Hamaguchi et al., the Brinkman index did not differ between the NAFLD and non-NAFLD groups at baseline, which is inconsistent with our results. This may be because the mean period of 414 days in Hamaguchi et al. was not long enough to investigate the influence of smoking. In addition, the results of our analysis are very important because a significant association between cigarette smoking and the development of NAFLD was present in a population in which a strong association between the development of NAFLD and metabolic syndrome risk factors was evident.

Risk factor modification such as weight loss and medication for insulin resistance and dyslipidemia should cure or prevent NAFLD. In this study, we found that cigarette smoking is a risk factor for NAFLD. Therefore, smoking cessation is likely to decrease the risk of NAFLD among current smokers. However, in our subanalysis of three groups that included new quitters, and continuing smokers and nonsmokers, smoking cessation seemed to convey a higher risk for developing NAFLD than continuing to smoke (Table 7). It has been reported that smoking is a risk factor for diabetes mellitus, but smoking cessation is also associated with substantial weight gain and may lead to a higher short-term risk of type 2 diabetes [17, 29]. Despite the fact that we were unable to clarify the effect of smoking cessation on the treatment or development of NAFLD because (1) our study population was too small, (2) the date when smoking was stopped was not considered, and (3) metabolic syndrome risk factors that arose or were cured during the 10-year period and the effects of treatment on these diseases were not fully considered, we speculate that smoking cessation without weight gain is likely to be beneficial for patients with NAFLD.

Subjects who drank $\leq$20 g/day of alcohol were included in our study. In a cross-sectional study in Japanese men, low alcohol consumption (40–140 g/week) significantly reduced the incidence of fatty liver (AOR = 0.824 [95% CI 0.683–0.994]) [20]. We have also found that alcohol intake may inhibit the development of fatty liver through an association of alcohol drinking pattern with obesity [48]. In the current study, intake of a small amount of alcohol had an inhibitory effect on NAFLD development in multivariate analysis that included the number of metabolic syndrome risk factors as a variable (Table 5). In animal models, cigarette smoking and alcohol intake have been shown to contribute to the development and exacerbation of fatty liver [49]. Thus, further studies of the apparent synergistic effect of alcohol intake and cigarette smoking on NAFLD are required in humans.

There are several limitations in this study. Firstly, ultrasonography is effective for diagnosing fatty liver, but detecting fatty liver in patients with $\leq$30% liver fat or in obese patients is relatively difficult [4]. Moreover, as simple fatty liver and NASH cannot be distinguished by ultrasonography, an association of cigarette smoking with fatty liver severity could not be shown. Secondly, although the frequency of NAFLD development in continuing smokers was higher than that in continuing nonsmokers in the limited group of subjects, the difference was not statistically significant in multivariate analysis (Table 5). This may be due to the modest sample size. Finally, our study was a follow-up study of a 10-year interval in which the data was obtained at only two points, in 1998 and in 2008. Longitudinal studies such as those involving the evaluation of NAFLD incidence and smoking patterns over time using several points during a 10-year period may provide more convincing evidence of the contribution of cigarette smoking to NAFLD development.

In conclusion, metabolic syndrome risk factors increase the risk of NAFLD. In this retrospective study, we found that cigarette smoking, a risk factor for metabolic syndrome, was also a risk factor for NAFLD development independent of metabolic syndrome risk factors. In addition, although NAFLD development became more likely as the Brinkman index increased, smoking cessation was also a likely risk factor for NAFLD development, which is partially explainable by an increase in BMI. Therefore, having never smoked is important for the prevention of NAFLD in nonsmokers, and additional treatment or prevention of metabolic syndrome risk factors may be necessary to encourage the cessation of cigarette smoking to treat or prevent NAFLD in smokers.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Alcohol drinking patterns and the risk of fatty liver in Japanese men

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Abstract
Background Alcohol is considered to be a major cause of fatty liver (FL). In contrast, however, recent investigations have suggested that moderate alcohol consumption is protective against FL. To clarify the role of alcohol consumption in FL development, we examined the association between drinking patterns and FL prevalence.
Methods We enrolled 9,886 male participants at regular medical health checks. Each subject's history of alcohol consumption was determined by questionnaire. The subjects were classified according to alcohol consumption as non-, light, moderate, and heavy drinkers (0, <20, 20–59, and ≥60 g/day, respectively). FL was defined by ultrasonography. Independent predictors of FL were determined by logistic regression analysis.
Results The prevalence of FL displayed a "U-shaped curve" across the categories of daily alcohol consumption (non-, 44.7%; light, 39.3%; moderate, 35.9%; heavy drinkers, 40.1%; $P < 0.001$). The prevalence of FL was associated positively with body mass index and other obesity-related diseases and inversely with alcohol consumption (light, odds ratio [OR] 0.71, 95% confidence interval [CI] 0.59–0.86; moderate, OR 0.55, CI 0.45–0.67; heavy, OR 0.44, CI 0.32–0.62) as determined by multivariate analysis after adjusting for potential confounding variables. In addition, examination of drinking patterns (frequency and volume) revealed that the prevalence of FL was inversely associated with the frequency of alcohol consumption (≥21 days/month) (OR 0.62, CI 0.53–0.71) but not with the volume of alcohol consumed.
Conclusions Our observations suggest that alcohol consumption plays a protective role against FL in men, and consistent alcohol consumption may contribute to this favorable effect.

Keywords Fatty liver · Alcohol consumption · Nonalcoholic fatty liver disease · Metabolic syndrome

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Introduction

Fatty liver (FL) disease is commonly divided into nonalcoholic (NAFLD) and alcoholic (AFLD) FL disease categories. NAFLD is an increasingly recognized condition, predominantly linked to metabolic syndrome, which, in turn, is associated with obesity and insulin resistance [1–4]. The clinical importance of NAFLD is due to its wide

spectrum of histological damage, ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), which can lead to cirrhosis, hepatocellular carcinoma, and hepatic failure [5]. Alcohol dehydrogenase-mediated ethanol metabolism generates a reduced form of nicotinamide adenine dinucleotide (NADH), which promotes steatosis by stimulating the synthesis of fatty acids and opposing their oxidation. The hepatic lipogenic pathway is activated after the consumption of 24 g of ethanol per day [6]. Alcohol intake is a risk factor for both hypertriglyceridemia and FL [7, 8], and daily intake as low as 20–30 g of alcohol per day may be sufficient to cause alcohol-induced liver disease in some cases.

In contrast, recent investigations have reported a protective role of moderate alcohol consumption against FL [9–13]. Light to moderate alcohol consumption is associated with lower cardiovascular mortality [14–16] and a reduced risk of developing type 2 diabetes [17–19]. Mechanisms proposed to explain this observation include improved lipid profiles, especially high-density lipoprotein-cholesterol [15], and increased insulin sensitivity [19–21]. The mechanism of protection against FL, however, remains unclear.

In this study, to elucidate the relationship between the quantity and patterns of alcohol consumption and FL prevalence, we conducted a cross-sectional study of a male Japanese population.

Subjects, materials, and methods

Study population

The study subjects were Japanese men aged 30–69 years, who participated in regular health check-ups from April 2000 to March 2007. Of the initial 10,283 candidates, 204 (2.0%) hepatitis B virus surface antigen (HBsAg)-positive and 195 (1.9%) hepatitis C virus antibody (HCV Ab)-positive subjects were excluded from the analysis (two subjects were positive for both HBsAg and HCV Ab). The remaining 9,886 men were enrolled in this study. Serological testing for HBsAg and HCV Ab was performed by enzyme immunoassay and enzyme-linked immunosorbent assay, respectively. This study, conducted at Kagoshima Kouseiren Medical Health Care Center, was approved by the ethics committee of the Kagoshima Prefectural Federation of Agricultural Cooperatives for Health and Welfare.

A diagnosis of FL was made, using ultrasonography (SSA-250A and SSA-700A; Toshiba, Tokyo, Japan; Logic 400; GE Healthcare Japan, Tokyo, Japan), from findings of bright liver (increased echogenicity) with liver–kidney contrast (increased echogenicity of the liver in comparison to the right kidney). Body mass index (BMI) was calculated from the equation: body weight (kg)/height^2 (m^2). Body composition was categorized according to the Western Pacific Region of WHO criteria pertaining to obesity (WPRO criteria): BMI <18.5 kg/m^2 (underweight), 18.5–22.9 kg/m^2 (normal weight), 23.0–24.9 kg/m^2 (overweight), and 25 kg/m^2 or more (obese). Venous blood samples were taken from all subjects before nine o'clock after an overnight fast and were analyzed immediately. Alanine aminotransferase (ALT), aspartate aminotransaminase (AST), and γ-glutamyl transpeptidase (γ-GTP) activities and the concentrations of total cholesterol, triglycerides, and glucose were measured by standard laboratory procedures. High-density lipoprotein cholesterol (HDL-C) levels were determined by direct homogeneous assay of serum samples using detergents (Sekisui Medical, Tokyo, Japan).

The subjects were investigated for the presence of concomitant metabolic abnormalities. Hypertension was defined as being present in patients on medication for hypertension, those with systolic blood pressure >130 mmHg, and/or those with diastolic blood pressure >85 mmHg. Dyslipidemia was defined as being present in patients on medication for dyslipidemia, those with total cholesterol >220 mg/dl, triglycerides >150 mg/dl, and/or HDL-C <40 mg/dl. Diabetes mellitus (DM) was considered to be present in patients on medication for DM and/or in those with fasting blood glucose >125 mg/dl. ALT or AST elevations were defined as levels over 30 IU/L.

Using a common questionnaire, a history of alcohol intake was determined by a public health nurse without knowledge of the status of FL. Each subject reported their current frequency of alcohol consumption (A, 0; B, 1–5; C, 6–10; D, 11–20; E, ≥21 days/month) and volume of alcohol drunk per day (a, <20; b, 20–39; c, 40–59; d, 60–99; e, ≥100 g/day). All participants except former drinkers were divided into four groups according to the frequency (A–E) and volume (a–e) of alcohol consumption, as follows: non-drinkers, A; light drinkers, B-a–e, C-a–c, D-a or -b, E-a; moderate drinkers, C-d or -e, D-c or -d, E-b or -c; and heavy drinkers, D-e, E-d or -e (daily volumes consumed by non-, light, moderate, and heavy drinkers were approximately 0, <20, 20–59, and ≥60 g/day, respectively).

Statistical analysis

The distributions of each variable were compared between FL subjects and non-FL subjects. Continuous variables, including age, BMI, ALT, AST, γ-GTP, triglycerides, and HDL-C, were analyzed by *t*-test, and categorical variables were examined by the χ^2 test. In addition, associations of alcohol consumption (non-, light, moderate, and heavy drinkers) with clinical factors and smoking status were

examined by analysis of variance (ANOVA) or the χ^2 test. Maximum likelihood odds ratios (ORs) for FL risk and their 95% confidence intervals (95% CIs) were calculated using logistic regression models. Statistical analyses were performed using STATA version 9.2 (StataCorp, TX, USA). All *P* values presented are two-sided.

Results

Subject description

Among the 9,886 subjects, 3,816 men (38.6%) met the criteria for FL (Table 1). The subjects' characteristic

Table 1 Characteristic features of study subjects

	All	Fatty liver		*P* value
		(−)	(+)	
Number (%)	9886 (100%)	6070 (100%)	3816 (100%)	
Age (years)	50.7 [50.5, 50.9]	51.4 [51.2, 51.7]	49.5 [49.2, 49.8]	<0.001*
30–39	12.0%	11.4%	12.8%	<0.001**
40–49	31.2%	28.2%	36.0%	
50–59	32.5%	32.6%	32.5%	
60–69	24.3%	27.8%	18.6%	
BMI	23.7 [23.6, 23.7]	22.5 [22.5, 22.6]	25.6 [25.5, 25.7]	<0.001*
<18.5	2.4%	3.9%	0.1%	<0.001**
18.5–22.9	37.7%	51.9%	15.2%	
23.0–24.9	26.9%	27.3%	26.4%	
25–	33.0%	17.0%	58.4%	
Laboratory data				
ALT (IU/L)	25.7 [25.5, 36.7]	21.6 [21.4, 21.8]	33.9 [33.3, 34.4]	<0.001*
AST (IU/L)	24.7 [24.5, 24.9]	23.4 [23.2, 23.6]	27.0 [26.7, 27.3]	<0.001*
γ-GTP (IU/L)	36.2 [35.6, 36.7]	30.7 [30.1, 31.3]	47.0 [45.9, 48.1]	<0.001*
Triglycerides (mg/dl)	120 [119, 122]	102 [100, 103]	156 [153, 159]	<0.001*
HDL-C (mg/dl)	53.9 [53.6, 54.2]	57.0 [56.7, 57.4]	49.3 [48.9, 49.6]	<0.001*
Presence of clinical manifestation				
Fatty liver	38.6%			
ALT elevation	32.7%	18.8%	54.8%	<0.001***
AST elevation	21.2%	15.3%	30.5%	<0.001***
Hypertension	46.5%	42.1%	53.5%	<0.001***
Dyslipidemia	55.9%	45.9%	71.8%	<0.001***
Diabetes mellitus	11.4%	7.9%	16.8%	<0.001***
Smoking status				
Never smoker	29.4%	29.7%	28.8%	0.001***
Former smoker	31.2%	29.9%	33.3%	
Current smoker	39.4%	40.4%	37.9%	
Alcohol consumption				
Never drinker	8.6%	7.7%	10.0%	<0.001***
Former drinker	2.9%	2.6%	3.3%	
Light drinker	45.9%	45.4%	46.7%	
Moderate drinker	39.1%	40.8%	36.4%	
Heavy drinker	3.5%	3.4%	3.6%	

Data are presented as geometric means [corresponding 95% confidence intervals] or proportions

BMI body mass index, *ALT* alanine aminotransferase, *AST* aspartate aminotransaminase, *γ-GTP* γ-glutamyl transpeptidase, *HDL-C* high-density lipoprotein cholesterol

* *P* values were obtained by *t* test

** *P* values for trend were obtained by likelihood ratio test using a logistic regression model

*** *P* values were obtained by χ^2 test

features and the differences in biological parameters in relation to FL are summarized in Table 1. There were 1,131 (11.4%) non-drinkers, including 284 former drinkers; 4,540 (45.9%) light drinkers, 3,868 (39.1%) moderate drinkers; and 347 (3.5%) heavy drinkers.

The subjects with FL were significantly younger than the the subjects without FL ($P < 0.001$). BMI was significantly higher in FL (+) subjects in comparison to FL (−) subjects ($P < 0.001$). ALT, AST, and two parameters typically increased in association with alcohol consumption, γ-GTP and triglycerides, were significantly higher in FL (+) subjects in comparison to those who were FL (−) (all $P < 0.001$). In contrast, HDL-C, another parameter correlated with alcohol consumption, was lower in the FL (+) subjects than in FL (−) subjects ($P < 0.001$).

There was a U-shaped association between the prevalence of FL and categories of alcohol consumption ($P < 0.001$; Table 2). The prevalence of FL was highest in never drinkers among the five categories of alcohol consumption. AST, γ-GTP, triglycerides, and HDL-C, which

Table 2 Comparison of characteristic features across the categories of alcohol consumption

	Never drinker	Former drinker	Light drinker	Moderate drinker	Heavy drinker	*P* value
Number	847 (100%)	284 (100%)	4,540 [100%)	3,868 (100%)	347 (100%)	
Age (years)	51.6 [50.9, 52.3]	54.0 [52.8, 55.2]	50.4 [50.1, 50.7]	50.7 [50.4, 51.0]	48.1 [47.3, 48.9]	<0.001*
30–39	11.5%	7.8%	14.0%	10.0%	11.8%	<0.001**
40–49	28.7%	23.9%	30.6%	32.4%	39.2%	
50–59	30.0%	27.8%	29.4%	36.4%	40.6%	
60–	29.9%	40.5%	26.0%	21.2%	8.4%	
BMI (kg/m^2)	23.7 [23.5, 24.0]	23.6 [23.2, 24.0]	23.7 [23.6, 23.8]	23.6 [23.5, 23.7]	23.9[23.5, 24.2]	0.031*
<18.5	2.6%	4.2%	2.5%	2.0%	2.9%	0.048**
18.5–22.9	37.4%	38.4%	37.0%	38.9%	34.0%	
23.0–24.9	24.6%	25.0%	26.6%	27.9%	28.2%	
25.0–	35.4%	32.4%	33.9%	31.2%	34.9%	
Laboratory data						
ALT (IU/L)	25.4 [24.6, 26.8]	28.0 [26.2, 29.8]	25.4 [25.0, 25.7]	25.6 [25.2, 26.0]	30.5 [28.9, 32.3]	<0.001**
AST (IU/L)	22.7 [22.2, 23.1]	24.3 [23.3, 25.4]	23.7 [23.5, 23.9]	26.0 [25.7, 26.3]	30.5 [29.1, 32.0]	<0.001**
Fatty liver (−)	21.1 [20.6, 21.6]	22.4 [21.3, 23.6]	22.4 [22.1, 22.6]	24.7 [24.4, 25.0]	28.5 [27.1, 30.0]	<0.001***
Fatty liver (+)	24.7 [23.9, 25.6]	27.0 [25.2, 28.9]	25.8 [25.5, 26.2]	28.5 [28.0, 29.1]	33.8 [31.1, 36.8]	<0.001***
γ-GTP (IU/L)	22.5 [21.6, 23.3]	25.8 [23.9, 27.9]	30.2 [29.6, 30.8]	48.1 [46.9, 49.2]	67.1 [61.4, 73.4]	<0.001**
Fatty liver (−)	18.1 [17.3, 18.9]	21.2 [19.3, 23.3]	25.3 [24.8, 26.0]	40.8 [39.6, 42.0]	57.0 [50.9, 63.8]	<0.001***
Fatty liver (+)	29.2 [27.6, 31.0]	33.4 [29.9, 37.3]	39.7 [38.5, 40.9]	64.5 [62.1, 67.0]	85.8 [74.9, 98.3]	<0.001***
Triglycerides (mg/dl)	116 [111, 120]	107 [100, 113]	115 [113, 116]	127 [124, 130]	147 [137, 158]	<0.001**
HDL-C (mg/dl)	48.2 [47.5, 48.9]	49.8 [48.3, 51.2]	52.4 [52.0, 52.7]	57.1 [56.6, 57.5]	58.2 [56.6, 59.7]	<0.001**
Presence of clinical manifestation						
ALT elevation	32.4%	37.3%	31.2%	33.0%	45.5%	<0.001**
AST elevation	13.8%	20.4%	17.1%	26.3%	37.2%	<0.001**
Dyslipidemia	60.3%	56.3%	53.8%	56.8%	62.5%	<0.001**
Fatty liver	45.1%	43.7%	39.3%	35.9%	40.1%	<0.001**
Hypertension	37.4%	44.7%	42.6%	52.2%	58.5%	<0.001**
Diabetes mellitus	11.3%	18.0%	10.2%	12.3%	11.5%	<0.001**
Smoking status						
Never smoker	37.5%	36.6%	36.8%	19.6%	15.6%	<0.001**
Former smoker	24.1%	39.8%	30.0%	33.6%	30.3%	
Current smoker	38.4%	23.6%	33.2%	46.8%	54.2%	

Data are presented as geometric means [corresponding 95% confidence intervals] or proportions

BMI body mass index, *ALT* alanine aminotransferase, *γ-GTP* γ-glutamyl transpeptidase, *AST* aspartate aminotransaminase, *HDL-C* high-density lipoprotein cholesterol

* *P* values obtained by analysis of variance (ANOVA) after logarithmic transformation of each data

** *P* values obtained by χ^2 test

*** *P* values obtained by likelihood ratio test

are known factors associated with alcohol consumption, were significantly increased across the categories with increasing alcohol consumption (all $P < 0.001$). In addition, AST and γ-GTP were significantly higher in FL (+) subjects in comparison with FL (−) subjects in each category of alcohol consumption, and these parameters increased across the categories with increasing alcohol consumption in subjects both with and without FL.

Independent predictors of fatty liver

Independent predictors significantly affecting the prevalence of FL were identified by logistic regression analysis (Table 3). FL risk tended to decrease with age and increase with BMI. A significant elevation of FL risk was observed in subjects with ALT elevation, hypertension, dyslipidemia, and DM. Multivariate analysis revealed that alcohol

Table 3 Predictive factors of fatty liver by logistic regression analysis

	All subjects		Limited subjects[c]
	Univariate OR [95% CI]	Multivariate OR [95% CI][a]	Multivariate OR [95% CI][b]
Age (years)			
30–39	1.0 (referent)	1.0 (referent)	1.0 (referent)
40–49	1.14 [0.99, 1.30]	1.27 [1.07, 1.52]	1.41 [0.82, 2.41]
50–59	0.89 [0.78, 1.02]	1.14 [0.95, 1.36]	0.92 [0.52, 1.64]
60–69	0.59 [0.51, 0.69]	0.84 [0.69, 1.02]	0.94 [0.50, 1.76]
P for trend	<0.001	<0.001	0.333
BMI (kg/m^2)			
<18.5	0.05 [0.01, 0.19]	0.06 [0.01, 0.25]	–[d]
18.5–22.9	1.0 (referent)	1.0 (referent)	1.0 (referent)
23.0–24.9	3.30 [2.93, 3.72]	2.42 [2.12, 2.75]	3.15 [2.04, 4.86]
>25	11.8 [10.5, 13.2]	6.01 [5.27, 6.84]	5.50 [3.40, 8.89]
P for trend	<0.001	<0.001	<0.001
Presence of clinical manifestation			
ALT elevation	5.23 [4.78, 5.73]	2.46 [2.19, 2.77][b]	–
Hypertension	1.59 [1.46, 1.72]	1.18 [1.06, 1.32]	–
Dyslipidemia	3.00 [2.75, 3.27]	1.19 [1.05, 1.35]	–
Diabetes mellitus	2.34 [2.06, 2.65]	1.88 [1.61, 2.20]	–
Smoking status			
Never smoker	1.0 (referent)	1.0 (referent)	1.0 (referent)
Former smoker	1.15 [1.04, 1.28]	1.07 [0.94, 1.22]	–
Current smoker	0.97 [0.88, 1.07]	0.83 [0.73, 0.95]	0.58 [0.39, 0.87]
P for heterogeneity	0.001	<0.001	
Alcohol consumption			
Never drinker	1.0 (referent)	1.0 (referent)	1.0 (referent)
Former drinker	0.91 [0.72, 1.24]	0.89 [0.63, 1.25]	–
Light drinker	0.79 [0.68, 0.91]	0.71 [0.59, 0.86]	0.71 [0.38, 1.34]
Moderate drinker	0.68 [0.59, 0.79]	0.55 [0.45, 0.67]	0.81 [0.40, 1.62]
Heavy drinker	0.81 [0.63, 1.05]	0.44 [0.32, 0.62]	0.54 [0.15, 2.03]
P for trend	<0.001	<0.001	0.525

P values were obtained by likelihood ratio test

ALT alanine aminotransferase, *BMI* body mass index

[a] Odds ratios (ORs) and corresponding 95% confidence intervals [95% CIs] were obtained by logistic regression models using variables in this table and serum levels of alanine aminotransferase, γ-glutamyl transpeptidase, triglycerides, and high-density lipoprotein cholesterol

[b] ORs and corresponding 95% CIs were obtained by logistic regression models using variables in this table and serum levels of γ-glutamyl transpeptidase, triglycerides, and high-density lipoprotein cholesterol

[c] Subjects were limited to those who had no history of ALT elevation, hypertension, dyslipidemia, and diabetes mellitus. Subjects who stopped drinking or who were ex-smokers were also excluded from the analysis ($n = 1{,}481$)

[d] There was no subject with fatty liver in this BMI category

consumption was inversely associated with FL risk, after adjusting for the effects of all variables in Table 3, and serum levels of ALT, γ-GTP, triglycerides, and HDL-C (*P* for trend <0.001). This association was still present when the study subjects were limited to those who were not former smokers or drinkers, and who had no history of ALT elevation, hypertension, dyslipidemia, and DM, although the ORs for alcohol consumption were not statistically significant.

Influence of drinking patterns on FL prevalence

The prevalence of FL was examined in 10 subgroups classified by the frequency of alcohol consumption (0, 1–10, 11–20, and >20 days/month) and volume of alcohol consumed on days subjects drank (0, <20, 20–59, and ≥60 g/day) (Fig. 1). The prevalence of FL in non-drinkers was 44.7%. The prevalences of FL in those who consumed alcohol on 1–10, 11–20, and ≥21 days/month were 41.8, 39.1, and 30.7% (<20 g/day); 52.8, 48.5, and 35.0% (20–59 g/day); and 54.9, 53.6, and 38.4% (≥60 g/day), respectively. The prevalence of FL decreased with increased frequency of alcohol consumption and increased with increasing volumes of alcohol consumed per day (Fig. 1, Table 4).

For current alcohol drinkers, the FL risk was examined based on alcohol drinking patterns, the frequency of alcohol consumption, and the daily volume of alcohol (Table 4). There was a significant inverse association between the frequency of alcohol consumption and the risk

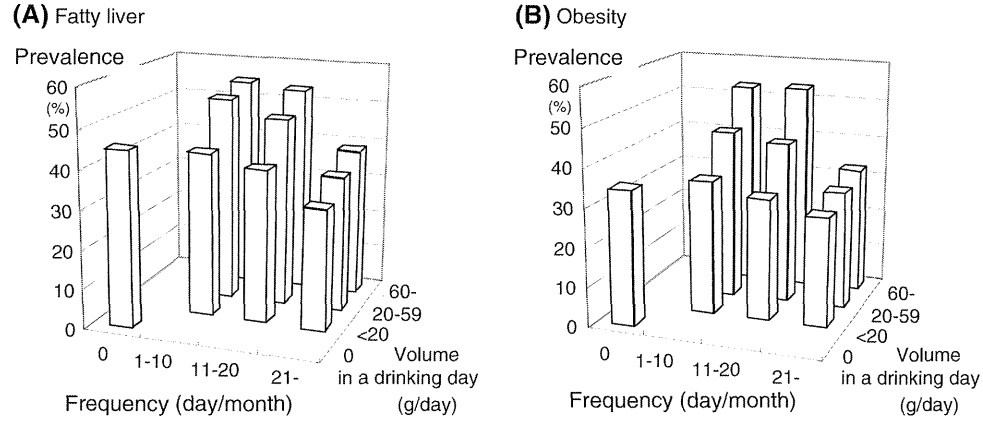

Fig. 1a,b Relationship of drinking patterns (frequency and volume in a drinking day) with the prevalence of fatty liver and obesity

Table 4 Estimated risk of fatty liver and obesity by alcohol drinking patterns among current alcohol drinkers

	Fatty liver		Obesity	
	Number (%)	OR [95% CI][a]	Number (%)	OR [95% CI][b]
Frequency (days/month)				
1–10 (*n* = 1,953)	921 (47%)	1.0 (referent)	775 (40%)	1.0 (referent)
11–20 (*n* = 863)	384 (45%)	0.92 [0.75, 1.12]	326 (38%)	1.05 [0.86, 1.27]
21– (*n* = 5,939)	2,005 (34%)	0.62 [0.53, 0.71]	1,765 (30%)	0.87 [0.76, 1.00]
P value	<0.001[#]	<0.001[##]	<0.001[#]	<0.033[##]
Alcohol volume (g/drinking day)				
1–20 (*n* = 3,438)	1,206 (35%)	1.0 (referent)	1,042 (30%)	1.0 (referent)
21–59 (*n* = 4,705)	1,823 (39%)	1.02 [0.90, 1.15]	1,566 (33%)	1.18 [1.05, 1.33]
60– (*n* = 612)	281 (46%)	0.83 [0.66, 1.04]	258 (42%)	1.41 [1.14, 1.74]
P value	<0.001[#]	0.378[##]	<0.001[#]	<0.001[##]

[#] *P* values were obtained by χ^2 test

[##] *P* values for trend were obtained by likelihood ratio test

[a] Odds ratios (ORs) and corresponding 95% confidence intervals [95% CIs] were obtained by logistic regression models using alcohol drinking patterns (frequency/daily alcohol consumption); age; body mass index; height; smoking status; the presence of alanine aminotransferase elevation, hypertension, dyslipidemia, and diabetes mellitus; and serum levels of alanine aminotransferase, γ-glutamyl transpeptidase, triglycerides, and high-density lipoprotein cholesterol

[b] ORs and 95% CIs were obtained by logistic regression models using alcohol drinking patterns (frequency/daily alcohol consumption); age; height; smoking status; the presence of fatty liver, alanine aminotransferase elevation, hypertension, dyslipidemia, and diabetes mellitus; and serum levels of alanine aminotransferase, γ-glutamyl transpeptidase, triglycerides, and high-density lipoprotein cholesterol

of FL (P for trend <0.001). On the other hand, alcohol volume was not related to the risk of FL (P for trend = 0.378).

Influence of drinking patterns on body composition

The association between alcohol drinking patterns and the prevalence of obesity, which is the most important risk factor for FL, are shown in Fig. 1b and Table 4. The prevalence of FL in non-drinkers was 34.6%. The prevalences of obesity in those who consumed alcohol on 1–10, 11–20, and ≥21 days/month were 34.3, 31.2, and 28.1% (<20 g/day); 43.7, 41.8, and 30.4% (20–59 g/day); and 53.0, 53.7, and 32.6% (≥60 g/day), respectively. As with the prevalence of FL, the prevalence of obesity decreased with increasing frequency of alcohol consumption and increased with the increasing alcohol volume in a drinking day (Table 4).

Logistic regression analysis (Table 4) revealed a significant association between the volume of alcohol in a drinking day and the risk of obesity (P for trend <0.001). In addition, consistent alcohol consumption tended to reduce the likelihood of obesity (OR 0.87, 95% CI 0.76–1.00, P for trend 0.033).

Discussion

This study demonstrated that FL in men was positively associated with factors including the presence of obesity, hypertension, dyslipidemia, and DM, but was negatively associated with age and alcohol consumption. Although our survey was not prospective in nature, these findings confirm that the major risk factors for FL are factors related to adiposity [1–4], not alcohol consumption, findings which agree with recent reports proposing a protective effect of alcohol intake [9–13]. We also confirmed that alcohol consumption tended to be negatively associated with FL in the limited number of subjects who had no history of ALT elevation, hypertension, dyslipidemia, or DM. In addition, our study may provide new evidence to help understand the role of alcohol drinking patterns in the pathogenesis of hepatic steatosis.

While alcohol consumption certainly may be a cause of FL in some cases [7, 8], it potentially plays a protective role against FL regardless of daily alcohol volume. Gunji et al. previously reported that "any drinking" might potentially be protective against FL; light (40–140 g/week) and moderate alcohol (140–280 g/week) consumption decreased the risk of FL, and the prevalence of FL was not increased even by heavy alcohol consumption [12]. Our study is also a report providing evidence of a significant inverse association between FL and alcohol consumption, even in heavy drinkers (≥60 g/day) (Table 3). We consider that alcohol consumption is a double-edged sword in the pathogenesis of hepatic steatosis. The difference in the results of our study and previous studies proposing alcohol consumption as a risk factor for FL may be due to differences in the ethnicity, age, BMI, and lifestyle (drinking style, type of alcohol, dietary habits, etc.) of the subjects in each study.

In the present study, we examined the relationship between drinking patterns (frequency of alcohol consumption and volume of alcohol in a drinking day) and FL. Consistent alcohol consumption (≥21 days/month) reduced the risk of FL independently (Table 4). In addition, consistent alcohol consumption may reduce the likelihood of obesity (Table 4), possibly contributing to a lowered risk of FL. Thus, consistent alcohol consumption may provide a protective effect on FL development in association with or without obesity. Conigrave et al. [22] reported that light to moderate alcohol consumption was inversely associated with an increased risk of DM in men only when consumed frequently (≥5 days/week). Consumption of alcohol on at least 3–4 days per week was associated with a decreased risk of myocardial infarction in men [23]. Consistent alcohol exposure may contribute to the favorable association with FL seen in the present study, as well as contributing to the favorable association with type 2 diabetes and ischemic heart disease reported in the studies cited above [22, 23], suggesting a common mechanism in these metabolic diseases.

We examined the relationship between alcohol volume in a drinking day and FL. Although the prevalence of FL increased with the increase in the daily volume of alcohol consumption (Fig. 1), no significant association between FL prevalence and the daily volume of alcohol consumption could be identified by logistic regression analysis after adjusting for BMI and other factors related to adiposity (Table 4). On the other hand, an increase in the daily volume of alcohol consumption was associated with an increased risk of obesity (Fig. 1; Table 4). We consider that excessive alcohol consumption in a drinking day may cause an alteration of body composition, most likely due to inadequate drinking and eating lifestyles, such as a prolonged duration of eating and increased calorie intake, probably resulting in the increasing prevalence of FL seen in the present study. These factors may influence the conflicting results reported about the relationship between alcohol consumption and the prevalence of FL.

Recent investigations have elucidated some of the mechanisms by which alcohol alters liver metabolism. Two critical nuclear transcription factors, sterol regulatory element binding protein (SREBP) [24] and peroxisome proliferator activated receptor alpha (PPARα) [25], are altered with alcohol consumption. You et al. [26] reported a role

for AMP activated protein kinase activity in the action of ethanol on the liver. In addition, disturbances in the cytokine network, including alterations in the tumor necrosis factor-α (TNF-α) [27] level, were shown to be involved in ethanol-induced steatosis. These pivotal factors, however, appear to be common in the pathogenesis of both NAFLD [28–30] and AFLD. Therefore, the inverse association between FL and alcohol consumption cannot be explained by these alterations alone.

It has been reported that moderate alcohol intake enhances insulin sensitivity [19, 20], contributing to a lower risk of DM. It was shown that moderate alcohol consumption was associated with a lower prevalence of both nonalcoholic steatohepatitis (NASH) and DM [11]. Sierksma et al. [21] hypothesized that the increase in adiponectin after chronic moderate alcohol consumption would cause an increase in insulin sensitivity in relatively insulin-resistant men. In addition, alcohol consumption also alters apolipoprotein profiles. Elevations in HDL cholesterol levels confer a lower risk of chronic heart disease [16]. Recently, studies examining the pathogenesis of NASH have demonstrated an association of hepatic apolipoprotein synthesis/secretion with the development of steatosis [31, 32]. Our study provides evidence that the risk of FL is decreased across the categories of alcohol consumption, despite an increase in serum triglyceride levels. Alcohol dehydrogenase-mediated ethanol metabolism generates a redox shift in the liver, which stimulates the synthesis of fatty acids. Subsequent removal of these fatty acids may be of benefit to prevent the development of FL.

Although the level of AST is considered to be higher than that of ALT in the majority of alcoholic liver diseases, the AST level was similar to that of ALT in moderate and heavy drinkers in our study (Table 2). In addition, our study demonstrated that liver injury assessed by AST and γ-GTP was positively associated with alcohol consumption regardless of the presence or absence of FL (Table 2). It was reported that the distribution of ratios of AST to ALT (AST/ALT) <1 and >1 was not different between healthy non-drinkers and moderate drinkers of normal weight or with obesity [33]. The ALT level was also similar to the AST level in moderate drinkers of normal weight, and it was observed that the effect of moderate alcohol consumption on liver-derived enzymes, including AST, ALT, and γ-GTP, increased with increasing BMI [33]. These results indicate that the relationships between alcohol consumption, BMI, and different serum liver-derived enzymes in drinkers should be considered. We should also pay attention to the finding that the absence of FL assessed by ultrasonography does not necessary rule out liver injury in drinkers.

There are several limitations in our study. Firstly, we did not enroll women subjects, although 7097 women subjects were investigated, because most of the women did not drink, or they drank 20 g/day at most; the number of subjects who drank more than 20 g/day was only 153 and the number of subjects with FL was 33. Thus, the sample size was insufficient to elucidate the association between alcohol drinking patterns and FL risk in women by the methods used in the present study. Secondly, subjects with other liver diseases, including autoimmune hepatitis and primary biliary cirrhosis, were not excluded. In addition, the association between alcohol consumption and FL prevalence was estimated by multivariate analysis after adjusting for age; BMI; the presence of hypertension, dyslipidemia, or DM; and smoking status. However, it is possible that additional factors for which we did not adjust may have influenced the results. One such factor is adult-onset type II citrullinemia (CTLN2) [34], an inherited metabolic disease caused by a deficiency of mitochondrial aspartate/glutamate carrier protein. Because of an impairment of cytosolic NADH oxidation, CTLN2 patients show both steatohepatitis and alcohol intolerance [35]. Thus, there may have been some differences in metabolic background, such as the capacity for alcohol oxidation or NAD^+/NADH metabolism, across the categories of alcohol consumption that could have affected the prevalence of FL. Thirdly, drinking habits are influenced by a polymorphism in the aldehyde dehydrogenase 2 (*ALDH2*) gene, and this polymorphism may affect our analysis. However, our study did not examine this polymorphism, and the alcohol-flushing response, which can be used to roughly estimate the presence of this polymorphism, was not investigated in the questionnaire. Furthermore, although treatment with an angiotensin II type 1 receptor blocker or peroxisome proliferator-activated receptor agonists is known to alleviate FL, we did not obtain information from the subjects about their use of these medications. Fourthly, the diagnosis of FL was made using abdominal ultrasonography, which defines the presence or absence of fatty steatosis. Diagnosis by ultrasonography may overlook a subset of advanced AFLD or NAFLD, so called "burnt-out steatohepatitis", in which fatty steatosis is reduced. Lastly, although direct interviews were carried out by trained medical staff, self-reported information on alcohol consumption may lead to under- or over-reporting. The influence of the type of alcoholic beverage was also not taken into account in this study. Further studies will be needed to clarify these issues.

In conclusion, this study demonstrates that the major risk factors for FL in Japanese men are factors related to adiposity, not alcohol consumption, and that consistent consumption of alcohol may play a protective role against FL. These results suggest that lifestyle modifications aimed at fighting central obesity and metabolic abnormalities should be the most important recommendations for the management of FL. In addition, it seems unlikely that the

risk of FL can be reduced by the discontinuation and/or reduction of alcohol consumption alone. Further studies are required to better understand FL pathogenesis and management.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

The complement component C3a fragment is a potential biomarker for hepatitis C virus-related hepatocellular carcinoma

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Abstract
Background Hepatocellular carcinoma (HCC) has a high mortality rate, and early detection of HCC improves patient survival. However, the molecular diagnostic markers for early HCC have not been fully elucidated. The aim of this study was to identify novel diagnostic markers for HCC.
Methods Serum protein profiles of 45 hepatitis C virus infection (HCV)-related HCC patients (HCV-HCC) were compared to 42 HCV-related chronic liver disease patients without HCC (HCV-CLD) and 21 healthy volunteers using the ProteinChip SELDI system. One of the identified proteins was evaluated as a diagnostic marker for HCC in patients with HCV.
Results Five protein peaks (4067, 4470, 7564, 7929, and 8130 m/z) had p-values less than 1×10^{-7} and were significantly increased in the sera of HCV-HCC patients compared to HCV-CLD patients and healthy volunteers. Among these proteins, an 8130 m/z peak was the most differentially expressed and identified as the complement component 3a (C3a) fragment. For HCV-HCC and HCV-CLD, the relative intensity of this C3a fragment had the best area under the ROC curve [0.70], followed by des-γ-carboxy prothrombin (DCP) [0.68], lectin-bound alpha fetoprotein (AFP-L3) [0.58] and AFP [0.53] for HCC. A combined analysis of the C3a fragment, AFP and DCP led to a 98% positive identification rate. In addition, the measurable C3a fragment in some HCC patients was not only significantly higher in the year of HCC onset compared to the pre-onset year, but also decreased after treatment.
Conclusions The 8130 m/z C3a fragment is a potential marker for the early detection of HCV-related HCC.

Keywords Hepatocellular carcinoma · Complement component C3a · Serum proteomics · Serum biomarkers · Proteinchip SELDI system · Hepatitis C virus

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Introduction

Hepatocellular carcinoma (HCC) is reportedly the third most frequent cause of global cancer-related deaths, and the incidence of HCC is increasing worldwide [1, 2]. The clearly established risk factor for HCC is chronic hepatitis C virus (HCV) infection [3].

To date, both ultrasonography and serum tumor markers such as the alpha fetoprotein (AFP), and des-γ-carboxy prothrombin (DCP) assay are the principle methods for screening and detecting HCC. Routine screening is the best method to detect early HCC and improve patient survival; however, elevated serum AFP and DCP levels have insufficient sensitivity and specificity, respectively. The sensitivity and specificity of serum elevated AFP levels were reported to range from 39–64% and 76–91%, while those of the serum elevated DCP levels were 41–77% and 72–98%, respectively [4–9]. In addition, it was recently reported that only a small percentage of small HCC tumors were diagnosed based on AFP and DCP [6, 10]. The lens culinaris agglutinin-reactive fraction of AFP (lectin-bound AFP or AFP-L3) has been reported to be elevated in the serum of HCC patients. Although AFP-L3 has a high range of specificity for detecting HCC, the sensitivity is low [11, 12]. The ability to detect early HCC, prior to the onset of clinical symptoms, leads to curative treatment and significantly improves the disease prognosis. Thus, additional biochemical markers are necessary for the specific detection of early HCC.

Serum profiling using a proteomic approach is thought to be a useful technique to detect or predict early HCC in chronic liver disease patients. Studies using the ProteinChip SELDI system, which is a powerful tool to discover new biomarkers, have shown that this method may be successfully used to diagnose HCC. Zinkin et al. [13], Schwegler et al. [14] and our research group [15] previously detected early HCC using the profile of several protein peaks that were identified by the ProteinChip SELDI system. Paradis et al. [16] reported the highest discriminating peak (8900 Da), which was identified as the V10 fragment of vitronectin. Furthermore, Lee et al. [17] described complement 3a, which had a molecular weight of approximately 8900 Da, as a novel marker of HCC. Therefore, using this proteomic approach to identify specific proteins may not only help establish simple methods to detect HCC, but also further our understanding of the molecular mechanisms of hepatocarcinogenesis and facilitate the development of novel cancer therapies. Therefore, this study assessed and compared the protein expression profiles in the sera of HCC patients in order to identify a more useful biomarker of HCC-associated HCV infection using proteomic approach.

Materials and methods

Samples

Eighty-seven patients [45 HCC patients and 42 patients with chronic liver diseases without HCC (CLD)] with HCV infection were selected to participate in this study (Table 1). These patients provided informed consent. Serum samples were collected by the Faculty of Medicine, University of Miyazaki (Miyazaki, Japan), and some patients were in a hyperendemic HCV area with a cohort study in Miyazaki [18]. The sera of all patients with and without HCC, which was confirmed by abdominal ultrasonography or computed tomography, were obtained prior to treatment. All of the sera samples from HCV-infected patients were analyzed in a previous study [15]. In addition, sera from 10 HCV-HCC patients who were diagnosed with HCC within 1 or 2 years and sera from five patients who had received radiofrequency ablation (RFA), percutaneous ethanol injection therapy (PEIT) and/or transarterial chemoembolization (TACE) for HCC were collected through a cohort study in Miyazaki. We also analyzed the sera of 21 healthy volunteers without HCC as controls. After freezing and thawing once, all samples were separated into 50–100 μl aliquots and refrozen at −80°C. The study protocol was approved by the Ethics Committee of the Faculty of Medicine, University of Miyazaki, Kagoshima University Graduate School of Medical and Dental Sciences, and Harvard School of Public Health and Boston University School of Public Health.

Table 1 Patient characteristics

	HCC[a]	CLD[b]	*p* value
Patients (male/female)	45 (40/5)	42 (40/2)	–
Age	73.6 [63–85]	61.8 [41–83]	<0.0001
PLT[c] ($\times 10^4$/ul)	12.5 ± 5.8	8.4 ± 4.6	0.001
Albumin (g/dl)	3.8 ± 0.8	4.2 ± 1.6	0.8
ALT[d] (IU/l)	57.7 ± 28.3	52.8 ± 37.5	0.7
AFP[e] (ng/ml)	311 ± 1144	51.6 ± 36.1 (38)	0.008
DCP[f] (mAU/ml)	235 ± 605 (44)	37.1 ± 59.8 (39)	<0.0001
HA[g] (ng/ml)	388 ± 446 (40)	280 ± 272 (27)	0.6
Diameter of HCC (mm)	23.2 [10–40]	–	–
TNM stage[h] (I/II/III/IV)	24/18/3/0	–	–

Data are shown as the means ± SD or means [range] (numbers)

[a] Hepatocellular carcinoma

[b] Chronic liver disease

[c] Platelet counts

[d] Alanine aminotransferase

[e] Alpha fetoprotein

[f] Des-γ-carboxy prothrombin

[g] Hyaluronic acid

[h] TNM; primary tumor/lymph node/distant metastasis

SELDI-TOF/MS analysis of sera

Expression difference mapping analysis profiles of the samples were obtained using weak cation-exchange (CM10) ProteinChip Arrays (Bio-Rad Laboratories). Arrays were analyzed by ProteinChip reader as previously reported [15]. In addition, the laser intensity ranged from 220 to 245, with a detector sensitivity of 8, and spectra ranging from 1300 to 150000 m/z were selected for analysis in this study.

Separation of candidate biomarker (8.1 k m/z)

The purification strategy was determined by the ProteinChip Arrays. Two hundred microliters of sera from HCV-HCC patients were diluted 5-fold into 50 mM Na-phosphate buffer, pH 7.0, and loaded onto a CM-Ceramic HyperD F spin column (Bio-Rad Laboratories). After equilibrating with the same buffer, the samples were eluted with a stepwise sodium chloride gradient from 0, 200, 300, and 1000 mM. The elution was desalinated and concentrated using a centrifugal concentrator (VIVA-SPIN, Vivascience, Hannover, Germany), and the purification progress was monitored using NP20 arrays. The flow-through fraction was dialyzed and then separated by 16.5% tricine one-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The SDS–PAGE samples were run in tricine sodium dodecyl sulfate buffer according to the manufacturer's instructions and then stained with Coomassie brilliant blue (CBB).

Identification of the candidate biomarker (8.1 k m/z)

Gel pieces containing the target 8.1 k m/z protein were excised. The excised bands were reduced and alkylated for 30 min at room temperature, and then digested with trypsin (Modified Sequence Grade, Roche Diagnostics, Basel, Switzerland) in Tris–HCl, pH 8.0, for 20 h at 35°. The reaction solution was applied to NP20 arrays and allowed to air dry. To identify the protein, the digested peptides were purified by high-performance liquid chromatography (HPLC; MAGIC 2002; Michrom Bioresources Inc., Auburn, CA) and analyzed by Q-Tof2 (Micromass; Waters Ltd., Hertsfordshire, UK). The HPLC solvent consisted of solvent A (2% acetonitlile/0.1% formic acid) and B (90% acetonitlile/0.1% formic acid). The digested peptides were separated with a linear gradient from 10 to 50% solvent B with a flow rate of 400 nl/min using HPLC [19]. Mass spectral data were searched with Mascot (http://www.matrixscience.com) to identify proteins based on the peptide mass [20, 21].

Immunodepletion assay

For immunodepletion, serum samples were prepared as follows. Sera (250 μl) from HCC patients were diluted 5-fold in 50 mM Tris–HCl buffer, pH 8.0, and loaded onto a CM-Sepharose Fast Flow spin column (GE Healthcare Bio-Sciences Corp., NJ). After equilibration with the same buffer, the samples were eluted with a stepwise sodium chloride gradient from 0, 500, and 1000 mM. The elution from each NaCl concentration was monitored using NP20 arrays. To prepare the antibodies for immunodepletion, 6 μl anti-human C3 antibody, which detected C3 and C3a expression, or anti-C4a antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was incubated with 20 μl Interaction Discovery Mapping (IDM) affinity beads (Bio-Rad Laboratories) and Protein A (Sigma Chemical Co, St. Louis, MO) over night at 4° with shaking. These beads were centrifuged, and the supernatant was discarded. The beads were washed with 50 mM phosphate buffer (pH 7.0), and 3 μl of the prepared serum sample was incubated with 15 μl IDM affinity beads with shaking for 2 h at 4°. As a negative control, 3 μl sample was incubated with IDM affinity beads and Protein A with an anti-C4a antibody or without antibody. After the incubation, the samples were cleared by centrifugation, and 5 μl of each supernatant was analyzed on NP20 ProteinChip arrays in a PBS II reader.

Cell culture and SELDI-TOF/MS analysis of culture supernatants

The human hepatocarcinoma cell line HuH-7 and human hepatoblastoma cell line HepG2 were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin G, and 100 mg/ml streptomycin sulfate (Invitrogen, Carlsbad, CA). Before starting the experiments, the cells were cultured on 96-well microplates in medium without FBS for 24 h. After washing with FBS-free media, the cells were cultured for 24 h with FBS-free media with or without 500 μg/ml of C3a (Calbiochem, San Diego, CA). The supernatants were collected by centrifugation and analyzed for the expression of 8.1 k m/z using the ProteinChip system.

Statistical analysis

Values are shown as the means ± SD. Statistical differences, including laboratory data and individual peaks in SELDI TOF/MS, were determined using the Mann–Whitney U test. Values of $p < 0.05$ were considered statistically significant. The discriminatory power for each putative marker was described via receiver operating characteristics

(ROC) area under the curve (AUC). These statistical analyses were performed using STATVIEW 4.5 software (Abacus Concepts, Berkeley, CA), SPSS software (SPSS Inc., Chicago, IL), JMP software, or Ciphergen ProteinChip Software, version 3.0.2.

Results

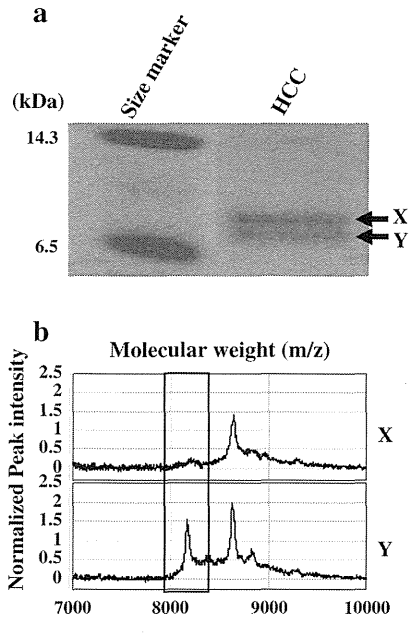
Profiling sera from HCC patients and healthy controls

We analyzed the sera of all patients with HCV-HCC or HCV-CLD and healthy controls without HCC using the CM10 ProteinChip array to identity the most differential protein peak. Peaks were automatically detected using the Ciphergen ProteinChip Software 3.0.2. following baseline subtraction as described previously [15, 22]. This analysis identified 178 protein peak clusters, as seen in the spectrum representations from the three groups (HCV-HCC, HCV-CLD, and healthy control) in the 3000- to 15000-m/z range. Peak expressions were increased for 18 proteins and decreased for 14 proteins in sera from HCV-HCC patients compared to HCV-CLD patients. Compared to healthy subjects, 68 protein peaks were increased, and 16 protein peak intensities were decreased in the sera of HCV-HCC patients. Five protein peaks (4067, 4470, 7564, 7929, and 8130 m/z) had a p-value less than 1×10^{-7} and were significantly increased in the sera of HCC patients compared to the sera of HCV-CLD patients and healthy volunteers. In particular, an 8130 m/z peak was the most significantly different peak and had the most differential expression profile between patients with HCV-HCC and with HCV-CLD.

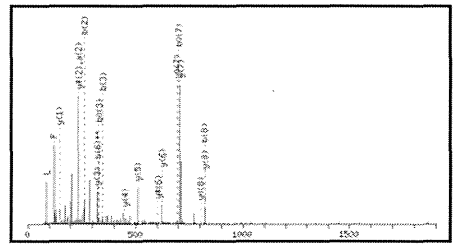
Purification and identification of the 8.1 k m/z peak

We optimized the adsorption and desorption conditions on the arrays using an HCV-HCC patient serum sample and healthy volunteer serum sample in order to determine a procedure to purify the target 8.1 k m/z protein. The optimal pH for retention of the 8.1 k m/z protein was a *pI* value of approximately 7.0 on the CM10 arrays, which indicates that weak cation-exchange sorbents and buffer pH should be fixed for further experiments. The target protein was eluted by increasing the sodium chloride concentrations in a Na-phosphate buffer and was eluted in the 1000 mM sodium chloride fraction. The concentrated serum protein that was eluted with 1000 mM sodium chloride was applied to SDS–PAGE for further separation. The 8.1 k m/z protein was identified and excised by in-gel trypsin digestion for identification. The peptide sequences were analyzed using liquid chromatography (LC)-MS/MS and then examined by a database search with Mascot. The digested peptides matched human complement C3a (Fig. 1).

After reacting the HCC sera with anti-complement C3a or anti-C4 antibodies or without antibody, the supernatants were analyzed by the SELDI ProteinChip system for immunodepletion. Analysis of the supernatant showed that only the 8.1 k m/z peak corresponding to complement C3a



c Complement component 3:
Total score: 70, Peptides matched: 2



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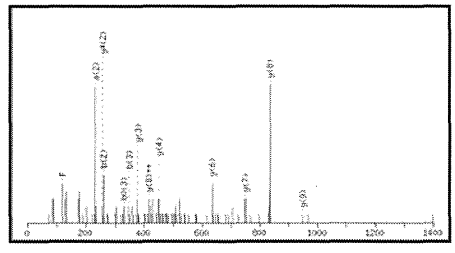

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Fig. 1 **a** Partially purified proteins were separated by SDS–PAGE using serum samples from HCV-HCC patients. The Coomassie-stained SDS-PAGE gel shows two clear bands at approximately 8 kDa (*X* and *Y*). **b** After each band (*X* and *Y*) was excised from the gel, the proteins were extracted and analyzed using the ProteinChip system. The target protein in the excised band was detected, and the 8.1 k m/z peak corresponded only to the "*Y*" band contained in gel. **c** The excised "*Y*" band was alkylated and digested using trypsin. The peptides were collected and subjected to LC–MS/MS analysis. The proteins, which were derived from complement C3a, were identified using a database search

was reduced. On the other hand, immunodepletion with a control anti-C4 antibody or without antibody did not reduce the 8.1 k m/z peak (Fig. 2).

Profiling the C3a of sera from patients with HCC and without HCC

The 8.1 k m/z peak was confirmed as the complement C3a fragment using an immunodepletion assay. However, C3a was stabilized as C3adesArg with a molecular weight of approximately 8.9 k m/z. Figure 3a, b compares the expression of the 8.1 k m/z peak in the sera of HCV-HCC or HCV-CLD patients and healthy controls. The intensities in HCC patient sera were significantly higher than those in the HCV-CLD patients or healthy controls. The expression of the 8.9 k m/z peak in HCV-HCC patients was also higher than that in HCV-CLD patients or healthy controls (Fig. 3c, d). Although the 8.9 k m/z peak was not identified as C3adesArg, it is possible that both the 8.1 and 8.9 k m/z peaks were specific tumor markers for HCC. Furthermore, we analyzed sera from 10 HCV-HCC patients who were diagnosed with HCC within 1 or 2 years and sera from five patients who had received curative treatments using RFA, PEIT, and TACE for HCC. The 8.1 k m/z C3a fragment in the HCV-HCC patients was significantly increased in the year of disease onset compared to the pre-onset year. After treatment, expression of the C3a fragment significantly decreased in all five of the patients who had measurable samples after treatment (Fig. 4a). In contrast, the 8.9 k m/z peak did not change regardless of the occurrence of HCC over time (Fig. 4b). Thus, the 8.1 k m/z C3a fragment appears to be the most discriminatory tumor marker for HCV-HCC.

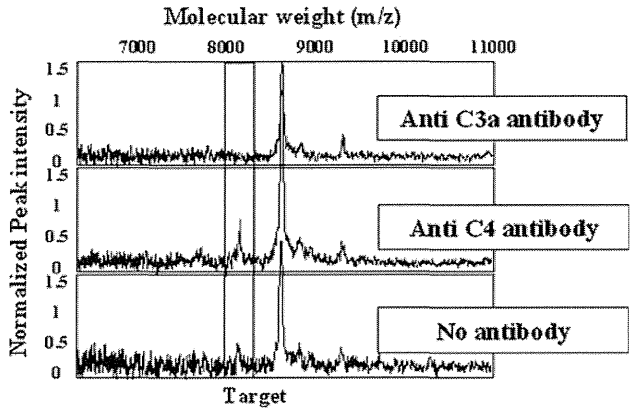

Fig. 2 Immunodepletion assay of the C3a fragment. Analysis of supernatant that had been immunodepleted with an anti-C3a antibody showed that only the 8.1 k m/z peak corresponding to complement C3a was reduced. Supernatants that had been immunodepleted with either a control anti-C4 antibody or without antibody did not have reduced 8.1 k m/z peaks by the ProteinChip system

Relationship between the C3a fragment and other tumor markers

AFP and DCP levels were measured in sera from 83 of 87 patients with HCV-associated liver disease. The recommended cutoff levels for these tumor markers, AFP and DCP, are 20 ng/ml and 40 mAU/ml, respectively. AFP-L3 in 26 patients with HCV-associated liver disease was also investigated among measurable samples in which AFP in a total 35 patients was higher than 20 ng/ml. The cutoff level of AFP-L3 was set at 10%. When samples from patients

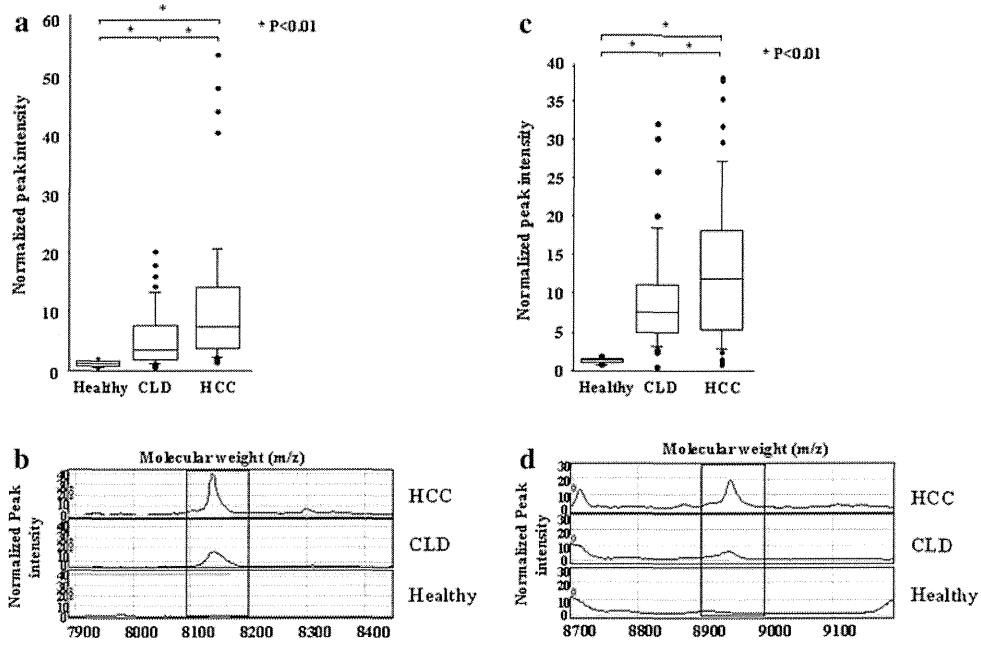

Fig. 3 **a** and **c** Comparisons of the expression profiles of the 8.1 and 8.9 k m/z peaks in HCV-HCC, HCV-CLD, and healthy sera. *Boxes* indicate the median ± 25th percentile. The *lower* and *upper bars* represent the 10th and 90th percentiles, respectively. **b** and **d** Representative spectra of the 8.1 and 8.9 k m/z peaks from patients in each group. The *horizontal axis* indicates the protein molecular weight, while the *vertical axis* designates the relative intensity

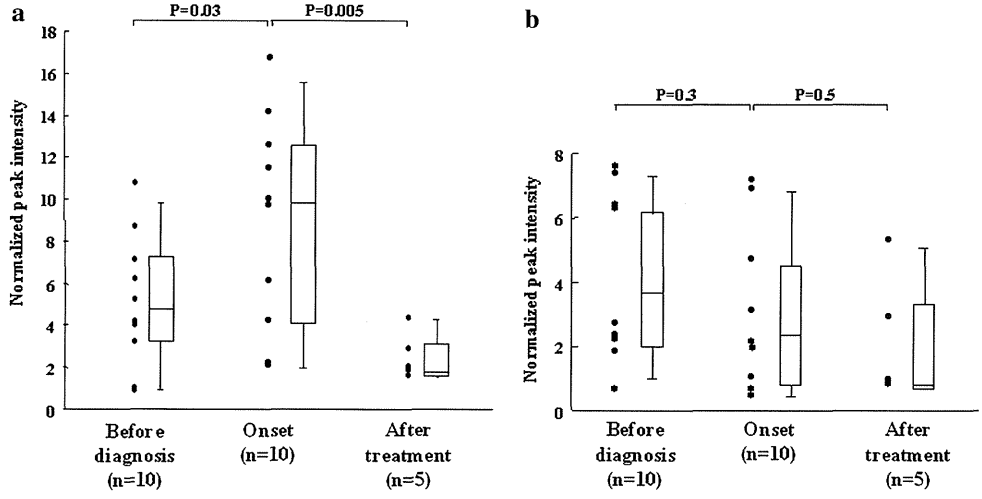

Fig. 4 Comparisons of the expression profiles of the 8.1 k m/z (**a**) and 8.9 k m/z (**b**) peaks in sera from HCV-HCC patients before diagnosis, during disease onset, and after treatment. The samples in the before diagnosis group included sera collected 1 or 2 years before the onset of HCC. *Boxes* indicate the median ± 25th percentile, the *lower bar* indicates the 10th percentile and the *upper bar* indicates the 90th percentile

Table 2 Diagnostic rates for hepatocellular carcinoma in the HCV infected patients

Markers	Sensitivity (%)	Specificity (%)	ROC AUC
AFP[a] (>20 ng/ml)	38 (17/45)	47 (18/38)	0.53
DCP[b] (>40 mAU/ml)	45 (20/44)	74 (29/39)	0.68
AFP-L3[c] (>10%)	58 (8/14)	50 (6/12)	0.58
C3a fragment (>3.5)	78 (37/45)	52 (22/42)	0.70
C3a fragment + AFP	91 (41/45)	26 (10/38)	0.72
C3a fragment + DCP	93 (41/44)	33 (13/39)	0.77
AFP + DCP	64 (28/44)	34 (12/35)	0.70
C3a fragment + AFP + DCP	98 (43/44)	20 (7/35)	0.80

[a] Alpha fetoprotein

[b] Des-γ-carboxy prothrombin

[c] Alpha fetoprotein, lectin lens culinaris agglutin-bound fraction

with HCV-HCC and HCV-CLD without HCC were compared, the sensitivity and specificity of AFP were 38 and 47%, whereas those of DCP were 45 and 74% and those of AFP-L3 were 58 and 50%, respectively. When the cutoff level for the relative intensity of the C3a fragment was set at 3.5, the sensitivity and specificity were 78 and 52%, respectively; the C3a fragment had the most sensitivity for the diagnosis of HCC. Furthermore, the ROC AUC of the C3a fragment, AFP, DCP, and AFP-L3 was 0.70, 0.53, 0.68, and 0.58, respectively (Table 2). There was no relationship between the C3a fragment and several other tumor and inflammation markers [AFP, DCP, AFP-L3, alanine aminotransferase (ALT), and high-sensitivity C-reactive protein (hs-CRP)], and each of these markers was independent of the diameter and number of tumors. The ROC AUC using AFP and DCP was highly similar to the ROC AUC with the C3a fragment alone. In addition, we investigated a combination assay that included the C3a fragment, AFP and DCP. This combination test, in which at least AFP, DCP, or the C3a fragment was positive, had a positive identification rate of 98%, although the specificity of this assay was too low at 20%. The ROC AUC of the combination test using AFP, DCP, and the C3a fragment was higher than those of any other markers. This result indicates that this combination assay using three markers is more useful than the combination assay using AFP ± DCP, which are measured worldwide to detect HCC (Table 2).

Profiling C3a expression in culture medium

C3a reacted with HCC cell lines, and the C3a peak in the culture medium was monitored by the ProteinChip system. The C3a fragment (approximately 8.1 k m/z) was not detected in the supernatants of HuH-7 and HepG2 cell cultures. However, the 8.9 k m/z peak was detected in the culture medium. This 8.9 k m/z peak was considered to be a stabilized form of C3a. This result indicated that the stabilized form of C3a (8.9 k m/z) was not undergoing proteasome-mediated degradation to yield the C3a fragment (8.1 k m/z) in these HCC cell lines.

Discussion

Because the HCC disease-associated mortality rate remains high, it is highly important to develop early diagnostic tools and treatments for HCC. Our study indicates that an 8.1 k m/z peak, which was identified as the C3a fragment by both peptide sequencing and an immunoassay, is up-regulated in the serum of HCC patients, 93% (42/45) of whom were TNM stage I or II. The C3a fragment in some HCC cases was also significantly higher in the year of HCC onset compared to the pre-onset year and decreased after curative treatment. Therefore, the C3a fragment appears to

be a promising simple tumor marker for the diagnosis of early HCC. In addition, a combination serum HCC diagnostic test that included AFP, DCP, and the C3a fragment had higher sensitivity than each individual marker. These results suggest that this combination test may be a useful HCC screening method, although the low specificity may pose challenges. Further examinations are needed to determine whether the C3a fragment or a combination test can be used to detect early HCC.

The results of our study demonstrated that the C3a fragment (8.1 k m/z) is a highly expressed novel tumor marker that is abundant in the sera of early HCC patients but not in the sera of healthy volunteers or HCV-CLD patients. A similar study by Lee et al. [17] used the ProteinChip SELDI system to show that C3a is a potential candidate biomarker for HCV-HCC. However, Lee et al. found that the molecular weight of C3a was represented by an approximately 8.9 k m/z peak. C3a has a very short half-life and is immediately cleaved into the more stable C3adesArg (8.9 k m/z), which is the anaphylatoxin C3a that lacks the C-terminal arginine and is stable state in the serum [23]. In our study, the 8.9 k m/z peak was also significantly different among HCV-HCC patients, HCV-CLD patients, and healthy volunteers (Fig. 3c, d). However, the discriminatory power of the 8.9 k m/z peak (ROC AUC was 0.60) was lower than the 8.1 k m/z peak (ROC AUC was 0.70) to distinguish between HCV-HCC and HCV-CLD. In addition, unlike the 8.1 k m/z peak, the levels of the 8.9 k m/z peak did not significantly increase with time as HCC progressed in 10 HCV-HCC cases (Fig. 4b). In contrast, Li et al. identified two proteins (8926 m/z and 8116 m/z) as complement component C3adesArg and a C-terminal truncated form of C3adesArg; the latter was a C-terminal truncation of C3adesArg that lacked the C-terminal sequence RASHLGLA (referred to as C3adesArgΔ8) in breast cancer patients [24]. However, these two biomarkers cannot be used to discriminate between breast cancers and benign tumors, and there were minimal differences in the peak intensities between breast cancer patients and healthy controls. Therefore, the C3a fragment with a molecular weight of 8.1 k m/z appears to be a potential diagnostic marker for HCC, although we cannot explain why the 8.1 k m/z fragment of C3a is overexpressed in HCC patients and did not confirm whether our C3a fragment (8.1 k m/z) is C3adesArgΔ8.

C3a, including C3adesArg, was also previously identified as a tumor marker for lymphoid malignancies, breast and colorectal cancers using the ProteinChip SELDI system [24–26]. Complement activation and subsequent deposition of complement components on tumor tissues has been demonstrated in cancer patients [27]. Malignant ovarian cells isolated from ascitic fluid samples had C3 activation products deposited on their cell surface [28]. Complement components are important mediators of inflammation and help regulate the immune response. C3a is biologically active and binds to mast cells and basophils, triggering the release of their vasoactive contents [29]. We investigated C3a expression by immunochemical examination of HCC tissues and Western blot analysis of proteins extracted from human HCC cell lines, including HepG2 and HuH-7. However, specific C3a expression, including the C3a fragment (8.1 k m/z), was not detected.

The complement system can be activated after exposure to tumor antigens [30]. It is speculated that small tumors can trigger a systematic reaction. Therefore, elevated C3a (8.9 k m/z) levels in the serum of HCV-HCC patients may reflect both a systematic immune response to HCV infection and non-specific tumor antigens rather than a specific immune response to HCC [24–26, 31]. In contrast, it is possible that overexpression of the C3a fragment (8.1 k m/z) is specific for HCC in addition to non-specific C3 activation.

In contrast to our results, Steel et al. [32] searched for HCC biomarkers using HCC-associated HBV-infected patient sera and found that the C-terminal fragment of complement C3 was down-regulated. Kawakami et al. [33] searched for characteristic alterations in the sera of HBV- and HCV-HCC-infected patients who had undergone curative radiofrequency ablation treatment and showed that C3 was up-regulated after treatment. In these studies, C3 was separated and identified using 2-DE of a mixture of proteins from a small number of patient sera samples, and this process identified various molecular weights for C3. In addition, we analyzed the sera of 25 patients with HCC-associated HBV infections, and the profile of several proteins was different between HCV- and HBV-infected patients. Although 35 protein peaks, including the C3a fragment, were overexpressed in the sera of both HCV-HCC and HBV-HCC patients compared to sera from healthy volunteers, the C3a fragment (8.1 k m/z) was particularly overexpressed in the sera of HCV-HCC patients and was not significantly different between HBV-HCC patients and HCV-CLD patients without HCC (data not shown). The biologic and pathogenic activities of HCV and HBV are different, and the molecular mechanisms underlying the development of hepatitis and hepatocarcinogenesis may differ between HBV and HCV infections [34–36]. Although the number of samples, cause of liver disease, and method of protein identification may affect these results, we speculate that the C3a fragment with a molecular weight of 8.1 k m/z is a candidate tumor marker for HCV-HCC but not HBV-HCC.

AFP, which is a commonly used HCC tumor marker, is elevated not only during HCC, but also during hepatocyte regeneration following liver damage. Previous reports revealed that AFP was abnormally elevated in the sera of patients with acute hepatitis, chronic hepatitis, and liver