaired fasting glucose, and 62% in patients with newly diagnosed and thus untreated diabetes [20]. This study revealed that there were a certain number of NAFLD subjects with normal range in various parameters. It is unclear whether NAFLD causes metabolic dysfunction or whether metabolic dysfunction is responsible for hepatic fat accumulation, or both. As shown in our study, there was a close relationship between the pathogenesis of NAFLD and that of glucose and lipid metabolism abnormalities.

Our study confirmed previous findings that various traditional metabolic parameters and aminotransferases may be normal in an appreciable proportion of patients with NAFLD, and therefore, are not sensitive enough for the diagnosis of NAFLD [7, 21–23]. It is widely accepted that

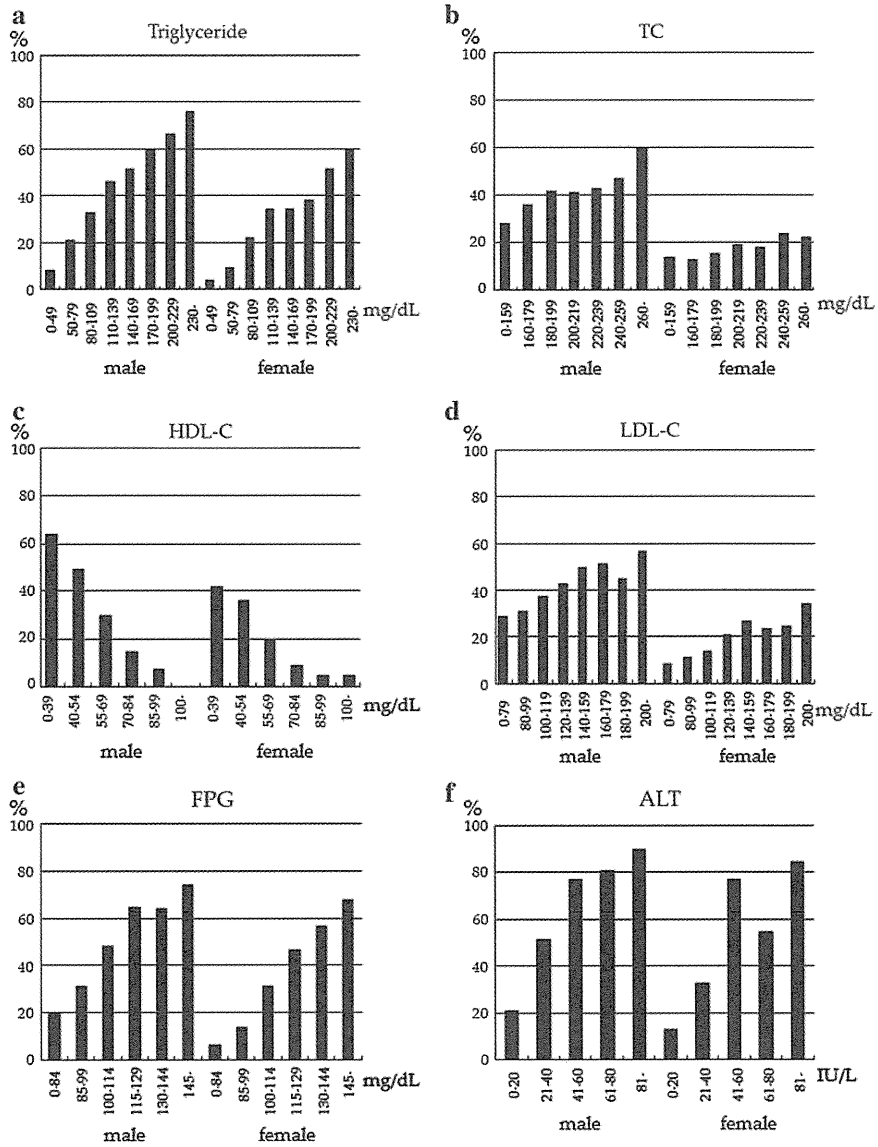

Fig. 3 Prevalence of NAFLD in various variables. The prevalence of NAFLD shows a linear increase with serum levels of triglycerides and low-density lipoprotein cholesterol (LDL-C) (**a**, **d**), whereas there is a linear decrease with high-density lipoprotein cholesterol (HDL-C) (**c**). The prevalence of NAFLD shows a linear increase with fasting plasma glucose (FPG) (<120 mg/dL), and this prevalence is approximately 60% and reaches a plateau with glucose ≥120 mg/dL, especially in males (**e**). The prevalence of NAFLD gradually increases with the elevation of alanine aminotransferase (ALT). There is an appreciable amount of NAFLD subjects with normal ALT levels (**f**)

Table 4 Distribution of subjects according to BAAT score

	BAAT score					Total
	0	1	2	3	4	
Non-NAFLD	1555	1708	290	13	0	3566
NAFLD	277	687	419	113	13	1509
Total	1832	2395	709	126	13	5075

serum aminotransferase levels are neither specific nor sensitive enough for diagnosis of NAFLD [2, 24, 25], which was consistent with the present study.

AAR was significantly lower in subjects with NAFLD compared with those without NAFLD. Similarly, lower values for AAR were found in NAFLD subjects compared

Table 5 Distribution of subjects according to FIB-4 index

	FIB-4 index			Total
	<1.3	≥1.3 but <2.67	≥2.67	
Non-NAFLD	2495	1011	60	3566
NAFLD	1117	352	40	1509
Total	3612	1363	100	5075

with those with alcoholic liver disease; thus, AAR can be used to differentiate between these conditions [26]. Another study demonstrated the significance of AAR, even within the spectrum of NAFLD, as a lower AAR was associated with a higher histopathological degree of hepatic steatosis in obese NAFLD subjects [27].

Although standard body weight is determined differently depending on ethnicity, obesity indicates excessive fat accumulation, and there is a relationship between the degree of obesity and the incidence of dyslipidemia, type 2 diabetes mellitus, and hypertension. For example, in Japan, a BMI of 22 is used to indicate the ideal body weight, because the incidence of obesity-related diseases is observed least frequently when the BMI is approximately 22.5 [28]. The incidence of obesity-related diseases is significantly increased in subjects with a BMI of more than 23 in Hong Kong [29]. In the current study, the prevalence of NAFLD showed a linear increase even though each variable was within the normal range. These results suggest that there is no threshold for the incidence of NAFLD and there are differences in the incidence of NAFLD among subjects.

In the present study, we focused on the gender difference for the relationship between the prevalence of NAFLD and metabolic abnormalities. It is well known that NAFLD and NASH exhibit age and sex differences in both prevalence and severity [30]. These age and gender differences are caused by differences in the prevalence of obesity and lifestyle-related diseases [31].

Computed tomography and magnetic resonance imaging are the most reliable procedures for measuring hepatic fat accumulation, but these procedures are not simple enough that they can be used for mass screening. Ultrasonography has many advantages for mass screening. Although ultrasonography is probably the least reliable of these three imaging methods for the quantitative assessment of the degree of hepatic steatosis, ultrasonography is simple and sensitive enough to evaluate hepatic fat accumulation when typical findings of hepatic steatosis are detected. A previous study indicated that the use of ultrasonography for diagnosing NAFLD had a sensitivity of 89% and specificity of 93% for the identification of fatty liver [32].

The prevalence of NASH in the general population is still not clearly documented. A recent study revealed that NASH was confirmed in 12.2% of a largely middle-aged population and 29.9% of patients with ultrasonographic fatty liver [9]. An autopsy study from the late 1980s found that the prevalence of NASH was 2.7% among lean subjects, rising to 18.5% among markedly obese patients [33]. More recently, three studies evaluating donor livers before transplantation found that the prevalence of NASH was 1.1–14% [34–36]. Since it is known that almost 10–20% of subjects with NAFLD have NASH, the prevalence of NASH is estimated to be 13% of the adult Japanese population, which is an extremely large number of potential patients [19]. However, no studies have estimated the prevalence of NASH in the Japanese general population.

In the present study, the prevalence of NAFLD with advanced fibrosis determined as a BAAT ≥3 was predicted as 8.3% in individuals with NAFLD and 2.8% in all subjects. There were 0.4% of subjects with a BAAT ≥3 in the cohort without NAFLD. Further analysis is required to clarify the characteristics of those subjects. The FIB-4 index was developed as a noninvasive panel to stage liver disease in subjects with human immunodeficiency virus and hepatitis C virus co-infection [14]. It has recently been demonstrated that its performance characteristics for the diagnosis of advanced fibrosis in NAFLD are better than those of other similar panels that do not require additional testing, and are comparable with several others that require additional tests [15]. In our study, the estimated prevalence of NASH according to the FIB-4 index was 1.9% in the whole cohort and it was 2.7% in subjects with NAFLD (cutoff ≥2.67). These results, which were predicted using representative scoring indices, suggest that there are potential patients with advanced NASH in the general population and the prevalence is similar to previous studies in Japan [31].

Recently, Sumida et al. [37] suggested a novel scoring system determined by serum ferritin, insulin, and type IV collagen 7S levels (NAFIC score) conducted with Japanese NAFLD patients. Although the scoring system is expected to accurately predict NASH with advanced liver fibrosis, we could not use the NAFIC score because of the lack of parameters.

Some limitations of this study should be noted. First, its cross-sectional design precluded any causal and temporal inferences about the relationships between the presence of NAFLD and various parameters. Second, the diagnosis of NAFLD was made by ultrasonography and exclusion of

other causes of chronic liver disease, but this was not confirmed by liver biopsy, and there were some limitations as mentioned above. Imaging modalities have several limitations in this respect. There might be some possible errors to examine due to the difference of ultrasonography equipment and examiners among each medical facility. The most important limitations of ultrasonography are that (1) it might detect only moderate to severe steatosis, which affects more than one-third of hepatocytes, and it cannot detect mild steatosis, (2) it is difficult to determine an accurate quantitative diagnosis, and (3) there might be differences in measurement deviations in each examiner in a multicenter study, even though a common ultrasonographic definition of NAFLD has been established [38]. Third, there was a lack of some important parameters required to evaluate the background of NAFLD such as waist circumference. We have previously demonstrated a relationship between visceral fat accumulation and development of insulin resistance in patients with NAFLD [39, 40]. Because the relationship between the pathogenesis of NAFLD and visceral fat accumulation is important, further studies are required to clarify the relationship between the prevalence of NAFLD and visceral fat accumulation and the prevalence of metabolic syndrome.

In conclusion, the present study showed that the prevalence of NAFLD is high in the general population in Japan and has increased compared with previous studies, especially in males, even though subjects are not obese. There is a linear relationship between the prevalence of NAFLD and various metabolic parameters, even in nonobese subjects. The prevalence of NASH with advanced fibrosis is estimated to be considerably high in subjects with NAFLD in Japan.

Acknowledgments The authors would like to thank Professor Kyuichi Tanikawa (International Institute for Liver Research) for excellent advice, Yukie Watanabe, Chieko Ogawa, Natsumi Izumi, Hisae Ariki, Ikuko Hirotaki, Reiko Sonoda, and all the co-medical staff at Saga Medical School Hospital, Eguchi Hospital, Kochi Medical School, and Kawamura Clinic for assistance, and members of the Japan Study Group of Nonalcoholic Fatty Liver Disease (JSG-NAFLD) for excellent advice. This work was supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (2010) (#22590741 to Y.E. and #20590785 to M.O.).

Conflict of interest None.

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