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Nonalcoholic steatohepatitis and increased risk of chronic kidney disease

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Abstract

Nonalcoholic fatty liver disease (NAFLD) and chronic kidney disease (CKD) share common features. Both are associated with visceral obesity, type 2 diabetes mellitus, metabolic syndrome, and insulin resistance. However, the relationship between NAFLD and CKD is poorly understood. We examined the prevalence of and risk factors for CKD in patients with NAFLD. We analyzed 174 Japanese patients with liver biopsy–proven NAFLD using a cross-sectional design. Chronic kidney disease was defined as estimated glomerular filtration rate less than 60 mL/min per 1.73 m^2 and/or overt proteinuria. Of 174 NAFLD patients, 92 (53%) exhibited histologic characteristics of nonalcoholic steatohepatitis (NASH), the progressive form of NAFLD; and 82 (47%) had non-NASH NAFLD. Chronic kidney disease was present in 24 (14%) of 174 NAFLD patients. The prevalence of CKD was significantly higher in NASH patients (19 of 92; 21%) than non-NASH patients (5 of 82; 6%). The presence of CKD was associated with a higher body mass index and the presence of hypertension and NASH. Our results demonstrated a high prevalence of CKD among patients with NASH.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is one of the most common causes of chronic liver disease. The incidence of NAFLD continues to increase, and the prevalence of NAFLD ranges from 17% to 33% in the general population of Western countries [1]. The spectrum of NAFLD ranges from a relatively benign accumulation of lipid (simple steatosis) to progressive nonalcoholic steatohepatitis (NASH) associated with fibrosis, necrosis, and inflammation [2-4]. Nonalcoholic steatohepatitis can progress to cirrhosis and hepatocellular carcinoma.

Chronic kidney disease (CKD) encompasses a spectrum of different processes associated with abnormal kidney function and a progressive decline in glomerular filtration rate (GFR). The prevalence of CKD in American adults was estimated to be 11% (19.2 million) [5]. Chronic kidney disease is increasingly recognized as a major risk factor for not only end-stage renal failure but also cardiovascular disease [6,7].

Nonalcoholic fatty liver disease and CKD share some common features, including visceral obesity, type 2 diabetes mellitus, hypertension, and metabolic syndrome [8-11]. Both diseases are also linked to an increased risk of cardiovascular disease [6,7,11]. Common factors underlying the pathogenesis of NAFLD and CKD include insulin resistance, oxidative stress, activation of the renin-angiotensin system, and inappropriate secretion of inflammatory cytokines [12,13]. However, the relationship between NAFLD and CKD is poorly understood; and the prevalence of and risk factors for CKD in patients with NAFLD remain unknown.

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Whereas laboratory test abnormalities and ultrasound or radiographic findings may be suggestive of NAFLD, histologic evaluation remains the only means of accurately assessing the degree of steatosis and the distinct necroinflammatory lesions and fibrosis of NASH; and it remains the only means of distinguishing NASH from simple steatosis [14]. In the present study, we therefore examined the association between liver biopsy–proven NAFLD and CKD using a cross-sectional design; and we investigated the risk factors associated with CKD in patients with NAFLD.

2. Methods

2.1. Patients

The study included a total of 174 Japanese patients with NAFLD who underwent liver biopsy between 2001 and 2009 at the Hospital of Kyoto Prefectural University of Medicine (Kyoto, Japan) and Nara City Hospital (Nara, Japan). The diagnosis of NAFLD was based on a liver biopsy showing steatosis in more than 5% of hepatocytes, along with exclusion of liver diseases of other etiology. Patients had to be older than 18 years. Exclusion criteria were as follows: patients consuming more than 20 g of alcohol per day; positive for hepatitis B virus surface antigen; positive for anti–hepatitis C virus antibody; other types of liver diseases, including primary biliary cirrhosis, autoimmune hepatitis, Wilson disease, or hemochromatosis; treated with drugs known to produce hepatic steatosis, including corticosteroids, high-dose estrogen, methotrexate, or amiodarone within 6 months of enrollment; and a history of gastrointestinal bypass surgery.

The Ethics Committees of the Kyoto Prefectural University of Medicine and Nara City Hospital approved this study. Informed consent was obtained from each patient in accordance with the Declaration of Helsinki.

2.2. Clinical assessment and laboratory tests

Body mass index (BMI) was calculated using the following formula: weight in kilograms/(height in meters)^2. *Obesity* was defined as a BMI of at least 25 according to the criteria of the Japan Society for the Study of Obesity [15]. Diabetes was defined as a fasting plasma glucose concentration of at least 126 mg/dL or a 2-hour plasma glucose concentration of at least 200 mg/dL during an oral glucose (75 g) tolerance test or the use of insulin or oral hypoglycemic agents to control blood glucose [16,17]. Hypertension was defined as a systolic blood pressure of at least 130 mm Hg, a diastolic blood pressure of at least 85 mm Hg, or the use of antihypertensive agents [18]. Dyslipidemia was defined as serum concentrations of triglycerides of at least 150 mg/dL or high-density lipoprotein (HDL) cholesterol less than 40 mg/dL and less than 50 mg/dL for men and women, respectively, or the use of specific medication [18].

Venous blood samples were taken in the morning after a 12-hour overnight fast. The laboratory evaluation included a blood cell count and the measurement of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γ-GTP), albumin, creatinine, total cholesterol, HDL cholesterol, triglyceride, and fasting plasma glucose. These parameters were measured using standard clinical chemistry techniques. Proteinuria was detected by dipstick examination. These clinical and laboratory data were collected at the time of liver biopsy.

Kidney function was estimated using the Japanese equation, which defines the estimated glomerular filtration rate (eGFR) as follows: eGFR = 194 × (serum creatinine$^{-1.094}$) × (age$^{-0.287}$) × 0.739 (if female) [19]. Chronic kidney disease was defined as eGFR less than 60 mL/min per 1.73 m^2 and/or overt proteinuria [20]. Both of these outcome measures had to be confirmed in a least 2 consecutive tests. Stage of CKD was defined according to the criteria proposed by the National Kidney Foundation [21].

2.3. Histopathologic examination

Liver biopsy specimens were obtained percutaneously from all patients for diagnostic purposes. The specimens were fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin, with Masson trichrome, and by silver impregnation. The sections were analyzed by experienced hepatopathologists (TO and YS) who were blinded to the laboratory parameters and clinical data. Patients with biopsy-established NAFLD were categorized as NASH or non-NASH [2,22]. Nonalcoholic steatohepatitis was defined as steatosis with lobular inflammation, hepatocellular ballooning, and Mallory hyaline (Mallory body) or fibrosis [2,14,23,24]. Patients whose liver biopsy specimens showed simple steatosis or steatosis with nonspecific inflammation were identified as having non-NASH NAFLD. The degree of fibrosis in NASH was evaluated and scored according to the criteria proposed by Brunt et al [24].

2.4. Statistical analysis

Results are presented as numbers with percentages in parenthesis for qualitative data or as the medians and ranges for quantitative data. Univariate comparisons were made using a χ^2 test for qualitative factors or a Mann-Whitney U test on ranks for quantitative factors with nonequal variance. Logistic regression analysis was used for multivariate analysis. P values < .05 from 2-sided tests were considered to be significant. Variables that achieved statistical significance on univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors. All statistical analyses were performed using SPSS 15.0 software (SPSS, Chicago, IL).

Table 1
Patient characteristics

Characteristic	Total (n = 174)	NASH (n = 92)	Non-NASH (n = 82)	P
Age (y)	54 (18-78)	62 (24-78)	49 (18-78)	<.001
Sex				.009
Male	102 (59%)	45 (49%)	57 (70%)	
Female	72 (41%)	47 (51%)	25 (30%)	
BMI (kg/m^2)	26.2 (18.6-43.4)	26.5 (19.1-39.4)	25.2 (18.6-43.4)	.02
Obesity	106 (61%)	61 (66%)	45 (55%)	.16
Diabetes	53 (31%)	33 (36%)	20 (24%)	.14
Dyslipidemia	84 (48%)	44 (48%)	40 (49%)	1.00
Hypertension	59 (34%)	39 (42%)	20 (24%)	.02
Platelet count (×10^4/μL)	21.8 (4.6-37.3)	18.9 (4.6-35.1)	24.2 (12.3-37.3)	<.001
AST (IU/L)	49 (10-447)	61 (10-447)	39 (16-151)	<.001
ALT (IU/L)	77 (12-358)	79 (16-316)	75 (12-358)	.14
γ-GTP (IU/L)	73 (19-1681)	76 (19-1681)	69 (19-568)	.59
Albumin (g/dL)	4.6 (2.9-5.5)	4.5 (2.9-5.2)	4.8 (4.0-5.5)	<.001
Fasting glucose (mg/dL)	102 (65-452)	103 (65-452)	99 (76-333)	.17
Total cholesterol (mg/dL)	202 (52-344)	192 (52-288)	217 (99-344)	.003
HDL cholesterol (mg/dL)	47 (25-79)	46 (25-79)	49 (35-77)	.12
Triglyceride (mg/dL)	136 (35-1454)	131 (42-1454)	139 (35-410)	.56
eGFR (mL/[min 1.73 m^2])	82.3 (46.5-161.8)	82.1 (46.5-161.8)	82.7 (53.8-137.9)	.18
Proteinuria	17 (10%)	12 (13%)	5 (6%)	.14
CKD	24 (14%)	19 (21%)	5 (6%)	.007
Stage[a]				.07
1	7 (4%)	6 (7%)	1 (1%)	
2	9 (5%)	5 (5%)	4 (5%)	
3	8 (5%)	8 (9%)	0 (0%)	
4	0 (0%)	0 (0%)	0 (0%)	
5	0 (0%)	0 (0%)	0 (0%)	

Values are median (range) or number (percentage). Where no other unit is specified, values refer to number of patients. All patients were of Japanese ethnicity.
[a] According to Levey et al [20].

3. Results

The characteristics of the 174 NAFLD patients included in the study are summarized in Table 1. Of these 174 NAFLD patients, 92 (53%) exhibited histologic characteristics of NASH; and 82 (47%) had non-NASH NAFLD. Five patients had liver cirrhosis (fibrosis stage 4). Patients with NASH, as compared with patients with non-NASH NAFLD, were significantly older, were more often female, had a higher BMI, more often had hypertension, had a higher AST, and had lower platelet count, albumin, and total cholesterol (Table 1).

Chronic kidney disease was present in 24 patients (14%), including 7 (4%) with stage 1, 9 (5%) with stage 2, and 8 (5%) with stage 3. The prevalence of CKD was significantly higher in patients with NASH (19 of 92; 21%) than in those with non-NASH NAFLD (5 of 82; 6%) (Table 1). Patients with NASH tended to have a more advanced stage of CKD than patients with non-NASH NAFLD, although the difference was not statistically significant (Table 1).

We evaluated the relationship between eGFR values and the histologic severity of NASH (fibrosis stage). The median (range) of eGFR values in NASH patients with fibrosis stage 1 (n = 37), 2 (n = 24), 3 (n = 26), and 4 (n = 5) was 79.4 (46.5-122.8), 82.2 (57.0-161.8), 83.8 (48.4-144.1), and 85.0 (66.5-97.6) mL/min per 1.73 m^2, respectively. The correlation between eGFR values and the fibrosis stage was not significant (P = .47).

Univariate correlations between variables and CKD are shown in Table 2. The presence of CKD was associated with a higher BMI and the presence of hypertension and NASH; but it was not associated with age, sex, the presence of diabetes or dyslipidemia, or levels of AST, ALT, or γ-GTP. Multivariate analysis revealed that the presence of hypertension correlated independently with the presence of CKD (Table 3).

Table 2
Univariate analysis of factors associated with CKD in NAFLD patients

Factor	No CKD (n = 150)	CKD (n = 24)	P
Age (y)	55 (18-78)	54 (31-78)	.19
Male	89 (59%)	13 (54%)	.66
BMI (kg/m^2)	25.6 (18.6-43.4)	28.3 (21.1-35.1)	.003
Diabetes	42 (28%)	11 (46%)	.10
Dyslipidemia	72 (48%)	12 (50%)	1.00
Hypertension	43 (29%)	16 (67%)	.001
AST (IU/L)	50 (10-210)	45 (21-447)	.82
ALT (IU/L)	78 (12-358)	65 (18-254)	.45
γ-GTP (IU/L)	77 (19-568)	72 (29-1681)	.87
NASH	73 (49%)	19 (79%)	.007

Values are median (range) or number (percentage). Where no other unit is specified, values refer to number of patients.

Table 3
Multivariate analysis of factors independently associated with CKD in NAFLD patients

Factor	Odds ratio	95% Confidence interval	P
BMI (kg/m^2)	1.09	0.98-1.21	.11
Hypertension	3.90	1.42-10.71	.008
NASH	2.46	0.82-7.42	.11

Data are from a total of 174 patients.

4. Discussion

Our results demonstrated a high prevalence (21%) of CKD among patients with NASH. The prevalence of CKD was significantly higher in NASH than in non-NASH NAFLD.

It is important to identify factors that increase the risk for CKD, even in individuals with normal GFR. In general, known risk factors include hypertension, diabetes, autoimmune disease, older age, African ancestry, a family history of renal disease, a previous episode of acute renal failure, or structural abnormalities of the urinary tract [25]. Our results showed that the risk factors associated with CKD in NAFLD patients include obesity (higher BMI), hypertension, and NASH. In particular, hypertension was an independent risk factor for CKD.

Nonalcoholic fatty liver disease is closely associated with obesity, hypertension, dyslipidemia, and type 2 diabetes mellitus, which are all features of the metabolic syndrome. This strongly supports the idea that NAFLD is the hepatic manifestation of the metabolic syndrome [8]. The presence of insulin resistance is recognized as the pathophysiologic hallmark of NAFLD. A recent study show that NAFLD is more prevalent in nondipper hypertensive patients than dipper hypertensive patients, and a high prevalence of NAFLD is associated with insulin resistance and low adiponectin in the nondippers [26]. Similarly, growing evidence suggests that the metabolic syndrome is an important factor in the pathogenesis of CKD [27]; and there is a positive relationship between insulin resistance and CKD [10].

Relatively few studies have evaluated NAFLD and the risk of CKD. Recent studies found that NAFLD is associated with an increasing incidence of CKD in type 2 diabetes mellitus patients [28] and in nonhypertensive and nondiabetic Korean men [29]. Although these findings are important, their interpretation is limited by the fact that the diagnosis of NAFLD was based on liver ultrasound imaging. Whereas ultrasound is the commonly used for diagnosing NAFLD in clinical practice, it cannot distinguish NASH from simple steatosis. Histologic evaluation remains the only means of diagnosing NASH. To our knowledge, our current study is the first to assess the association between NASH and CKD.

A recent report showed a positive relationship between microalbuminuria and liver fibrosis in nondiabetic patients with NAFLD [30]. In this study, however, we did not find an association between eGFR values and the degree of liver fibrosis in NASH patients. The association of liver fibrosis with low eGFR or proteinuria remains to be verified in future prospective studies using a larger number of samples.

The underlying mechanisms by which NASH increases the risk for CKD remain to be elucidated. It may simply reflect the coexistence of underlying known risk factors. Alternatively, NASH may be a stimulus for further increases in whole-body insulin resistance, leading to the development of CKD. Another possible underlying mechanism is increased oxidative stress and chronic subclinical inflammation. The possible mediators linking NASH and CKD include reactive oxygen species, tumor necrosis factor–α, and other proinflammatory cytokines [11,31].

Certain limitations should be considered in the interpretation of our findings. First, the cross-sectional study design hinders the ability to draw inferences regarding causality between NASH and CKD. Second, an eGFR was used rather than more precise measures to identify and classify kidney disease. However, equations that estimate GFR for the evaluation of renal function are recommended for epidemiological studies and for clinical practice [20]. Third, the dipstick urinalysis has a lower sensitivity and specificity in the diagnosis of proteinuria than 24-hour urine collection or measurement of the albumin-to-creatinine ratio in a random spot collection. Nevertheless, in most cases, screening with urine dipsticks is considered acceptable for detecting proteinuria [21]. Fourth, this was a hospital-based study and therefore may be influenced by selection bias. Finally, the study did not include a control group of nonsteatotic subjects.

In summary, this study shows that it is important to assess the risk of CKD in NASH/NAFLD patients. In addition, our findings suggest that preventing and treating obesity, hypertension, and NASH may help prevent NAFLD patients from developing CKD. Moreover, our results suggest that a higher BMI and the presence of hypertension and NASH are associated with an increasing prevalence of CKD in NALFD patients. Further prospective studies are warranted to establish the causal relationship between NASH and CKD.

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The fatty acid composition of plasma cholesteryl esters and estimated desaturase activities in patients with nonalcoholic fatty liver disease and the effect of long-term ezetimibe therapy on these levels

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ABSTRACT

Background: The aim of this study was to investigate the relationship between fatty acid composition of plasma cholesteryl esters (CEs) and estimated desaturase activity and the development and progression of nonalcoholic fatty liver disease (NAFLD). The study also assessed the effect of ezetimibe on CE levels.
Methods: Plasma CEs fatty acid composition was analyzed in 3 groups: patients with a NAFLD activity score (NAS) ≤ 4 (n = 31) or NAS ≥ 5 (n = 32) and normal controls (n = 25). The estimated desaturase activities were calculated using ratios of 16:1n−7/16:0 (D9-16D), 18:1n−9/18:0 (D9-18D), 18:3n−6/18:2n−6 (D6D) and 20:4n−6/20:3n−6 (D5D).
Results: Compared with controls, the levels of palmitate, palmitoleate, γ-linoleate, D9-16D and D6D were significantly increased, whereas levels of linoleate and D5D were significantly decreased. Patients with NAS ≥ 5 had significantly higher palmitate levels than patients with NAS ≤ 4. The levels of these fatty acids, especially palmitate and palmitoleate, correlated with NAFLD-related lipid, metabolic, and inflammatory parameters. Long-term therapy with ezetimibe caused significant improvements in the levels of these fatty acids, estimated desaturase activity index and NAFLD-related parameters.
Conclusions: Our results suggest that fatty acids and desaturase activity associate with the development and progression of NAFLD, and that ezetimibe may be a novel treatment for this disorder.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is one of the most common causes of chronic liver injury in the world [1–3]. NAFLD is a metabolic condition which encompasses a wide spectrum of liver disease, ranging from simple steatosis to non-alcoholic steatohepatitis (NASH). Although the intricacies of the molecular and cellular mechanisms responsible for progression from simple steatosis to NASH have not been fully elucidated, hyperlipidemia, insulin resistance and oxidative stress are known to be major contributing factors in the initiation and progression of NAFLD [4–6]. It has been proposed that steatosis, the earliest and most prevalent stage of NAFLD, often referred to as the "first hit", increases the vulnerability of the liver to a "second hit" that in turn lead to the inflammation, fibrosis and cellular death characteristic of NASH.

Excessive accumulation of lipid substrates in the liver has serious adverse effects on cell functions and is termed lipotoxicity [7]. Studies of lipid accumulation in tissue have usually involved measuring triglycerides (TG) content, although recent studies have shown clearly that the deleterious effects are due not only to TG accumulation but also to other lipid metabolites such as palmitate, diacylglycerols (DAG) and ceramide [7–11]. Recent studies have shown NAFLD is also characterized by increased DAG, free cholesterol, decreased phosphatidylcholine (PC), and altered n−3 and n−6

Abbreviations: CEs, Cholesteryl esters; DAG, Diacylglycerols; HOMA-R, Homeostasis model assessment of insulin resistance; IRI, Immunoreactive insulin; NAS, NAFLD activity score; emIDL, Electronegative charge modified-LDL; NAFLD, Nonalcoholic fatty liver disease; NASH, Non-alcoholic steatohepatitis; oxLDL, Oxidized LDL; PC, Phosphatidylcholine; PUFA, Polyunsaturated fatty acid.

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polyunsaturated fatty acid (PUFA) metabolism [12,13]. However, it is not yet established whether these changes are reflected by circulating lipidome levels and also whether or not NASH is associated with a distinct lipidomic profile. A change in the proportions of fatty acids in the diet is reflected mainly by serum triglyceride levels within the first hours. On the other hand, the fatty acids composition of serum cholesterol esters (CEs) is related to the average dietary fatty acid composition during the last 3 to 6 weeks and also reflects endogenous fatty acid metabolism [14]. Fatty acid composition is used as an indicator of disease risk, because its alteration has been related to metabolic disease and cardiovascular disease [15,16]. Low concentrations of linoleic acid (18:2n−6) and high concentrations of palmitic (16:0), palmitoleic (16:1n−7) and dihomo-g-linolenic (20:3n−6) acids in plasma lipid esters have been reported to be associated with metabolic syndrome [17,18]. However, to our knowledge, only two studies have assessed the relationship between plasma fatty acid composition between histopathologically-proven NAFLD/NASH [19,20]. Desaturases are involved in the endogenous synthesis of PUFAs. The delta 9, 6, and 5 desaturases (D9D, D6D and D5D) introduce a double bond at specific position on long-chain fatty acids. D9D synthesizes monounsaturated fatty acids (MUFA), palmitoleic (16:1n−7) and oleic acids from palmitic (16:0) and stearic (18:0) acids, respectively. D5D and D6D catalyze the synthesis of long-chain n−6 and n−3 PUFAs. In human studies, the estimated desaturase activities are generally used, since it is not possible to directly measure desaturase activities in human. Therefore, the estimated desaturase activities of D9D, D6D and D5D calculated by the plasma ratio of 16:1n−7/16:0, 18:3n−6/18:2n−6 and 20:4n−6/20:3n−6, respectively, can be used as surrogates of the measure of the true desaturase activity [14].

The aim of this study was to assess whether the levels of fatty acid components of plasma CEs and their estimated desaturase activities were associated with the development and progression of NAFLD. We also investigated the effect of long-term ezetimibe, a cholesterol absorption inhibitor, on CE levels, as it has been reported that this drug causes significant reduction in the absorption of several saturated fatty acids in diet-induced obese and diabetic mice [21].

2. Patients and methods

2.1. Patients

The study protocol was approved by the ethics committee of Saiseikai Suita Hospital and the Kyoto Prefectural University of Medicine. Informed consent was obtained from all subjects prior to enrollment in the study. A total of 63 patients at Saiseikai Suita Hospital and Kyoto Prefectural University Hospital who had been diagnosed histologically with NAFLD between 2007 and 2009 were evaluated in the study.

All liver biopsy specimens were examined by 2 experienced pathologists blinded to the patients' clinical and laboratory data and liver biopsy sequence. In this study, the NAFLD activity score (NAS) system was used to classify NAFLD into 2 groups; NAS ≤4 (n=31, "simple steatosis" and "borderline NASH") and NAS ≥5 (n=32, "definite NASH"). The NAS system was reported as a reliable scoring system for diagnosing NASH by Kleiner et al. [22]. Prior to evaluation of liver histology we excluded patients with an alcohol intake exceeding 20 g/day and those who reported signs, symptoms and/or history of known liver disease including viral, genetic, autoimmune, and drug-induced liver disease, and previous use of anti-diabetic medication including insulin-sensitizing agents such as metformin and pioglitazone.

After enrollment, all the patients were asked to adhere to a dietary plan tailored to their energy requirements and metabolic control. The dietary plans were formulated by a registered dietitian and/or medical doctor using the current Japan Diabetes Society recommendations (JDSR) and were maintained throughout the study. Blood samples were obtained in the morning after an overnight fasting. Blood samples were also obtained from 25 age- and sex-matched normal control subjects who were on the clinical staff at our hospitals. To investigate the efficacy of long-term ezetimibe therapy on CE levels, all patients received ezetimibe (10 mg/day) for 24 months. Fatty acid composition in plasma CEs and liver histological examination was determined before and after the 24-month ezetimibe therapy.

2.2. Analysis of fatty acid composition in plasma CEs

Blood was drawn into tubes containing ethylenediaminetetraacetic acid disodium salt. Plasma was separated by centrifugation of the samples at 1600×*g* for 15 min at 4 °C and then stored at −80 °C until assayed. Total lipid was extracted from plasma by using the method of Bligh and Dyer [23]. CEs were separated by thin-layer chromatography on silica gel plates (Silica Gel 60, Merck, Darmstadt, Germany) using a solvent system of petroleum ether:ethyl ether:acetic acid (80:20:1, v/v/v). The spot corresponding to the CEs was scraped from the plate and transmethylated with 2 ml of acetyl chloride:methanol (5:50, v/v) at 90 °C for 2 h. Heptadecanoic acid (17:0) was used as an internal standard. Fatty acid methyl esters were quantified using a model GC14A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a 25-m×0.5-mm capillary column (HR-SS-10, Shinwa Chemical Industries, Ltd., Kyoto, Japan).

2.3. Estimation of desaturase activity

Desaturase and elongase activities were estimated as the ratio product of individual precursor fatty acids in plasma CEs according to the following criteria: D9-16D = 16:1n−7/16:1, D9-18D = 18:1n−9/18:1, D6D = 18:3n−6/18:2n−6 and D5D = 20:4n−6/20:3n−6 [14].

2.4. Other laboratory investigations

Plasma glucose (PG) was measured by the glucose oxidase method and HbA1c determined by high performance liquid chromatography (HPLC: Arkray Inc., Kyoto, Japan). Plasma insulin (immunoreactive insulin: IRI) concentrations were measured by an immunoradiometric assay (Insulin-RIAbead II, Abbott, Japan). The homeostasis model assessment of insulin resistance (HOMA-R) was calculated from fasting insulin and plasma glucose levels by the following equation: HOMA-R = fasting IRI (μU/ml)×fasting PG (mg/dl)/405. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol (T-Ch), high-density lipoprotein cholesterol (HDL-Ch), low-density lipoprotein cholesterol (LDL-Ch) and triglyceride (TG) were measured by enzymatic methods using a chemical autoanalyzer (Hitachi Co., Tokyo, Japan). Serum type IV collagen 7S was measured by a radioimmunoassay kit (Mitsubishi Chemical Group, Tokyo, Japan), serum high-sensitivity C-reactive protein (hs-CRP) was measured by latex particle-enhanced nephelometry (Dade Behring, Tokyo, Japan) and serum oxidized LDL (oxLDL) was measured by an enzyme-linked immunoassay (ELISA) kit (Kyowa Medex Co., Ltd., Tokyo, Japan).

Net electronegative charge modified-LDL (emlDL) was analyzed using an agarose gel electrophoresis lipoprotein fraction system, according to the manufacturer's instructions (Chol/Trig Combo System™; Helena Labs, Saitama, Japan). The percentage frequency of emlDL was calculated on a computer using the migration distance (b) of the LDL fraction in the test samples and the migration distance (a) of normal control sera according to the following formula: emlDL density = [b − a/a]×100% [24]. Production of LDL with an increased net electronegative charge, caused by modification of lysine residues by either acetylation, carbamylation, glycation, glycoxidation or oxidation all result in increased uptake of the lipids by macrophages via the scavenger receptor system [25–27]. This uptake is thought to

be a key process in the formation of foam cells, the hallmark of early atherosclerotic lesions.

2.5. Statistical analysis

All statistical analyses were performed using Statview version 5.0 (Abacus Concepts, Berkeley, CA), with data expressed as the means ± SD. When the data were not normally distributed, logarithmical transformation was performed. Differences between the groups were determined by one-way ANOVA with Scheffé's multiple comparison test. The nonparametric Wilcoxon signed rank test was used to compare data of pre- and post-ezetimibe treatment. The risk of progression of NAFLD was estimated by logistic regression analysis with the standardized (SD = 1.0) odds ratio (OR) and 95% confidence interval (CI) being calculated. Spearman's correlation coefficients were used to evaluate the relationship between NAFD-related metabolic risk factors and plasma CEs fatty acids and index of estimated desaturase activity. A P-value <0.05 was considered statistically significant.

3. Results

3.1. Subject characteristics

The baseline clinical and laboratory characteristics of the normal controls and two patient groups classified according to the NAS scores are shown in Table 1. Compared with normal controls, both patient groups (NAS ≤4 and NAS ≥5) had significantly higher values of body mass index (BMI), waist circumference (WC), visceral fat area (VFA), systolic blood pressure (sBP), diastolic blood pressure (dBP), HbA1c, PG, IRI, HOMA-R, AST, ALT, TG, T-Ch, LDL-Ch, oxLDL, emlDL, type IV collagen 7S, leptin and hsCRP, and significantly lower HDL-Ch and adiponectin levels. As expected, a significant proportion of NAFLD subjects had features of the metabolic syndrome. The patients with a NAS ≥5 had significantly higher serum emlDL and type IV collagen 7S levels than patients with NAS ≤4 (both P<0.05). There was no significant difference in any other clinical or laboratory parameter between the two patient groups.

3.2. Fatty acid composition in plasma CEs

The fatty acid composition of the plasma CEs and their estimated desaturase activity was shown in Table 2. In both patient groups, the levels of palmitic acid (C16:0), palmitoleic acid (16:1n−7) and γ-linoleic (C18:3n−6) acid were significantly higher, and linoleic acid (C18:2n−6) was significantly lower, compared to controls. Oleic (18:1n−9) and dihomo-γ-linoleic acid (C20:3n−6) levels in patients with a NAS ≤4 were significantly higher than in control subjects. In contrast, the levels of arachidoric (C20:4n−6), eicosapentaenoic (C20:5n−3) and docosahexaenoic (C22:6n−3) acids showed no significant differences between the groups. The levels of oleic and dihomo-γ-linoleic acid in the patients with NAS ≥5 were also higher than in control subjects, although this difference was not statistically significant. The levels of palmitic acid in patients with a NAS ≥5 were significantly higher in patients with a NAS ≤4 (P<0.05). No other significant difference in fatty acid composition of plasma CEs was measured between the two groups. Logistic regression analysis showed that high palmitic acid levels were a risk factor for the development (OR = 1.980, 95% CI = 1.109–3.536, P = 0.0209) and progression (OR = 2.040, 95% CI = 1.180–3.526, P = 0.0107) of NAFLD. Both patient groups had significantly higher levels of D9-16D and D6D activities, and significantly lower levels of D5D activity compared to controls. There was no significant difference in D9-16D, D9-18D, D6D and D5D activity between patients with a NAS ≤4 or NAS ≥5 (Table 3).

3.3. Relationship between NAFLD-related metabolic factors and CE fatty acid composition and estimated desaturase activity

As shown in Table 4, there were significant and positive associations between palmitic acid and ALT, TG, T-Ch, emlDL and hsCRP, while palmitoleic acid showed positive associations with BMI, VFA, IRI, ALT,

Table 1
Baseline clinical and laboratory characteristics of the healthy control subjects and two patient groups classified according to NAS scores.

	Controls (n = 25)	NAS ≤4 (n = 31)	NAS ≥5 (n = 32)
Male/female	14/11	16/15	18/14
Age (y)	52.2 ± 9.2	53.3 ± 10.4	54.5 ± 11.1
Body mass index (kg/m²)	22.6 ± 2.8	27.5 ± 3.1*	27.3 ± 3.4*
Waist circumference (cm)	80.1 ± 3.7	93.6 ± 5.8*	92.1 ± 5.9*
Visceral fat area (cm²)	96.7 ± 34.6	158.5 ± 41.5*	152.9 ± 40.2*
Systolic blood pressure (mm Hg)	118 ± 14	137 ± 8*	140 ± 18*
Diastolic blood pressure (mm Hg)	77 ± 9	88 ± 11*	89 ± 12*
HbA1c (%)	4.9 ± 0.3	6.4 ± 0.7*	6.3 ± 0.8*
Fasting glucose (mg/dl)	93 ± 32	125 ± 31*	121 ± 34*
Fasting insulin (μU/ml)	4.8 ± 1.6	11.9 ± 4.7*	13.3 ± 7.6*
HOMA-R	1.1 ± 08	3.7 ± 2.1*	3.9 ± 2.7*
Aspartate aminotransferase (U/l)	24 ± 9	38 ± 16*	40 ± 22*
Alanine aminotransferase (U/l)	25 ± 11	49 ± 23*	58 ± 25*
Triglyceride (mg/dl)	96 ± 56	166 ± 85*	160 ± 94*
Total cholesterol (mg/dl)	188 ± 33	218 ± 36*	216 ± 34*
HDL cholesterol (mg/dl)	61 ± 22	51 ± 7*	48 ± 12*
LDL cholesterol (mg/dl)	110 ± 25	134 ± 31*	131 ± 32*
Oxidized LDL (U/ml)	8.7 ± 3.0	13.8 ± 2.5*	14.1 ± 2.9*
Electronegative charge modified-LDL (ecd)	1.2 ± 3.0	3.4 ± 3.2*	6.4 ± 3.5*, #
Type IV collagen 7S (ng/dl)	3.8 ± 0.7	4.1 ± 1.6	5.2 ± 1.9*, #
Adiponectin (mg/ml)	8.5 ± 3.6	5.4 ± 2.9*	5.7 ± 3.1*
Leptin (ng/ml)	2.4 ± 1.1	4.1 ± 3.0*	3.6 ± 2.7*
High-sensitivity CRP (ng/ml)	388 ± 244	585 ± 377*	788 ± 457*
75 g OGTT (NGT/IGT/DM)	25/0/0	7/17/7*	7/15/10*

Data are expressed as the mean ± standard deviation.
ecd, electronegative-charge density; OGTT, oral glucose tolerance test; NGT, normal glucose tolerance; GT, impaired glucose tolerance; DM, diabetes mellitus.
* P<0.05 vs. controls.
# P<0.05 vs. NAS ≤4.

Table 2
Plasma cholesterol esters fatty acid composition and estimated desaturase activities in the control subjects and two patient groups classified according to NAS scores.

Fatty acids (% of total fatty acids)	Controls (n = 25)	NAS ≤4 (n = 31)	NAS ≥5 (n = 32)
C14:0 (miristic acid)	0.55 ± 0.14	0.57 ± 0.11	0.57 ± 0.23
C16:0 (palmitic acid)	11.14 ± 1.05	11.83 ± 0.99*	12.55 ± 1.03*, #
C16:1n−7 (palmitoleic acid)	2.38 ± 0.77	3.24 ± 1.00**	3.13 ± 1.02*
C18:0 (stearic acid)	1.04 ± 0.25	1.09 ± 0.29	1.09 ± 0.30
C18:1n−9 (oleic acid)	17.82 ± 2.60	19.61 ± 2.53*	19.20 ± 2.41
C18:2n−6 (linoleic acid)	53.20 ± 4.64	48.08 ± 4.41*	48.67 ± 4.32*
C18:3n−6 (γ-linoleic acid)	0.59 ± 0.18	0.85 ± 0.25**	0.82 ± 0.24**
C18:3n−3 (α-linoleic acid)	0.77 ± 0.38	0.76 ± 0.32	0.77 ± 0.36
C20:3n−6 (dihomo-γ-linoleic acid)	0.63 ± 0.18	0.79 ± 0.22*	0.75 ± 0.23
C20:4n−6 (arachidonic acid)	5.54 ± 1.24	5.71 ± 0.95	5.65 ± 1.18
C20:5n−3 (eicosapentaenoic acid)	2.19 ± 0.69	2.15 ± 0.57	2.13 ± 0.72
C22:6n−3 (docosahexaenoic acid)	0.90 ± 0.27	0.86 ± 0.31	0.84 ± 0.30
Estimated desaturase index			
D9-16D (16:1n−7/16:0)	0.21 ± 0.07	0.28 ± 0.09*	0.26 ± 0.08*
D9-18D (18:1n−9/18:0)	17.13 ± 4.13	17.99 ± 4.06	17.77 ± 4.01
D6D (18:3n−6/18:2n−6)	0.011 ± 0.007	0.019 ± 0.011**	0.017 ± 0.009**
D5D (20:4n−6/20:3n−6)	9.05 ± 2.60	7.22 ± 2.45*	7.48 ± 2.38*

Data are expressed as the mean ± standard deviation.
* P<0.05 vs. controls.
** P<0.01 vs. controls.
# P<0.05 vs. NAS ≤4.

Table 3
Spearmen's correlation between 17 clinical variables and fatty acid composition of plasma cholesterol esters nonalcoholic fatty liver disease (n = 63).

	C16:0	C16:1 n−7	C18:1 n−9	C18:2 n−6	C18:3 n−6	C20:3 n−6	D9-16D[a]	D6D[b]	D5D[c]
Body mass index (kg/m^2)	NS	0.257*	NS	−0.252*	NS	0.275*	0.252*	0.252*	−0.318**
Waist circumference (cm)	NS	NS	NS	NS	NS	NS	0.260*	NS	−0.266*
Visceral fat area (cm^2)	NS	0.255*	NS	NS	NS	NS	0.264*	0.260*	−0.263*
Fasting glucose (mg/dl)	NS	NS	NS	NS	NS	NS	NS	NS	NS
Fasting insulin (μU/ml)	NS	0.261*	NS	NS	NS	NS	NS	0.263*	−0.277**
Aspartate aminotransferase (U/l)	NS	NS	NS	NS	NS	NS	NS	NS	NS
Alanine aminotransferase (U/l)	0.259*	0.258*	NS	NS	NS	NS	NS	NS	NS
Triglyceride (mg/dl)	0.253*	0.305**	NS	−0.251*	0.288**	0.265*	0.313**	0.281**	−0.260*
Total cholesterol (mg/dl)	0.252*	0.260*	NS	NS	NS	NS	0.259*	0.251*	−0.255*
HDL cholesterol (mg/dl)	NS	−0.254*	NS	NS	NS	NS	NS	NS	0.271*
LDL cholesterol (mg/dl)	NS	NS	NS	NS	NS	NS	NS	NS	−0.260*
Oxidized LDL (U/ml)	NS	NS	NS	NS	NS	NS	NS	NS	NS
Electronegative charge modified-LDL (ecd)	0.266*	NS	NS	−0.254*	NS	NS	NS	NS	−0.270*
Type IV collagen 7S (ng/dl)	NS	NS	NS	NS	NS	NS	NS	NS	NS
Adiponectin (mg/ml)	NS	NS	NS	NS	NS	NS	NS	NS	0.262*
Leptin (ng/l)	NS	NS	NS	NS	NS	NS	NS	NS	NS
High-sensitivity CRP (ng/ml)	0.255*	NS	NS	NS	NS	NS	NS	NS	NS

* $P<0.05$.
** $P<0.01$.
[a] D9-16D = 16:1n-7/16:1.
[b] D6 = 18:3n-6/18:2n-6.
[c] D5D = 20:4n-6/20:3n-6.

TG, and inverse associations with HDL-Ch. Linoleic acid was significantly and inversely associated with BMI, TG and emlDL. Significant and positive associations were also observed between γ-linoleic acid and TG, dihomo-γ-linoleic acid and BMI and TG, between D9-16D and BMI, WC, VFA, TG and T-Ch, and between D6D and BMI, VFA, TG and T-Ch. D5D showed significant inverse associations with BMI, WC, VFA, IRI, TG, T-Ch, LDL-Ch and emlDL, and positive associations with HDL-Ch and adiponectin.

3.4. Effect of long-term ezetimibe therapy on CE fatty acid composition, estimated desaturase activity and histological changes

We next investigate the effect of long-term ezetimibe therapy on CE fatty acid composition and estimated desaturase activity. As shown in Fig. 1, a significant decrease was observed in miristic acid (−3.9%, $P<0.05$), palmitic acid (−7.0%, $P<0.01$), palmitoleic acid (−10.3%, $P<0.01$), oleic acid (−4.1%, $P<0.05$), dihomo-γ-linoleic acid (−6.1%, $P<0.01$) and D9-16D activity (−9.8%, $P<0.01$), associated with a significant increase in linoleic acid (9.2%, $P<0.01$) and D5D activity (+10.0%, $P<0.01$). Long-term ezetimibe therapy also caused significant decreases in the levels of VFA (−3.5%, $P<0.05$), ALT (−4.8%, $P<0.05$), TG (−4.1%, $P<0.05$), T-Ch (−10.5%, $P<0.01$), LDL-Ch (−11.5%, $P<0.01$), emlDL (−20.5%, $P<0.01$), and hsCRP (−4.5%, $P<0.05$). Follow-up liver biopsies were performed on 33 patients at the end of the 24 months of EZ treatment. Table 4 shows the histologic changes before and after treatment. The mean level of steatosis grade (from 2.3 ± 0.7 to 1.9 ± 0.8, $P=0.0003$), necroinflammatory grade (from 1.9 ± 0.7 to 1.8 ± 0.7, $P=0.0456$), ballooning score (from 1.4 ± 0.5 to 1.3 ± 0.5, $P=0.0253$) and NAS score (from 5.6 ± 1.6 to 5.1 ± 1.8, $P=0.0007$) improved significantly during the study. Of 33 patients, 24 had a one or more point improvement in the NAS score, 8 had no change and one had a one-point increase. In contrast, the mean level of fibrosis stage level did not change significantly (from 2.0 ± 0.8 to 2.1 ± 0.9, P = NS).

Table 4
Histological changes in 33 patients with NAFLD.

	Baseline	After treatment	*P*-value
Steatosis grade	2.3 ± 0.7	1.9 ± 0.8	$P=0.0003$
0	0 (0)	1 (3)	
1	5 (16)	9 (31)	
2	14 (44)	16 (44)	
3	14 (41)	7 (22)	
Necroinflammatory grade	1.9 ± 0.7	1.8 ± 0.7	$P=0.0456$
1	10 (30)	12 (36)	
2	16 (48)	16 (45)	
3	7 (21)	5 (18)	
Fibrosis stage	2.0 ± 0.8	2.1 ± 0.9	$P=0.6547$
0	1 (3)	1 (33)	
1	6 (18)	8 (24)	
2	17 (52)	12 (42)	
3	9 (27)	12 (30)	
4	0 (0)	0 (0)	
Ballooning score	1.4 ± 0.5	1.3 ± 0.5	$P=0.0253$
0	0 (0)	1 (3)	
1	19 (64)	22 (70)	
2	14 (36)	10 (27)	
NAS score	5.5 ± 1.6	5.0 ± 1.8	$P=0.0007$

4. Discussion

In this study, we demonstrated patients with NAFLD had significantly higher levels of palmitic acid, palmitoleic acid, oleic acid, γ-linoleic acid, D9-16D and D6D, and significantly lower levels of D5D compared with normal controls. We did not observe significant differences in total, n−3 and n−6-PUFA levels or n−6/n−3 ratio between our three study groups, despite an increase in the ratio

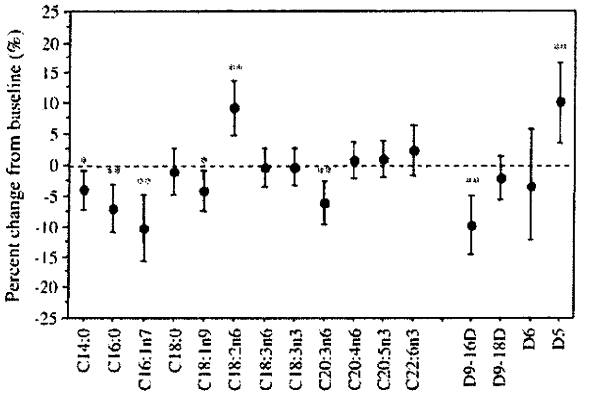

Fig. 1. Effects of ezetimibe on plasma cholesterol fatty acid component and estimated desaturase index in patients with nonalcoholic fatty liver disease. Percentage changes from baseline are least-square means adjusted for the baseline level. Error bars indicate the 95% confidence interval. *$P<0.05$, **$P<0.01$ vs. baseline.

having been reported previously in liver lipids of patients with NASH [12,13]. Our findings essentially agree with the report of Puri et al. [19] that the levels of palmitic acid, palmitoleic acid, oleic acid, and D9-16D were increased significantly in NAFLD across multiple plasma lipid classes, including CEs. Similar findings have also been reported by de Almeida et al. [20].

A noteworthy finding in our study was that patients with a NAS ≥ 5 had significantly higher palmitic acid levels than patients with a NAS ≤ 4, and that these levels were significantly and positively correlated with, not only TG, T-Ch and emIDL, but also ALT and hsCRP. Furthermore, logistic regression analysis showed that a high level of palmitic acid was predictive of NAFLD progression. Joshi-Barve et al. [9] demonstrated that palmitic acid induced production of interleukin-8 by hepatocytes, leading ultimately to liver injury. Several recent studies have also demonstrated palmitic acid induces apoptosis in liver cells [28–30]. Taken together, these earlier data and the findings of the present study suggest strongly that increased levels of palmitic acid are involved in the development and progression of NAFLD.

Stearoyl-CoA desaturase 1 (SCD1; alternatively known as D9D) is the final step in de novo lipogenesis and converts saturated fatty acids to monounsaturated fatty acids, whereas Δ5- and Δ6-desaturases participate in the metabolism of PUFAs [31]. In this study, higher levels of D9-16D and D6D activity and lower levels of D5D activity were observed in patients with NAFLD. Miyazaki et al. [32] have shown that the biosynthesis of hepatic CEs and triglycerides is highly dependent on the expression of the SCD1 gene, while Attie et al. [33] reported that D9D activity was correlated with serum TG levels in human subjects. In this study, we observed significant and positive correlations between D9-16D activity and TG, T-Ch and metabolic syndrome-related anthropometric parameters. With regard to the activities of D5D and D6D, it has been reported that they are both related to insulin sensitivity and insulin levels [34–37]. We also found that insulin resistance parameters correlated positively with D6D levels and negatively with D5D levels. However, no significant difference in the levels of these estimated desaturase activities was observed between NAS ≤ 4 and NAS ≥ 5. Taken together, it is possible to speculate that increasing levels of D9-16D and D6D and decreasing levels of D5D may be major factors in the development and progression of liver steatosis.

Ezetimibe, a lipid-lowering drug, selectively inhibits intestinal cholesterol absorption by binding to Niemann–Pick C1 Like 1 (NPC1L1) protein [38]. Recently, Labonté et al. [21] reported that ezetimibe caused significant reductions in absorption of several saturated fatty acids in diet-induced obese and diabetic mice. In the current study, we demonstrated for the first time that long-term therapy with ezetimibe was also associated with significant decreases in the levels of miristic acid, palmitic acid, palmitoleic acid, oleic acid, dihomo-γ-linoleic acid and D9-16D activity, and significant increases in linoleic acid levels and D5D activity. Several recent studies in an experimental NAFLD model have shown that ezetimibe monotherapy not only protects against diet-induced hyperlipidemia, but also attenuates liver steatosis in an experimental NAFLD model [39,40]. In this study, liver histologic findings were also improved in steatosis grade, necroinflammatory grade, ballooning score and NAS score, although the mean level of fibrosis stage level did not change significantly. In addition, we also found long-term ezetimibe therapy not only decreased T-Ch, LDL-Ch and TG but also emIDL, the most atherogenic lipoprotein that has a strong relationship to insulin resistance. Our findings therefore suggest that ezetimibe may be a useful therapy for NAFLD-related dyslipidemia and hepatic insulin resistance.

In conclusion, the results of the present study suggest that fatty acids composition and desaturase activity associate with the development and progression of NAFLD, and that ezetimibe may be a novel treatment for NAFLD. An appropriately designed, large-scale, prospective clinical study, including histopathological evaluation, is necessary to confirm our findings.

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PEG10 is a probable target for the amplification at 7q21 detected in hepatocellular carcinoma

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Abstract DNA copy number aberrations in human hepatocellular carcinoma (HCC) cell lines were investigated using a high-density oligonucleotide microarray, and a novel amplification at the chromosomal region 7q21 was detected. Molecular definition of the amplicon indicated that *PEG10* (paternally expressed gene 10), a paternally expressed imprinted gene, was amplified together with *CDK14* (cyclin-dependent kinase 14; previously PFTAIRE protein kinase 1, *PFTK1*) and *CDK6* (cyclin-dependent kinase 6). An increase in *PEG10* copy number was detected in 14 of 34 primary HCC tumors (41%). *PEG10*, but not *CDK14* or *CDK6*, was significantly overexpressed in 30 of 41 tumors (73%) from HCC patients, compared with their nontumorous counterparts. These results suggest that *PEG10* is a probable target, acting as a driving force for amplification of the 7q21 region, and may therefore be involved in the development or progression of HCCs.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy in men and the eighth most common in women worldwide; it is estimated to cause approximately half a million deaths annually [1]. Although the risk factors for HCC, which include hepatitis B virus, hepatitis C virus, and alpha-toxin, are well characterized, the molecular pathogenesis of this widespread type of cancer remains poorly understood [2].

Amplification of DNA in certain regions of chromosomes plays a crucial role in the development and progression of human malignancies, specifically when protooncogenic target genes within those amplicons are overexpressed. Oncogenes that are often amplified in cancers include *MYC*, *ERBB2*, and *CCND1*. The recent introduction of high-density oligonucleotide microarrays designed for typing of single nucleotide polymorphisms (SNPs) facilitates high-resolution mapping of chromosomal amplifications, deletions, and losses of heterozygosity [3,4].

To identify genes potentially involved in HCC, we investigated DNA copy number aberrations in human HCC cell lines using high-resolution SNP arrays and found a novel amplification at the chromosomal region 7q21. Recurrent amplifications at 7q21 have been observed in human neoplasms [5]. Gains of 7q21 have been associated with the aggressiveness of several tumors, including HCC [6], colorectal cancer [7], prostate cancer [8], Burkitt lymphoma [9], and esophageal squamous cell carcinoma [10]. These data suggest that this chromosomal region may harbor one or more protooncogenes (henceforth referred to as *target genes*) whose overexpression following amplification might contribute to the initiation or progression of HCC. The actual target gene that drives the 7q21 amplification in HCC remains unclear, however, and we therefore conducted a molecular definition study of the amplicon to identify such genes. Three putative oncogenes, *CDK14* (cyclin-dependent kinase 14; previously *PFTK1*, PFTAIRE protein kinase 1), *CDK6* (cyclin-dependent

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kinase 6), and *PEG10* (paternally expressed gene 10), were identified in the 7q21 amplicon.

The serine/threonine-protein kinase PFTAIRE-1 protein (also known as PFTK1) is a member of the cell division cycle-2 (CDC2)-related protein kinase family [11] and acts as a cyclin-dependent kinase that regulates cell cycle progression and cell proliferation [12]. CDK6 is activated in response to increased expression of D-type cyclins in the early G1 phase of the cell cycle and inactivates the retinoblastoma protein by phosphorylation, thereby activating the transcriptional complex E2F-DP1 that regulates the genes for S-phase onset [13]. *PEG10* has been characterized as a paternally expressed, maternally silenced gene [14]. Several research groups have recently reported overexpression of *PEG10* in HCC [15–19].

2. Materials and methods

2.1. Cell lines and tumor samples

A total of 20 HCC cell lines were examined: JHH-1, JHH-2, JHH-4, JHH-5, JHH-6, JHH-7, SNU354, SNU368, SNU387, SNU398, SNU423, SNU449, SNU475, Huh-1, Huh7, Hep3B, PLC/PRF/5, Li7, HLE, and HLF [20]. All cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Paired tumor and nontumor tissues were obtained from 36 HCC patients who underwent surgery at the Hospital of Tokyo Medical and Dental University. All specimens were frozen immediately in liquid nitrogen and were stored at −80°C until required. Genomic DNA was isolated using a Puregene DNA isolation kit (Gentra, Minneapolis, MN), and total RNA was obtained using Trizol reagent (Invitrogen, Carlsbad, CA). Thirty-four tumor samples were available for DNA analyses, and 41 paired tumor and nontumor samples were available for mRNA analyses.

Prior to the study, informed consent was obtained and the study was approved by ethics committees.

2.2. SNP array analysis

DNA copy number changes were analyzed by the GeneChip Mapping 100K array set (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions as described previously [21]. In brief, 250 ng of genomic DNA was digested with a restriction enzyme (*Xba*I or *Hin*dIII), ligated to an adaptor and amplified by polymerase chain reaction (PCR). Amplified products were fragmented, labeled by biotinylation, and hybridized to the microarrays. Hybridization was detected by incubation with a streptavidin–phycoerythrin conjugate, followed by scanning of the array; analysis was performed as previously described [22]. After appropriate normalization of mean array intensities, signal ratios were calculated between HCC cell lines and anonymous normal references, and copy numbers were inferred from the observed signal ratios based on the hidden Markov model using CNAG software (Copy Number Analyzer for Affymetrix GeneChip mapping arrays) [23]. The CNAG software is available at http://www.genome.umin.jp.

2.3. Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) was performed using five bacterial artificial chromosomes (BACs) as probes, as described previously [24]: RP11-66P5, RP11-412F4, RP11-316P4, RP11-28O23, and RP11-958G24 (Invitrogen, Carlsbad, CA). The BACs were selected based on their homology to locations in the human genome according to the database provided at the University of California, Santa Cruz, Genome Bioinformatics Web site (http://genome.ucsc.edu/).

2.4. Real-time quantitative PCR

Genomic DNA and mRNA were quantified using a real-time fluorescence detection method, as described previously [21]. The primers used for PCR (Table 1) were designed using Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) on the basis of sequence data obtained from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) database. *GAPDH* was used as endogenous control for mRNA levels, and the long interspersed nuclear element 1 (LINE-1) was used as an endogenous control for genomic DNA levels.

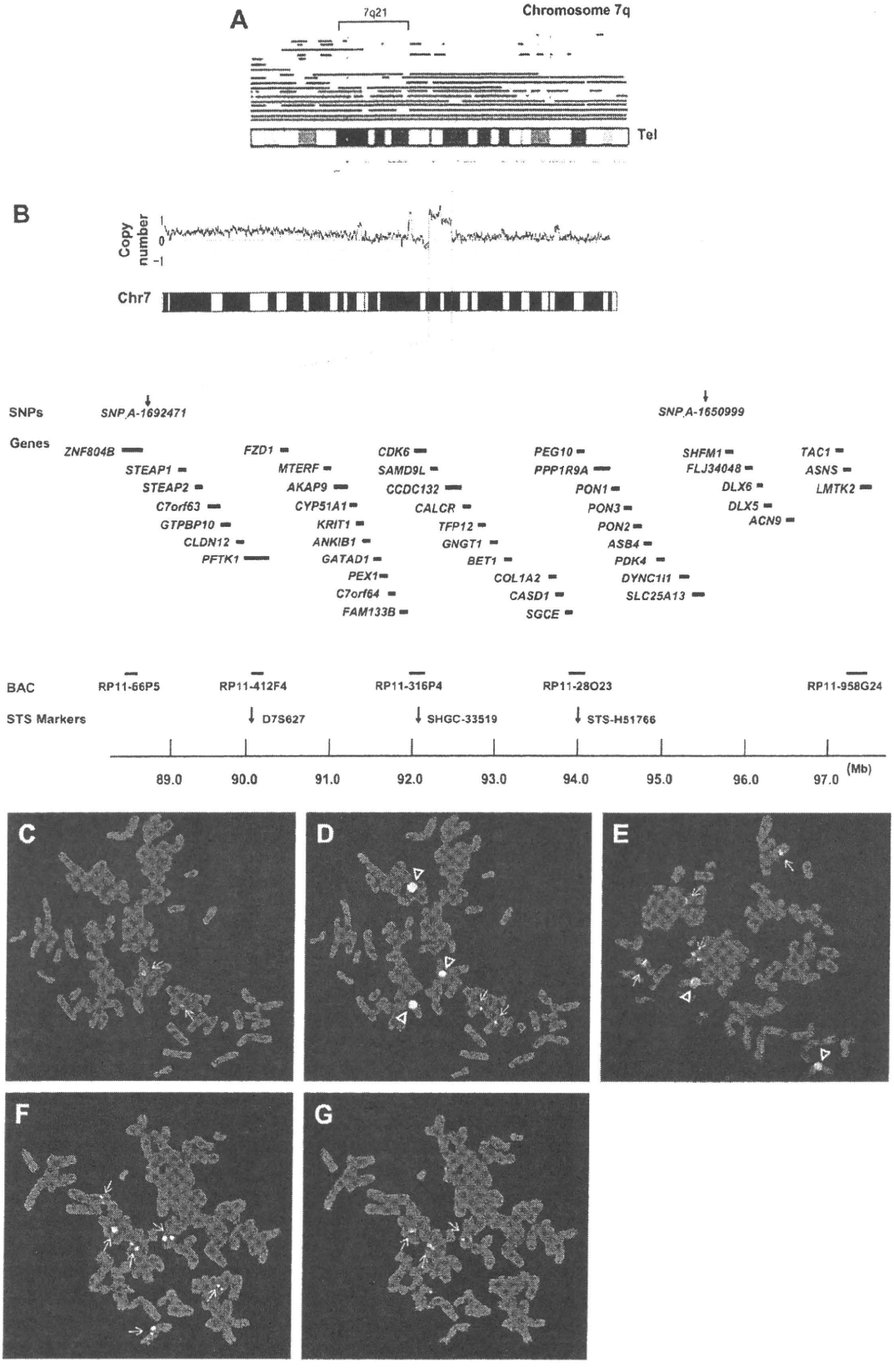
2.5. Statistical analysis

The Wilcoxon signed-rank test was performed using SPSS 15.0 software (SPSS, Chicago, IL). P values of <0.05 were considered significant.

Table 1
Primer sequences used for polymerase chain reaction with sequence-tagged site (STS) markers for the three genes investigated

Gene	STS marker	Forward primer	Reverse primer
CDK14 genomic DNA	D7S627	5′-AAACCAAGAACATTCCAG-3′	5′-ACACATCACATTCTCACC-3′
CDK14 mRNA		5′-CCAAGGAGTTGCTGCTTTTC-3′	5′-GAATGAACTCCAGGCCATGT-3′
CDK6 genomic DNA	SHGC-33519	5′-AAGTCAGAAGGAAAAAAGCTTACTG-3′	5′-TGAGATGTGTTAAAGTAGGTTTTCA-3′
CDK6 mRNA		5′AGCCCAAGATGACCAACATC-3′	5′-AGGTCAAGTTGGGAGTGGTG-3′
PEG10 genomic DNA	STS-H51766	5′-AAAGTTTACATACATTTATGAAGGG-3′	5′- TTCCAGACTGCACCATATAG-3′
PEG10 mRNA		5′-CAGGCCTGAAAAGAAAGTGC-3′	5′-AATGCTTTGTGGAAGCCATC-3′

The gene *CDK14* was previously assigned the symbol *PFTK1* (http://www.genenames.org).


A
7q21
Chromosome 7q
Tel
B
Copy number
1
0
−1
Chr7
SNPs
SNP_A-1692471
SNP_A-1650999
Genes
ZNF804B
STEAP1
STEAP2
C7orf63
GTPBP10
CLDN12
PFTK1
FZD1
MTERF
AKAP9
CYP51A1
KRIT1
ANKIB1
GATAD1
PEX1
C7orf64
FAM133B
CDK6
SAMD9L
CCDC132
CALCR
TFP12
GNGT1
BET1
COL1A2
CASD1
SGCE
PEG10
PPP1R9A
PON1
PON3
PON2
ASB4
PDK4
DYNC1I1
SLC25A13
SHFM1
FLJ34048
DLX6
DLX5
ACN9
TAC1
ASNS
LMTK2
BAC
RP11-66P5
RP11-412F4
RP11-316P4
RP11-28O23
RP11-958G24
STS Markers
D7S627
SHGC-33519
STS-H51766
89.0
90.0
91.0
92.0
93.0
94.0
95.0
96.0
97.0
(Mb)
C
D
E
F
G

3. Results

3.1. Detection of the 7q21 amplicon

Twenty HCC cell lines were screened for DNA copy number aberrations by GeneChip Mapping 100K array analysis. The copy number detection algorithm CNAG allowed assessment of copy number and identification of genomic gains and deletions using the hidden Markov model [23]. Gains at the chromosomal region 7q21 were frequently found in 13 of the 20 cell lines (65%) (Fig. 1A). Of these cell lines, JHH-4 cells exhibited a high-level copy number gain indicative of gene amplification at 7q21 (Fig. 1B). The estimated extent of the amplification in JHH-4 cells is 9 Mb. This chromosomal region lies between the Affymetrix markers SNP_A-1692471 and SNP_A-1650999 (supplementary Table S1) and includes 35 known or predicted protein-coding genes. The 7q21 region may harbor one or more genes that, when activated by amplification, play a role in carcinogenesis. Because we identified three putative oncogenes (i.e., *CDK14*, *CDK6*, and *PEG10*) in the 7q21 amplicon, we chose to focus further analysis on these three genes.

To confirm amplification of *CDK14*, *CDK6*, and *PEG10*, we performed FISH analyses on JHH-4 cells using the BACs RP11-66p5, RP11-412F4, RP11-316P4, RP11-28O23, and RP11-958G24 as probes. RP11-412F4 (containing *CDK14*) (Fig. 1D) and RP11-316P4 (containing *CDK6*) (Fig. 1E) generated amplified FISH signals, and RP11-28O23 (containing *PEG10*) (Fig. 1F) showed an increase in the number of FISH signals. In contrast, neither RP11-66P5 nor RP11-958G24, which correspond to chromosomal regions outside of the amplicon, showed an amplified signal or an increase in the number of FISH signals (Fig. 1C, 1G). These data confirm that *CDK14*, *CDK6*, and *PEG10* are amplified in JHH-4 cells.

3.2. DNA copy number and expression level of CDK14, CDK6, and PEG10 in HCC cell lines

To further analyze the potential role of *CDK14*, *CDK6*, and *PEG10* in HCC, we determined the DNA copy number of these three genes in 20 HCC cell lines by real-time quantitative PCR. For this analysis, copy number changes were counted as gains if the copy number for a given tumor cell type exceeded the mean plus 2 standard deviations of the level of the gene in normal cells. A copy number gain of *CDK14*, *CDK6*, and *PEG10* was observed in 13 (65%), 12 (60%), and 14 (70%) of the 20 cell lines, respectively (Fig. 2A). JHH-4 cells showed the highest copy number gain of each gene.

A common criterion for designation of a gene as a putative target of amplification is that gene amplification leads to its overexpression [25]. To determine whether *CDK14*, *CDK6*, and *PEG10* are overexpressed, we determined the mRNA level of these three genes in the 20 HCC cell lines by real-time quantitative PCR. Both *CDK6* and *PEG10*, but not *CDK14*, were overexpressed in JHH-4 cells, relative to the other cell lines (Fig. 2B). These findings suggested that *CDK6* and *PEG10* are candidate targets for the 7q21 amplification.

3.3. DNA copy number and expression level of CDK14, CDK6, and PEG10 in primary HCC tumors

To determine whether the amplification of *CDK14*, *CDK6*, and *PEG10* that was observed in JHH-4 cells was relevant to primary human carcinomas, we first determined the copy number of the three genes in 34 primary HCCs, using a method similar to that used for the HCC cell lines. A copy number gain of *CDK14*, *CDK6*, and *PEG10* was observed in 8 (24%), 16 (47%), and 14 (41%), respectively, of the 34 tumors (Fig. 3).

We then further examined the expression of the three genes in paired tumor and nontumor tissues from the 41 HCC patients by real-time quantitative PCR. Patient and tumor characteristics are summarized in Table 2. *PEG10* was significantly overexpressed in 30 of the 41 tumors (73%), compared with their nontumorous counterparts (Wilcoxon signed-rank test, $P < 0.001$) (Fig. 4). In contrast, expression of *CDK14* or *CDK6* was not upregulated in HCC tumors (Fig. 4). Taken together, these results suggest that *PEG10* is the most likely target for the 7q21 amplicon in HCC.

Fig. 1. Map of the amplicon at 7q21 in the human hepatocellular carcinoma (HCC) cell line JHH-4. (A) Recurrent copy number gains on the 7q arm as assessed using a GeneChip mapping 100K array (Affymetrix, Santa Clara, CA). Copy number gains are indicated by red horizontal lines above the chromosome ideogram: high-level gains (amplifications) are shown by bright red lines, whereas simple gains are shown by dark red lines. Copy number losses are indicated by green lines under the chromosome ideogram. Each horizontal line represents an aberration detected in a single HCC cell line. The cytobands in 7q are shown. (B) Copy number profile of chromosome 7 in JHH-4 cells. Copy number values were determined by GeneChip mapping 100K array analysis. Shown are the position of the Affymetrix single-nucleotide polymorphism (SNP) probes, the 35 genes included within the amplicon, the five bacterial artificial chromosomes (BACs) used as probes for fluorescence in situ hybridization (FISH) experiments, and the three sequence-tagged site (STS) markers used for real-time quantitative polymerase chain reaction (PCR) based on the University of California, Santa Cruz, Genome Bioinformatics database (http://genome.ucsc.edu/). (C-G) Representative images of FISH on metaphase chromosomes from JHH-4 cells, using the following BAC probes: paired RP11-66P5, containing *ZNF804B* (red) (C) and RP11-412F4, containing *CDK14* (green) (D); single RP11-316P4, containing *CDK6* (red) (E); and paired RP11-28O23, containing *PEG10* (green) (F) and RP11-958G24, containing *LMTK2* (red) (G). Arrows indicate normal signals; arrowheads indicate amplified signals. The set of images shows two normal signals (C), three amplified signals plus two normal signals (D), two amplified signals plus four normal signals (E), six normal signals (F), and three normal signals (G). *Note:* In these figures the gene *CDK14* is identified by the previously approved symbol, *PFTK1*.

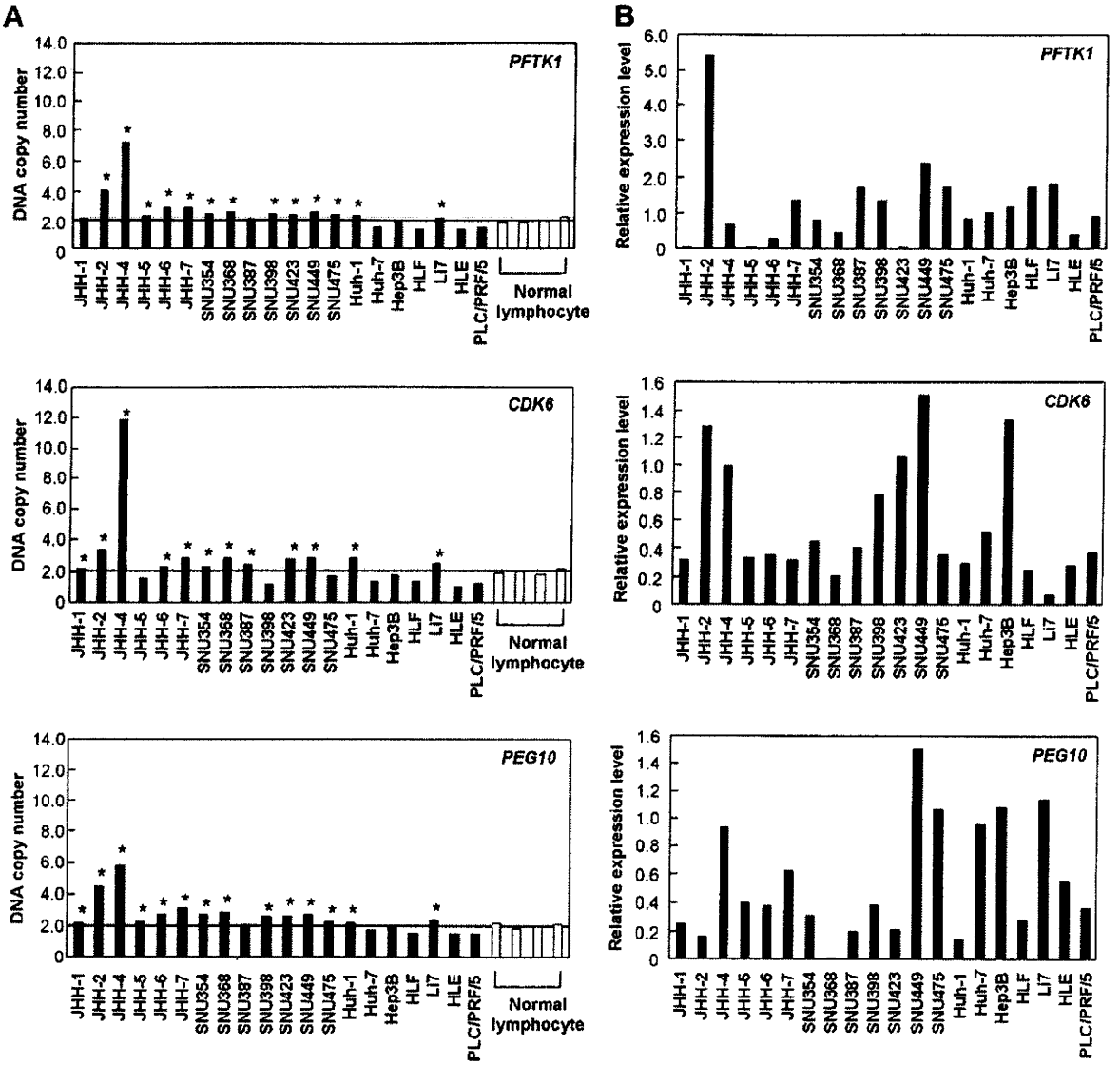


Fig. 2. DNA copy number and expression level of *CDK14* (previously *PFTK1*), *CDK6*, and *PEG10* in HCC cell lines. (A) DNA copy number of *CDK14*, *CDK6*, and *PEG10* in 20 HCC cell lines and four normal peripheral blood lymphocytes as measured by real-time quantitative PCR with reference to LINE-1 controls. Values are normalized such that the average copy number in genomic DNA derived from four normal lymphocytes has a value of 2 (solid horizontal line). A value corresponding to the mean +2 S.D. of the copy number of normal lymphocytes was used as the cutoff value for copy number gain (dotted line). Asterisks indicate cell lines showing copy number gain. (B) Relative expression levels of *CDK14*, *CDK6*, and *PEG10* in 20 HCC cell lines as determined by real-time quantitative PCR. The results are presented as the expression level of each gene relative to a reference gene (*GAPDH*), to correct for variations in the amount of RNA.

4. Discussion

The high-resolution SNP array analysis reported in this study identified amplification at the chromosomal region 7q21 in JHH-4 HCC cells. A copy number gain at this region was frequently observed, not only in HCC cell lines, but also in primary HCCs. Of the three genes identified in the amplicon (i.e., *PEG10*, *CDK6*, and *CDK14*), subsequent experiments suggested that *PEG10* is the most likely target for the amplicon, in that the *PEG10* transcript was both overexpressed in JHH-4 cells and significantly upregulated in primary HCC tumors, compared with their nontumorous counterparts. In contrast, although the highest level of copy number gain was found at the *CDK6* locus in JHH-4 cells and primary HCC tumors, *CDK6* expression was not upregulated in primary HCC tumors.

Contrary to these data, a recent report indicated that expression of *CDK14* was higher in HCC tumors than in

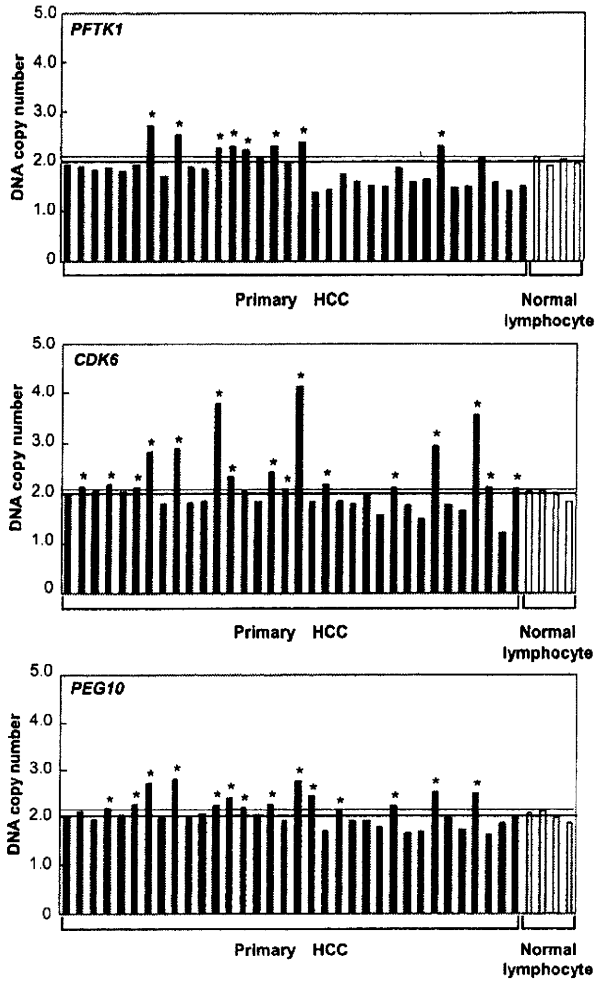


Fig. 3. DNA copy number of *CDK14* (previously *PFTK1*), *CDK6*, and *PEG10* in primary HCC tumors. The DNA copy number of each gene in 34 primary HCC tumors and four normal peripheral blood lymphocytes was determined as already described (for Fig. 2A). Asterisks indicate primary tumors showing copy number gain.

Table 2
Patient and tumor characteristics

Characteristics	Value[a]
Sample size[b]	n = 41
Sex	
Male	33
Female	8
Median age, yr (range)	67 (35–79)
Etiology of liver disease	
Hepatitis B virus	9
Hepatitis C virus	21
Other	11
Median tumor size, cm (range)	5.0 (1.9–26)
Tumors, single or multiple	
Single	26
Multiple	15
Tumor differentiation	
Well	7
Moderate	20
Poor	14
Stage[c]	
I	1
II	15
III	14
IV	11
Background liver tissue	
Normal	4
Chronic hepatitis	18
Liver cirrhosis	19
Child–Pugh classification	
A	40
B	1
C	0
Median α-fetoprotein, ng/mL (range)	14.9 (0.9–114,859)

[a] Where no other unit is specified, values refer to number of patients.
[b] All patients were of Japanese ethnicity.
[c] International Union Against Cancer tumor–node–metastasis (UICC TNM) classification of malignant tumor.

adjacent nontumorous liver tissues and that upregulation of the gene correlated with both advanced metastatic HCCs and microvascular invasion [26]. Further studies are required to clarify the potential role of *CDK14* in HCC.

PEG10 was first identified as an imprinted gene that is paternally expressed and maternally silenced [14]. It has been suggested that *PEG10* is derived from a retrotransposon that was previously integrated into the mammalian genome [14]. The overexpression of *PEG10* in HCC observed in this study is consistent with the previously reported overexpression of *PEG10* in tumors, including HCC [15–19], and in B-cell leukemia [27,28]. Furthermore, several lines of evidence suggest that *PEG10* may be important for the regulation of cell proliferation and cell death: *PEG10* is overexpressed in regenerating mouse liver [16], knockdown of *PEG10* inhibits the proliferation of cancer cells [29], and the PEG10 protein inhibits cell death mediated by SIAH1, a mediator of apoptosis [15]. The importance of *PEG10* for cell regulation is further suggested by the fact that targeted disruption of the mouse *Peg10* gene results in early embryonic lethality due to defects in the placenta [30].

The exact mechanism by which *PEG10* signals is unclear, but it is known to interact with members of the TGF-β receptor family [31]. Further evidence of a potential role for *PEG10* in cell growth and carcinogenesis is that its expression can be regulated by the protooncogene *MYC* [29], by E2F transcription factors that modulate the cell cycle [32], and by the sex hormone androgen [33].

Although the exact mechanism of *PEG10* function in tumors remains to be elucidated, and the findings in this study must be verified in future studies using a larger sample number, the data presented in this work suggest a role for amplification and overexpression of *PEG10* in hepatocarcinogenesis.

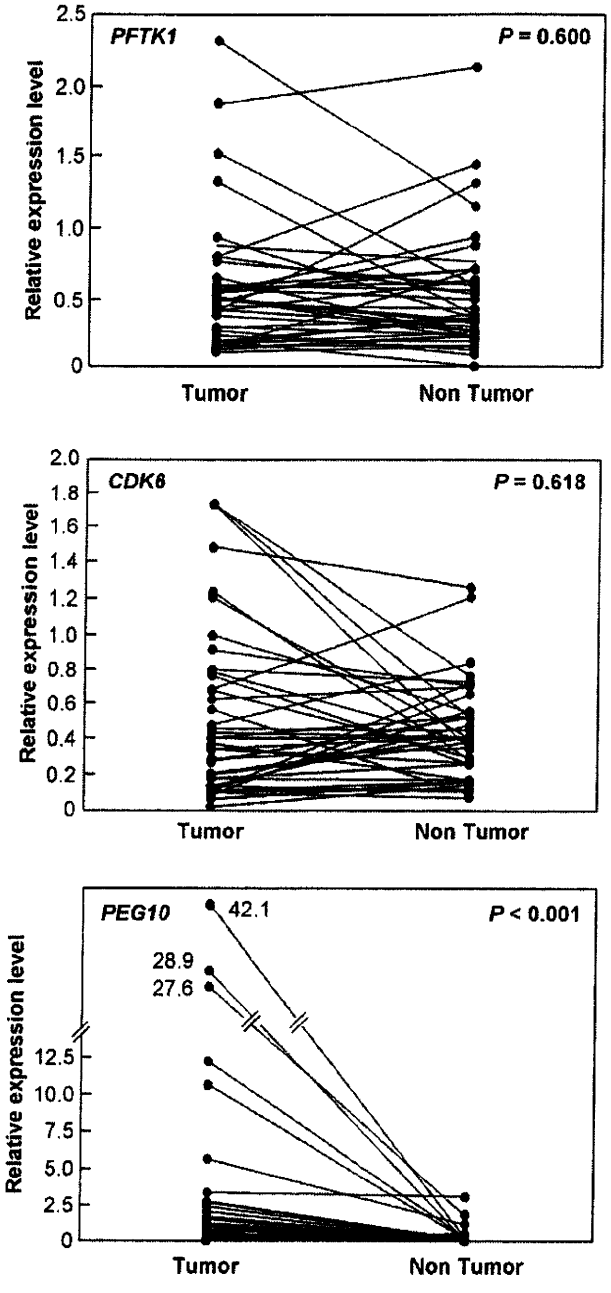


Fig. 4. Expression levels of *CDK14* (previously *PFTK1*), *CDK6*, and *PEG10* in paired tumor (T) and nontumor tissues (NT) from 41 patients with primary HCC. The expression level of each gene was determined as already described (for Fig. 2B). *CDK14*, *CDK6*, and *PEG10* were overexpressed in 22 (54%), 21 (51%), and 30 (73%) of the 41 tumors, respectively, compared with their nontumorous counterparts.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at 10.1016/j.cancergencyto.2010.01.004.

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