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Simple scoring system for predicting cirrhosis in nonalcoholic fatty liver disease

Takaomi Kessoku, Yuji Ogawa, Masato Yoneda, Kento Imajo, Yoshio Sumida, Yuichiro Eguchi, Hideki Fujii, Hideyuki Hyogo, Masafumi Ono, Yasuaki Suzuki, Takumi Kawaguchi, Kazuaki Chayama, Saiyu Tanaka, Kazuma Fujimoto, Keizo Anzai, Toshiji Saibara, Michio Sata, Yoshito Itoh, Atsushi Nakajima, Takeshi Okanoue; Japan Study Group of NAFLD (JSG-NAFLD)

Takaomi Kessoku, Yuji Ogawa, Masato Yoneda, Kento Imajo, Atsushi Nakajima, Department of Gastroenterology and Hepatology, Yokohama City University Graduate School of Medicine, Yokohama 236-0004, Japan
Yoshio Sumida, Yoshito Itoh, Department of Molecular Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine, Kyoto 602-0841, Japan
Yuichiro Eguchi, Department of General Medicine, Saga Medical School, Saga 849-8501, Japan
Hideki Fujii, Department of Hepatology, Osaka City University, Osaka 558-8585, Japan
Hideyuki Hyogo, Kazuaki Chayama, Department of Medicine and Molecular Science, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima 730-0053, Japan
Masafumi Ono, Toshiji Saibara, Department of Gastroenterology and Hepatology, Kochi Medical School, Kochi 783-8505, Japan
Yasuaki Suzuki, Department of Gastroenterology, Nayoro City General Hospital,, Hokkaido 096-8511, Japan
Takumi Kawaguchi, Michio Sata, Department of Gastroenterology, Kurume University School of Medicine, Kurume 830-0011, Japan
Saiyu Tanaka, Department of Internal Medicine, Nara City Hospital, Nara 634-8522, Japan
Kazuma Fujimoto, Keizo Anzai, Department of Internal Medicine, University Hospital, Saga 849-8577, Japan
Takeshi Okanoue, Department of Gastroenterology and Hepatology, Saiseikai Suita Hospital, Osaka 565-0862, Japan
Author contributions: Kessoku T contributed to the design, statistical analysis, manuscript development and final revision of the paper; Ogawa Y, Yoneda M, Imajo K, Sumida Y, Eguchi Y, Hyogo H, Ono M, Kawaguchi T, Saibara T, Sata M, Itoh Y, Nakajima A and Okanoue T contributed to the design, the concept, acquisition, the interpretation data, drafting and revising of the manuscript; Fujii H, Suzuki Y, Chayama K, Tanaka S, Fujimoto K and Anzai K contributed to their practical and intellectual input; All authors read and approved the final manuscript.
Correspondence to: Masato Yoneda, MD, PhD, Department of Gastroenterology and Hepatology, Yokohama City University Graduate School of Medicine, 3-9 Fuku-ura, Kanazawa-ku, Yokohama 236-0004, Japan. dryoneda@yahoo.co.jp
Telephone: +81-45-7872640 Fax: +81-45-7843546
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Abstract

AIM: To investigate a simple noninvasive scoring system for predicting liver cirrhosis in nonalcoholic fatty liver disease (NAFLD) patients.

METHODS: A total of 1048 patients with liver-biopsy-confirmed NAFLD were enrolled from nine hepatology centers in Japan (stage 0, 216; stage 1, 334; stage 2, 270; stage 3, 190; stage 4, 38). The weight and height of the patients were measured using a calibrated scale after requesting the patients to remove their shoes and any heavy clothing. Venous blood samples were obtained in the morning after the patients had fasted overnight for 12 h. Laboratory evaluation was performed in all patients. Statistical analysis was conducted using SPSS version 12.0. Continuous variables were expressed as mean ± SD.

RESULTS: The optimal cutoff value of platelet count, serum albumin, and aminotransferase/alanine aminotransferase ratio (AAR) was set at $< 15.3\ 10^4/\mu L$, < 4.0 g/dL, and > 0.9, respectively, by the receiver operating characteristic curve. These three variables were combined in an unweighted sum (platelet count = 1 point, serum albumin = 1 point, AAR = 1 point) to form an easily calculated composite score for predicting cirrhosis in NAFLD patients, called the PLALA (platelet, albumin, AAR) score. The diagnosis of PLALA $\geqslant 2$ had sufficient accuracy for detecting liver cirrhosis in NAFLD patients.

CONCLUSION: The PLALA score may be an ideal scoring system for detecting cirrhosis in NAFLD patients with sufficient accuracy and simplicity to be considered for clinical use.

Key words: Nonalcoholic fatty liver disease; Cirrhosis; Fibrosis; Platelet; Albumin; Alanine aminotransferase ratio

Core tip: Nonalcoholic fatty liver disease (NAFLD) is an important cause of chronic and progressive liver injury. We aimed to develop a simple noninvasive scoring system for predicting liver cirrhosis in NAFLD patients. These three variables were combined in an unweighted sum [platelet count = 1 point, serum albumin = 1 point, aminotransferase (AST)/alanine aminotransferase (ALT) ratio = 1 point] to form an easily calculated composite score, called the PLALA (platelet, albumin, AST/ALT ratio) score. The diagnosis of PLALA $\geqslant$ 2 had sufficient accuracy for detecting liver cirrhosis in NAFLD patients. The PLALA score may be an ideal scoring system for detecting cirrhosis in NAFLD patients.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is an important cause of chronic liver injury in many countries[1,2]. NAFLD represents a spectrum of conditions that are characterized histologically by macrovesicular hepatic steatosis, and the diagnosis is made after excluding a history of consumption of alcohol in amounts sufficient to be considered harmful to the liver. NAFLD range over a wide spectrum, extending from simple steatosis, which is generally benign, through to nonalcoholic steatohepatitis (NASH) to liver cirrhosis, end-stage liver disease, and even hepatocellular carcinoma despite the absence of significant alcohol consumption[3-7]. The probability of developing advanced fibrosis and hepatocellular carcinoma is significantly greater in individuals with steatohepatitis than in those with simple steatosis. Data collected from the United States have shown that the prevalence of NAFLD has increased steady in recently years, despite other diseases remaining at steady states. Natural history studies suggest that fibrosis progression occurs in 32%-37% of patients over 3-6 years[8-10], and up to 12% of patients will progress to cirrhosis over 8-10 year[11]. If these patients with NAFLD progress liver cirrhosis, they need to be kept under the surveillance data for early detection of HCC and gastroesophageal varices, similar to the case, such as hepatitis C[12-14].

Liver biopsy as a confirmation tool of NASH can reveal the histologic activity of steatosis, inflammation, and fibrosis. It is frequently used for diagnosis as the gold standard tool in patients with NASH[1,3,15]. However, it is difficult to perform liver biopsy for every patient with NAFLD to ascertain the presence of NASH and determine the stage and grade of the disease[16]. The estimated number of patients with NAFLD has reached 80-100 million in the United States, and the corresponding number of patients in Japan has been estimated at 10-20 million. The prevalence of NAFLD and nonalcoholic steatohepatitis (NASH) is increasing and is becoming a major target disease not only in Western countries, but also in Japan. Therefore, alternative diagnostic methods, noninvasive procedures such as transient elastography, have recently been developed. However, these are not appropriate for health check-ups because they cannot be used in patient with ascites, thick subcutaneous fat, narrow intercostal spaces, and hepatic atrophy.

Therefore, the aim of this study was to develop a mass screening system for general physicians, which can be used for predicting liver cirrhosis in NAFLD patients, using routine laboratory parameters.

MATERIALS AND METHODS

Patients

1048 NAFLD patients who underwent liver biopsy were enrolled between 2002 and 2011 from institutes affiliated with the Japan Study Group of NAFLD (JSG-NAFLD), represented by the following 10 hepatology centers: Yokohama City University, Asahikawa Medical College, Kurume University, Nara City Hospital, Hiroshima University, Saga Medical School, Osaka City University, Kyoto Prefectural University of Medicine, Kochi Medical School, and Saiseikai Suita Hospital. The study was conducted with the approval of the Ethics Committee of all hepatology centers. Liver biopsy was available in all NAFLD patients for the purpose of diagnosis and staging of NASH. Macrovesicular steatosis affecting at least 5% of the hepatocytes was observed in all the cases, with displacement of the nuclei to the edges of the cells[17]. The exclusion criteria included history of hepatic disease such as chronic hepatitis C or concurrent active hepatitis B (seropositive for hepatitis B surface antigen); autoimmune hepatitis; primary biliary cirrhosis; Wilson disease; hemochromatosis; α1-antitrypsin deficiency; sclerosing cholangitis; hepatic injury caused by substance abuse, or current or past consumption of > 20 g of alcohol daily. Informed consent for evaluation of liver histology was obtained from all the enrolled patients, and the present study was performed in accordance and compliance with the Ethic Principles of the 1975 Declaration of Helsinki.

Table 1 Characteristics of patients in the estimation and validation groups

Variables	Fibrosis stages 0-3 (non-cirrhosis)[1]	Fibrosis stage 4 (cirrhosis)[1]	*P* value[2]
Age (yr)	51.1 ± 15.0	63.9 ± 9.6	< 0.0010
n	1010	38	-
BMI (kg/m^2)	27.8 ± 4.9	28.6 ± 3.9	0.3648
AST (IU/L)	58.3 ± 38.2	70.2 ± 74.7	0.0721
ALT (IU/L)	92.2 ± 64.4	67.5 ± 65.6	0.0208
AAR	0.71 ± 0.29	1.15 ± 0.41	< 0.0010
ALP (IU/L)	258.8 ± 94.9	312.2 ± 155.6	0.0012
GGT (IU/L)	88.2 ± 93.8	95.4 ± 73.0	0.6408
ChE (IU/L)	383.0 ± 95.3	298.7 ± 127.0	< 0.0010
Albumin (g/dL)	4.42 ± 0.41	3.69 ± 0.47	< 0.0010
Ferritin (ng/mL)	255.3 ± 249.8	227.5 ± 198.0	0.5677
Fasting glucose (mg/dL)	113.3 ± 38.9	124.0 ± 57.2	0.1116
Fasting insulin (μU/mL)	14.8 ± 13.8	18.4 ± 10.5	0.1938
HOMA-IR	4.31 ± 5.10	6.05 ± 5.64	0.1015
Hemoglobin (g/dL)	14.5 ± 1.6	13.2 ± 1.5	< 0.0010
Platelet (× 10^4/μL)	22.6 ± 6.53	12.08 ± 4.40	< 0.0010
Hyaluronan (ng/mL)	54.5 ± 81.0	250.7 ± 191.0	< 0.0010
Collagen Ⅳ (ng/mL)	4.70 ± 3.60	8.14 ± 1.80	< 0.0010

[1]Results are presented as numbers for qualitative data or as mean ± SD for quantitative data; [2]*P* values were calculated by the *t* test or the χ^2 test. ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; AAR: AST/ALT ratio; BMI: Body mass index; ChE: Cholinesterase; GGT: γ-glutamyl transpeptidase; HOMA-IR: Homeostasis model assessment-insulin resistance.

Anthropometric and biochemical measurements

Body mass index (BMI) was calculated as body weight (kg) divided by height (m^2). Fasted Human blood was collected from all biopsy-proven patients in the morning after overnight for 12 h. In patients with NAFLD, the blood cell counts and the serum levels of aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ-glutamyl transpeptidase, albumin, ferritin, cholinesterase (ChE), fasting plasma glucose, fasting immunoreactive insulin, hyaluronan, and collagen Ⅳ were measured consecutively in the each hospital's laboratory. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated for NAFLD patients as using the following formula: fasting insulin (μU/mL) × fasting plasma glucose (mg/dL)/405.

Histological evaluation

All patients had undergone percutaneous liver biopsy under ultrasound guidance. Liver biopsies were obtained with 16 or 18 gauge needle biopsy apparatus. The number of biopsy specimen fragments was one or two. Liver tissue specimens were fixed in formalin, embedded in paraffin and stained, and analyzed independently by an expert pathologist who was blinded to the clinical data. Fatty liver was defined as the presence of > 5% steatosis. In addition to steatosis, the diagnosis of NASH was histologically confirmed as the presence of lobular inflammation, hepatocyte injures including hepatocyte ballooning cells or perisinusoidal/pericellular fibrosis in zone 3 of the hepatic acini[4,18,19]. The grading and staging of NASH was assessed by Brunt's modified semi-quantitative system, which classifies inflammatory activity into 3 grades [grade 1, mild; grade 2, moderate; grade 3, severe, and the stage of fibrosis into a 4 stages (stage 1, zone3 perivenular and/or perisinusoidal fibrosis; stage 2, stage 1 with periportal fibrosis; stage 3, bridging fibrosis; and stage 4, cirrhosis)]. The individual parameters of fibrosis were scored independently according to the NASH Clinical Research Network (CRN) scoring system developed by the NASH CRN[20].

Statistical analysis

Statistical analysis was performed by using SPSS software version 12.0 for windows (SPSS, Chicago, IL, United States). Continuous variables were expressed as mean ± SD. Qualitative data were represented as numbers, with the percentages indicated within parentheses. The statistical significance of differences between the two groups in the quantitative data were assessed using the *t*-test or the χ^2 test. Data sets involving more than two independent groups were assessed by Kruskal-Wallis test, because the variables were often not normally distributed. The diagnostic performance was assessed by analysis of receiver-operating characteristic (ROC) curves, and the ROC curve was a plot of sensitivity versus (1-specificity) for all possible cutoff values. The probabilities of a true-positive (sensitivity) and true-negative (specificity) were determined for selected cutoff values, and the area under the ROC curve (AUROC) was calculated for each index. Statistical significance was defined as a $P < 0.05$.

RESULTS

Patient and laboratory characteristics of enrolled subjects

Using a multicenter database, 1048 biopsy-proven cases of NAFLD were investigated (fibrosis stage 0, 216; stage 1, 334; stage 2, 270; stage 3, 190; stage 4, 38). The clinical laboratory data and liver biopsy specimens with characteristics of individuals with fibrosis stages 0-3 and stage 4 (cirrhosis) are shown in Table 1. The age, AST/ALT ratio (AAR), ALP, hyaluronan, and collagen Ⅳ were significantly higher, and ChE, albumin, hemoglobin, and platelet were significantly decreased in NAFLD patients with liver cirrhosis (fibrosis stage 4), compared with those with no cirrhosis (fibrosis stages 0-3).

Multiple logistic regression analysis of factors related to fibrosis: Fibrosis stage 0-3 vs fibrosis stage 4 (cirrhosis)

Multiple logistic regression analysis is performed by using age, AAR, serum ChE, albumin, hemoglobin, platelet, hyaluronan, and collagen Ⅳ, which were significantly increased or decreased in NAFLD patients with liver cirrhosis (fibrosis stage 4) compared with NAFLD patients without cirrhosis (fibrosis stages 0-3), by univariate analysis ($P < 0.0001$) (Table 2). On the factors using multiple logistic regression analysis associated with fibrosis stage 0-3 compared with stage 4, AAR ($P = 0.0427$), serum albumin level ($P < 0.001$), and platelet ($P < 0.001$) were the

Table 2 Multiple logistic regression analysis of factors associated with stage 0-3 compared with stage 4 (cirrhosis)

Variables	OR	95%CI	*P* value
Age (yr)	0.976	0.935-1.018	0.2610
AAR	0.122	0.054-0.279	0.0427
Cholinesterase (IU/L)	1.000	0.995-1.005	0.9200
Albumin (g/dL)	5.977	2.430-14.703	< 0.0001
Hemoglobin (g/dL)	0.988	0.761-1.283	0.9263
Platelet ($\times 10^4/\mu L$)	1.282	1.169-1.405	< 0.0001
Hyaluronan (ng/mL)	1.000	0.997-1.003	0.8879
Type IV collagen 7s	0.941	0.883-1.004	0.0671

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; AAR: AST/ALT ratio.

factors related to progression to cirrhosis.

ROC curve for differentiating stage 4 fibrosis based on albumin, platelet, and AAR

We performed ROC curve analysis for differentiating fibrosis stage 4 (cirrhosis) based on albumin, platelet, and AAR (Figure 1). For detecting cirrhosis (fibrosis stage 4) compared with non-cirrhosis (fibrosis stage 0-3), the AUROC for AAR, albumin, and platelet was 0.843, 0.898, and 0.918, respectively. Based on the ROC curve, the cut-off level for the diagnosis of AAR, albumin, and platelet was set at ≥ 0.9, ≤ 4.0 g/dL, and $\leq 15.3 \times 10^4$/mL, respectively. Sensitivity, specificity, negative predictive value (NPV), and positive predictive value (PPV) of AAR, albumin, and platelet were 76.3%/84.2%/81.6% (sensitivity), 82.9%/84.6%/88.6% (specificity), 98.9%/99.3%/99.2% (NPV), and 13.9%/17.0%/21.2% (PPV), respectively.

PLALA score (platelet, albumin, AAR)

By multiple logistic regression analysis, three variables remained significant, including platelet, albumin, and AAR. Thus, these three variables, platelet $< 15.3 \times 10^4/\mu L$, albumin < 4.0 g/dL, and AAR > 0.9, were combined to form an easily calculated composite score for predicting NAFLD with cirrhosis, called the PLALA score. The three variables were given a score of 1 point each (Figure 1), and a score of 0-3 was calculated. Figure 2 shows the percentage of patients with cirrhosis (fibrosis stage 4) with a platelet $< 15.3 \times 10^4/\mu L$, albumin < 4.0 g/dL, and AAR > 0.9.

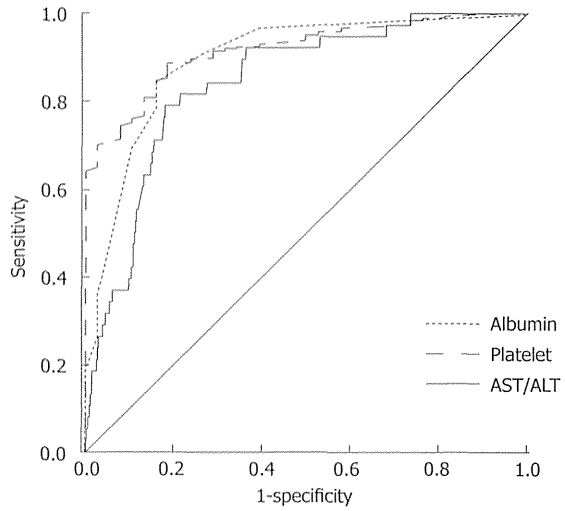
The diagnostic accuracy of the scoring system in distinguishing patients with and without cirrhosis was confirmed in 1048 patients. The percentage of patients with cirrhosis (fibrosis stage 4) with a PLALA score of 0, 1, 2, and 3 was 0%, 13%, 29%, and 58%, respectively (Figure 3). When using a PLALA score of 2 as a cutoff, the sensitivity, specificity, NPV, and PPV were 86.8%, 90.8%, 99.5%, and 26.2%, respectively. All of these data were superior to those of platelet, albumin, and AAR.

DISCUSSION

We developed a simple scoring system to differentiate cirrhosis from non-cirrhosis in NAFLD patients. The three variables platelet, albumin, and AAR were combined in an unweighted sum (platelet $< 15.3 \times 10^4/\mu L$; 1 point, serum albumin < 4.0 g/dL; 1 point, and AAR > 0.9; 1 point) and formed an easily calculated composite score for predicting cirrhosis in NAFLD patients, called the PLALA score. A PLALA score (2 and 3) was useful for detecting liver cirrhosis in NAFLD patients (sensitivity, 86.8%; specificity, 90.8%; NPV, 99.5%; PPV, 26.2%).

Bhala *et al*[21] reported that 2.4% of patients with NAFLD followed up for approximately 85.6 ± 54.5 mo developed HCC, and 66.7% of the patients with NAFLD-associated HCC had cirrhosis (fibrosis stage 4). Hashimoto *et al*[22] reported that 88% of patients with NASH-associated HCC had advanced fibrosis (stage 3 or 4). Therefore, advanced fibrosis was recognized as an important risk factor for HCC. Furthermore, HCC was the major cause of mortality in NASH patients with advanced fibrosis[22]. It is important to closely follow cirrhosis patients with NASH. In the field of NAFLD, various scoring systems have been reported, for example NAFIC[23], FIB4 index[24], HAIR[25], BAAT[26], BARD[13], NAFLD fibrosis score[27], and N score (Nippon)[28]. These scoring systems can differentiate NASH from NAFLD or differentiate advanced fibrosis (stages 3 or 4) from mild fibrosis (stage 0-2). However, the PLALA score developed in our study, with the three variables platelet, albumin, and AAR, differentiates cirrhosis (stage 4) from non-cirrhosis in the NAFLD patients (stages 0-3), and it is easy to calculate.

The platelet is one of the most commonly reported parameters associated with clinically significant portal



Variables	Cutoff values	Score values
Platelet ($\times 10^4/\mu L$)	< 15.3×10^4/mL	1 point
Albumin (mg/dL)	< 4.0 g/dL	1 point
AAR	> 0.9	1 point

Figure 1 Receiver operating characteristic curve. Receiver operating characteristic curve for differentiating fibrosis stage 4 based on albumin, platelet, and AAR; aspartate aminotransferase (AST)/alanine aminotransferase (ALT) ratio. PLALA score (platelet, albumin, AAR).

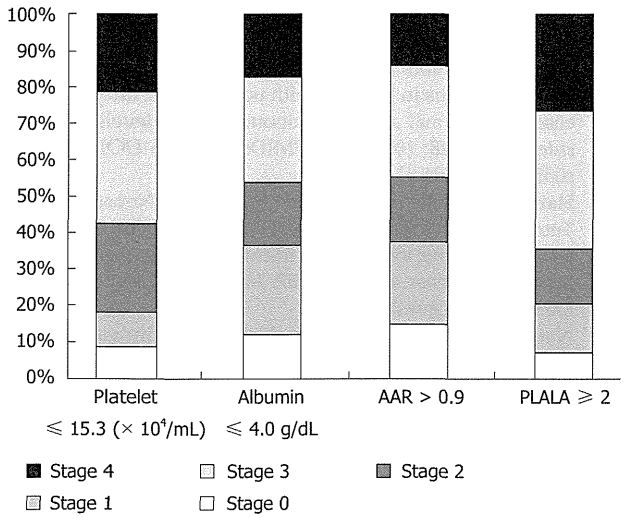


Figure 2 The percentage of patients with cirrhosis. The percentage of patients with cirrhosis (stage 4) with a platelet < 15.3 $\times 10^4/\mu L$, albumin < 4.0 g/dL, and alanine aminotransferase ratio; aspartate aminotransferase (AST)/alanine aminotransferase (ALT) ratio (AAR) > 0.9.

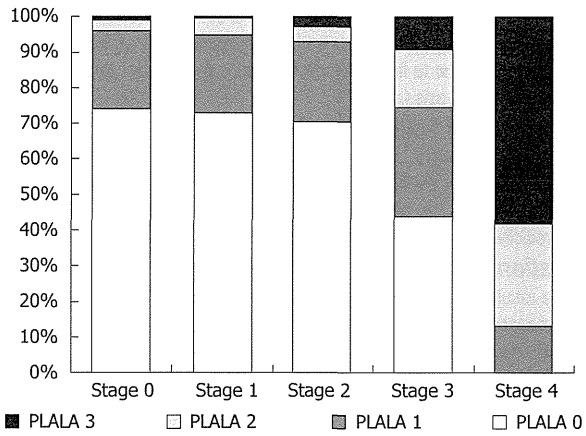


Figure 3 PLALA score and fibrosis stage of 1048 biopsy-proven nonalcoholic fatty liver disease patients.

hypertension in compensated cirrhosis patients[29]. The levels of serum albumin reflect the protein-synthesizing capacity of the liver. Patients with advanced cirrhosis almost always have hypoalbuminemia caused by decreased protein synthesis in the hepatocytes. AAR reflects fibrosis of the liver[30,31]. When only one of three values is positive, factors that may cause false-positive low cutoff values for platelet are older age (decreased platelet production), idiopathic thrombocytopenic purpura, idiopathic portal hypertension, and drugs. Factors that may cause false-positive low levels of serum albumin are loss of urinary albumin due to renal dysfunction (*e.g.*, nephritic syndrome and diabetic nephropathy), severe burns, and inadequate protein intake. The possibility of false-positive values for AAR > 0.9 is within the normal range of AST and ALT.

Liver cirrhosis simultaneously induces liver fibrosis, portal hypertension, and decreased production of albumin. Thus, PLALA score includes: platelet, which reflects portal hypertension; albumin, which reflects protein production; and AAR, which reflects liver fibrosis. A PLALA score of 2 or 3 points is highly diagnostic for liver cirrhosis in patients with false-positive results for NAFLD. If these NAFLD patients with liver cirrhosis have early detection of HCC and portal hypertension, such as gastroesophageal varices, it is important for them to need to be kept under surveillance.

This study had several limitations. The study had a largely retrospective design. The proportion of patients with advanced fibrosis was small. Therefore, in contrast to the NPVs, the PPVs did not have sufficient accuracy for the diagnosis of advanced fibrosis. Therefore, it would seem appropriate to consider liver biopsy in all patients with values above the cutoff of the selected index, PLALA (2 and 3). We previously reported, possibly for the first time, that transient elastography and acoustic radiation force impulse elastography can be used to measure the severity of fibrosis in patients with NAFLD[32,33]. It is possible that a combination of transient elastography and the aforementioned scoring systems may provide better performance than each of them used alone, although this needs to be verified. The patients were recruited from multiple hepatology centers in Japan with a particular interest in the study of NAFLD; therefore, the possibility of some referral bias cannot be ruled out. Patient selection bias could also have existed, because liver biopsy might have been considered for NAFLD patients who were likely to have NASH and progression of fibrosis. Thus, the findings may not represent NAFLD patients in the community at large. We also acknowledge that the pathological diagnosis was mainly determined using liver tissues derived from percutaneous liver biopsies, which are prone to sampling errors and/or inter-observer variability[34,35]. There is a possibility that our results might not be adaptable for NAFLD patients of other races, because all participants were Japanese. Because of these limitations, the present results need to be validated in independent populations by other investigators.

In conclusions, the PLALA score may be an ideal scoring system for detecting cirrhosis in NAFLD patients, because it is easy to use, cost effective, and accurate. Therefore, we consider that this scoring system is useful for mass screening by general physicians, using routine laboratory parameters.

COMMENTS

Background

Nonalcoholic fatty liver disease (NAFLD) is one of the most common causes of chronic liver damage in many countries around the world. Although liver biopsy is useful as the gold standard method for diagnosis of nonalcoholic steatohepatitis (NASH) with cirrhosis, it is difficult to perform liver biopsy for every patient with NAFLD. However, noninvasive markers for predicting cirrhosis in NAFLD patients have not yet been well established.

Research frontiers

In the field of NAFLD, various scoring systems related liver fibrosis have been reported, for example FIB4 index, NAFLD fibrosis score. These scoring systems can differentiate advanced fibrosis (stage 3 or 4) from mild fibrosis (stage

0-2). However, NASH-related cirrhosis (stage 4) is cause of most complication, especially hepatocellular carcinoma and portal hypertension. Therefore, the research hotspot is how to find noninvasive scoring systems for detecting with NASH-associated cirrhosis (stage 4).

Innovations and breakthroughs

The PLALA score developed with the three variables, differentiates cirrhosis (stage 4) from non-cirrhosis in the NAFLD patients (stages 0-3). When using a PLALA score of 2 as a cutoff, the sensitivity, specificity, negative predictive value, and positive predictive value were 86.8%, 90.8%, 99.5%, and 26.2%, respectively.

Applications

The study results suggest that PLALA score is to develop a mass screening system for general physicians, which can be used for predicting liver cirrhosis in NAFLD patients, using routine laboratory parameters.

Terminology

NAFLD is mainly represents a spectrum of liver disease from simple steatosis to nonalcoholic fatty steatohepatitis, which can progress to cirrhosis and hepatocellular carcinoma, despite the absence of significant alcohol consumption. PLALA score is constructed from platelet, Alb, AAR. These three variables were combined to form an easily calculated composite score for predicting NAFLD with cirrhosis.

Peer review

The manuscript aimed to develop a simple noninvasive scoring system for predicting liver cirrhosis in nonalcoholic fatty liver disease patients by using early available clinical and biochemical variables. This article is interesting, original and well written, and gives good clues to the readers.

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Original Article

Questionnaire survey on lifestyle of patients with nonalcoholic steatohepatitis

Haruka Noto, Katsutoshi Tokushige,* Etsuko Hashimoto, Makiko Taniai and Keiko Shiratori

Tokyo Women's Medical University, Department of Medicine and Gastroenterology, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan

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Lack of exercise and excessive food intake are known to be the important causes of nonalcoholic steatohepatitis (NASH). To elucidate the relationship between lifestyle and NASH, we surveyed exercise and dietary habits, comparing them among 171 biopsy-proven NASH patients, 29 nonalcoholic fatty liver (NAFL) patients and 49 normal subjects. Dietary habits including the duration of dinner time, amount of rice at dinner, and weekly frequencies of meat, fries, Chinese noodles, sweets, and instant food consumption were significantly different in male NASH patients compared to normal male subjects. In women, differences were seen in the amount of rice at dinner, frequency of eating out, and proclivity for sweets. In male NASH patients, the frequency of physical exercise was significantly lower. The lifestyle tendencies of NASH were almost similar to those of NAFL. In the comparison between obese NASH and non-obese NASH, no clear lifestyle differences were found. In conclusion, the most striking result of this survey was that the lifestyle of males contributed significantly to the development of NASH. These results point to treatment of NASH in males. In female NASH patients, lifestyle differences were minimal, and the effects of other factors such as genetic background will need to be investigated.

Key Words: NASH, lifestyle, exercise, gender, dietary habit

In recent years, with increases in overeating, Western food intake, and the use of automobiles instead of walking in Japan, the rates of obesity and lifestyle-related diseases such as type 2 diabetes mellitus have been on the rise. Non-alcoholic fatty liver disease (NAFLD) has recently been recognized as a leading cause of abnormal liver function.[1] Its spectrum ranges from fatty liver alone, usually a benign and non-progressive condition, so-called nonalcoholic fatty liver (NAFL), to non-alcoholic steatohepatitis (NASH), which may progress to liver cirrhosis and hepatocellular carcinoma.[2-4] Patients with NASH are usually complicated by insulin resistance syndrome.[5] Diet and lack of exercise are strongly related to the development of insulin resistance.[6,7] It has been reported that high-fat Western food and glycemic load are positively associated with fat deposits and insulin resistance.[8] On the other hand, even moderate exercise is effective for the improvement of insulin resistance.[9] However, there are few reports about the association of detailed lifestyle variables, such as food preferences and exercise habits, with the development or progression of NASH/ NAFLD.

In addition, we were interested in the difference between obese-NASH and non-obese NASH. In case of non-obese NASH, it is not clear whether genomic background or lifestyle has more impact on the occurrence of NASH.

The aim of this study was to clarify the kind of lifestyle variables involved in the development and occurrence of obese and non-obese NASH.

Materials and Methods

One hundred seventy-one Japanese patients histologically diagnosed with NASH at Tokyo Women's Medical University between 1995 and 2010 were evaluated, along with 49 healthy subjects serving as controls. The diagnosis of NASH was established based on the following criteria: (1) Histologically, macrovesicular steatosis affecting at least 5% of hepatocytes with lobular inflammation, ballooning degeneration and/or perivenular or/and pericellular fibrosis.[10,11] (2) Intake of less than 140 g of ethanol per week, as confirmed by physicians and family members. (3) Appropriate exclusion of other liver diseases such as alcoholic liver disease, viral hepatitis, autoimmune hepatitis, drug-induced liver disease, primary biliary cirrhosis, primary sclerosing cholangitis, and metabolic liver diseases. In all NASH patients, liver biopsy was performed. Fibrosis was scored using a 5-grade scale: F0, normal connective tissue; F1, foci of perivenular or pericellular fibrosis in zone 3; F2, perivenular or pericellular fibrosis confined to zones 2 and 3, with or without portal/periportal fibrosis; F3, bridging or septal fibrosis; F4, cirrhosis.[10,11] Steatosis was graded on a scale of 1 to 3: 1, mild (affecting 10–33% of hepatocytes); 2, moderate (33–66% of hepatocytes); 3, severe (>66% of hepatocytes). Ballooning degeneration was graded on a scale of 1 to 2: 1 mild or moderate; 2, severe. In addition, the NAFLD activity score (NAS) was assessed.[12] We added 29 NAFL patients. Six patients were diagnosed by liver biopsy and 23 patients by NAFIC score.[13] Liver biopsy showed steatosis without ballooning degeneration, or NAFIC scores were 0 or 1. There was no HCC case in NASH and NAFL. The 49 Japanese control subjects were matched for age and gender with the NASH patients. All control subjects previously having undergone ultrasonographic examination confirming the absence of steatosis were confirmed by blood test to have normal liver function and to be free of viral hepatitis infection. The NASH patients and control subjects were asked about their dietary habits and exercise routines.

The survey included the following questions: Question (Q.) 1) Do you have breakfast every day? Q.2) How long do you take to have dinner? Q.3) How many bowls of rice do you eat at dinner? Q.4) How many times per week do you eat meat dishes? Q.5) How many times per week do you eat fries? Q.6) How many times per week do you eat Chinese noodles? Q.7) How many times per week do you eat out? Q.8) How many times per week do you eat instant food? Q.9) How many times per week do you eat fast food? Q.10) How many cans of coffee or juice do you drink per week? Q.11) How many times per week do you eat sweets? Q.12) How many times per week do you eat cake? Q.13) Do you do exercise at least once a week? Q.14) Do you prefer to use the elevator or stairs? Q.15) Do you travel to work by car or train? These questions were

*To whom correspondence should be addressed.
E-mail: ktoku@pg7.so-net.ne.jp

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answered on paper forms, and the answers of NASH patients, NAFL patients and normal subjects were compared.

Obesity was defined as a BMI>25 kg/m^2 according to the Japanese Obesity Association criteria. Diagnosis of type II diabetes mellitus (DM) was based on the WHO criteria. The diabetes criteria of WHO is almost the same as Japanese criteria. Briefly, fasting plasma glucose ≥126 mg/dl or 2-h plasma glucose of ≥200 mg/dl. Dyslipidemia was diagnosed if the patient was currently on treatment with lipid-lowering medications or had elevated serum levels of total cholesterol (>220 mg/dl) and/or triglycerides (>150 mg/dl) on at least 3 occasions. Hypertension was diagnosed if the patient was receiving antihypertensive therapy or had a blood pressure >140/90 mmHg on at least 3 occasions.

Brinkman index, the number of cigarettes smoked per day multiplied by the years of smoking, was used for smoking assessment.

In addition, the answers were compared between mild fibrosis NASH patients (F0–2) and severe fibrosis (F3–4) NASH patients, between NAS 5 points or over and NAS 4 points or under,[12] between mild steatosis (S1–2) and severe steatosis (S3), between mild-moderate ballooning degeneration and severe ballooning degeneration, and between obese NASH (BMI >25 kg/m^2) and non-obese NASH. We also compared non-obese NASH or non-obese NAFL with non-obese control subjects.

Statistical analysis. The results were expressed as mean percentage or times. The Mann-Whitney U test or χ^2 test was used to find differences between NASH patients or NAFL patients and control subjects. A p value of <0.05 was considered to be statistically significant in all analyses.

Results

Table 1 shows the profile of NASH, NAFL patients and control subjects. There were no age differences among the male NASH patients, male NAFL patients and male control, nor among the female NASH patients, female NAFL patients and female controls. In both male and female NASH patients, the prevalence of obesity, DM, and body weight gain ≥10 kg compared to their 20-year-old body weights were significantly higher than in the respective control subjects. There is no significant differences between male NASH and male NAFL, and between female NASH and female NAFL.

Comparison of lifestyle among NASH, NAFL and control subjects. The results of the questionnaire survey are shown below (Table 2). Q.1) Skipping breakfast: no difference between NASH and control subjects in both females and males. Q.2) Time required for dinner: a higher percent of <30-min dinners in male NASH patients than in male controls (male NASH, 71%, male control, 43%); no difference in females. Q.3) Number of bowls of rice at dinner: percent who eat >one bowl significantly higher in NASH patients in both females and males than controls (male NASH, 56%, male controls, 29%, female NASH, 18%, female controls, 0%). Q.4) Meat dishes: percent who eat meat 3 times or

Table 1. Patient profile

	Male			Female		
	NASH	NAFL	Control	NASH	NAFL	Control
Number	93	16	21	78	13	28
Age (average)	45	49	46	58	61	54
Obesity (BMI >25) (%)	77.4**	68.8$^{▲▲}$	9.5	59**	69.2$^{▲▲}$	3.6
DM (%)	25.8*	12.5	4.8	42.3**	30.8$^{▲}$	3.6
HT (%)	32.3	56.3$^{▲}$	14.3	42.3**	46.2$^{▲▲}$	3.6
Dyslipidemia (%)	34.4	50$^{▲}$	14.3	44.9**	69.2$^{▲}$	10.7
Ethanol (g/week) (average)	29.2	62.1	62.7	7.6	9.4	54.4
Brinkman index (average)	198	109.1	63	57	93.8	4
+10 kg weight gain (%) (compare to their twenties)	50.5*	56.3$^{▲}$	23.8	62.8**	53.8$^{▲▲}$	7.1
Serum ALT (IU/L)	108 ± 84	73 ± 56	23 ± 8	86 ± 81	32 ± 19	21 ± 7

NASH vs Control *p<0.05, **p<0.01; NAFL vs Control $^{▲}p$<0.05, $^{▲▲}p$<0.01. There is no difference between NASH and NAFL.

Table 2. The results of the questionnaire survey among NASH, NAFL , and control subjects

	Male			Female		
	NASH	NAFL	Control	NASH	NAFL	Control
Q.1) Having breakfast everyday	59/93 (63%)	10/16 (63%)	13/21 (62%)	54/78 (69%)	9/13 (69%)	22/28 (79%)
Q.2) Time required for dinner (<30 min)	66/93 (71%)*	10/16 (63%)	9/21 (43%)	42/78 (54%)	8/13 (62%)	11/28 (39%)
Q.3) Rice at dinner (>1 bowl of rice)	52/93 (56%)*	7/16 (44%)	6/21 (29%)	14/78 (18%)*	2/13 (15%)$^{△}$	0/28 (0%)
Q.4) Meat dishes (≥3 times per week)	67/93 (72%)**	8/16 (50%)	8/21 (38%)	39/78 (50%)	8/13 (62%)	20/28 (71%)
Q.5) Fries (≥3 times per week)	44/93 (47%)**	3/16 (19%)	1/21 (5%)	19/78 (24%)	2/13 (15%)	4/28 (14%)
Q.6) Chinese noodles (≥3 times per week)	48/93 (52%)**	6/16 (38%)	4/21 (19%)	31/78 (40%)	4/13 (31%)	12/28 (43%)
Q.7) Eating out (≥3 times per week)	50/93 (54%)	9/16 (56%)	8/21 (38%)	30/78 (39%)*	2/13 (15%)	4/28 (14%)
Q.8) Instant food (≥2 times per week)	41/93 (44%)**	6/16 (38%)$^{△}$	1/21 (5%)	19/78 (24%)	1/13 (8%)	5/28 (18%)
Q.9) Fast food (≥once per week)	41/93 (44%)	5/16 (31%)	5/21 (24%)	31/78 (40%)	3/13 (23%)	6/28 (21%)
Q.10) Canned coffee or juice (≥3 bottles per week)	17/93 (18%)	1/16 (6%)	3/21 (14%)	5/78 (6%)	1/13 (8%)	0/28 (0%)
Q.11) Sweets (≥0.5 times per week)	73/93 (79%)**	10/16 (63%)	7/21 (33%)	59/78 (76%)*	6/13 (46%)	12/28 (43%)
Q.12) Cakes (≥once per week)	27/93 (29%)	3/16 (19%)	6/21 (29%)	27/78 (35%)	2/13 (15%)	8/28 (29%)
Q.13) No regular exercise	73/93 (79%)*	12/16 (75%)	12/21 (57%)	59/78 (76%)	10/13 (77%)	16/28 (57%)
Q.14) Elevator/escalator (prefer to use)	66/93 (71%)*	10/16 (63%)	10/21 (48%)	55/78 (71%)	8/13 (62%)	18/28 (64%)
Q.15) Commuting method (percentage of cars)	10/93 (11%)**	4/16 (25%)	10/21 (48%)	2/78 (3%)**	1/13 (8%)	6/28 (21%)

Q, question; NASH vs Control *p<0.05, **p<0.01; NAFL vs Control $^{△}p$<0.05, $^{△△}p$<0.01. There is no difference between NASH and NAFL.

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more per week significantly higher in male NASH patients than in male controls (male NASH, 72%; male control, 38%); no difference in females. Q.5) Fries: percent who eat fries 3 times or more per week significantly higher in male NASH patients than in male controls (male NASH, 47%; male control, 5%); no difference in females. Q.6) Chinese noodles: percent who eat Chinese noodles 3 times or more per week significantly higher in male NASH patients than in male controls (male NASH, 52%; male control, 19%); no difference in females. Q.7) Eating out: percent who eat out 3 times or more per week higher in female NASH patients than in female controls (female NASH, 39%; female control, 14%); no difference in males. Q.8) Instant foods: percent of eating instant foods 2 times or more per week significantly higher in male NASH patients than in male controls (male NASH, 44%; male control, 5%); no difference in females. Q.9) Fast food: percent who eat fast food once a week, no difference. Q.10) Coffee or juice cans: no difference between NASH and control subjects. Q.11) Sweets: frequency of eating sweets, higher in both male and female NASH patients than in respective controls (male NASH, 79%; male control, 33%; female NASH, 76%; female controls, 43%). Q.12) Cakes: frequency of eating cake once or more per week, no difference between NASH and control subjects. Q.13) Regular exercise: frequency of no exercise in male NASH patients significantly higher than in male controls (male NASH, 79%; male control, 57%); no difference in females. Q.14) Elevator: frequency of using elevator in male patients significantly higher than in male controls (male NASH, 71%; male control, 48%). Q.15) Commuting method: percentage of cars significantly lower in NASH patients in both females and males than in controls (male NASH, 11%: male control, 48%; female NASH, 3%; female control, 21%).

A comparison of NASH with NAFL showed no significant difference (Table 2).

Multivariate analysis was used for the comparison between male NASH and male controls, and 1) frequency of >one bowl of rice at dinner (Q.3) (p value, 0.007; Odds ratio, 25.9; 95% confidence interval 2.40–28.1), 2) frequency of eating meat 3 times or more per week (Q.4) (p value, 0.018; Odds ratio, 30.7; 95% confidence interval 1.80–523.5), and 3) frequency of using elevator (Q.14) (p value, 0.048; Odds ratio, 16.06; 95% confidence interval 1.03–250.7) were selected as independent variables.

Relationship between lifestyle and histological findings. When comparing the lifestyle factors between severe fibrosis (F3–F4) and mild fibrosis (F0–F2) of NASH, the frequency of eating cakes (Q.12) was higher in both male and female patients with severe fibrosis than in patients with mild fibrosis (Fig. 1). The other factors showed no differences between severe fibrosis and mild fibrosis.

Among NASH, the male NASH patients with 5 NAS points and over had high frequencies of rice (Q.3), Chinese noodles (Q.6) and instant food (Q.8). The female NASH patients with 5 NAS points and over had high frequencies of Chinese noodles (Q.6), and instant food (Q.8).

Regarding the relationship with steatosis grade, the male NASH patients with severe steatosis (S3) had high frequency of rice (Q.3). The female NASH patients with severe steatosis had high frequency of ≥10 kg body weight gain, and rice (Q.3). Regarding the relationship with ballooning, there were no significant differences in both male and female groups.

Lifestyle of non-obese NASH. Table 3 compares the questionnaire answers of obese NASH and non-obese NASH patients. The average age was younger in male obese NASH than in non-obese NASH. Average Brinkman index was higher in female obese NASH. In female NASH, obese patients had a higher prevalence of body weight gain (10 kg gain since their twenties). In male obese NASH patients, the amount of rice (Q.3) was larger and intake of instant food (Q.8) was smaller. Other questions were not different between obese NASH and non-obese NASH.

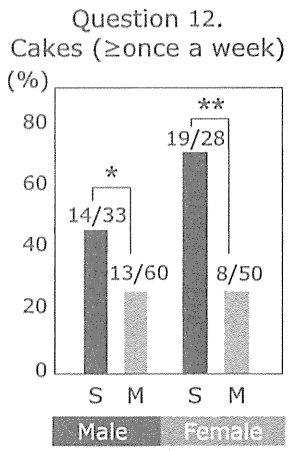

Fig. 1. The comparison of the frequency of eating cakes between severe fibrosis and mild fibrosis in NASH. The frequency of eating cakes once or more per week was significantly higher in NASH patients with severe fibrosis than in NASH patient with mild fibrosis. S; severe fibrosis, M; mild fibrosis, *p<0.05, **p<0.01.

Table 4 compares the questionnaire answers of non-obese NASH, non-obese NAFL, or non-obese control subjects. In males, non-obese NASH patients had a higher prevalence of body weight gain (≥10 kg gain since their twenties), larger intake of fries (Q.5), Chinese noodles (Q.6), and instant food (Q.8), compared to non-obese control. In females, DM, dyslipidemia, and ≥10 kg body weight gain were significantly different between non-obese NASH and non-obese control subjects. But there were no significant differences between non-obese NASH and non-obese NAFL.

Discussion

Our results suggested that dietary and exercise habits contributed to the development of male NASH. Yatsuji *et al.*[14] reported that, in Japan, about 55% of male NASH patients are under 45 years old, whereas only about 10% of female NASH patients are in this age category. Age and gender influence appetite and food preference, and other lifestyle factors. Overeating is rare in older women. Our data from single nucleotide polymorphism (SNP) analysis of the adiponectin gene revealed that the frequencies of adiponectin SNP sites were significantly different between NAFLD patients and controls in females only, not in males.[15] Several papers have reported the SNP of patatin-like phospholipase domain-containing protein 3 (PNPLA-3) gene played important role in occurrence and progression of NASH.[16-18] In a meta analysis, the influence of PNPLA-3 SNP was more powerful in female NAFLD patients.[16] These data suggest that our survey results are reasonable, pointing to the strong possibility that female NASH is influenced not so much by lifestyle but rather by genomic background. In addition, Yatsuji *et al.*[14] reported that DM was more prevalent in older female NASH patients. In our study DM was more common in female than male NASH patients. As many studies have suggested that genomic background is involved in the pathogenesis and occurrence of type 2 DM,[19,20] this data also suggest the role of genomic background in female NASH/NAFLD. In males, by contrast, our data suggested that overeating and less exercise play more important roles in NASH.

The importance of exercise was re-confirmed by our study. Streenivasa *et al.*[21] reported that daily aerobic exercise helped in normalizing ALT levels in NAFLD. Even if exercise is done at

Table 3. The comparison between obese NASH and non-obese NASH

	Male NASH		Female NASH	
	Obese	Non-obese	Obese	Non-obese
Number	73	20	46	32
Age (average)	43*	53	57	60
Ethanol (g/week) (average)	33	15	9	6
Brinkman index (average)	218	126	96*	0
DM	18/73 (25%)	6/20 (30%)	20/46 (44%)	13/32 (41%)
HT	27/73 (37%)	3/20 (15%)	19/46 (41%)	16/32 (50%)
Dyslipidemia	26/73 (36%)	5/20 (25%)	21/46 (46%)	14/32 (44%)
10 kg BW gain (compare to their 20's)	40/73 (55%)	7/20 (35%)	39/46 (85%)**	9/32 (28%)
Q.1) Having breakfast everyday	48/73 (66%)	11/20 (55%)	28/46 (61%)	26/32 (81%)
Q.2) Time required for dinner (<30 min)	54/73 (74%)	12/20 (60%)	26/46 (57%)	16/32 (50%)
Q.3) Rice at dinner (>1 bowl of rice)	45/73 (62%)*	7/20 (35%)	10/46 (22%)	4/32 (13%)
Q.4) Meat dishes (≥3 times per week)	54/73 (74%)	13/20 (65%)	25/46 (54%)	14/32 (44%)
Q.5) Fries (≥3 times per week)	35/73 (48%)	9/20 (45%)	10/46 (22%)	9/32 (28%)
Q.6) Chinese noodles (≥3 times per week)	38/73 (52%)	10/20 (50%)	20/46 (44%)	13/32 (41%)
Q.7) Eating out (≥3 times per week)	39/73 (53%)	11/20 (55%)	20/46 (44%)	10/32 (31%)
Q.8) Instant food (≥2 times per week)	32/73 (44%)*	15/20 (75%)	4/46 (9%)	4/32 (13%)
Q.9) Fast food (≥once per week)	33/73 (45%)	8/20 (40%)	18/46 (39%)	13/32 (41%)
Q.10) Canned coffee or juice (≥3 bottles per week)	14/73 (19%)	3/20 (15%)	5/46 (11%)	0/32 (0%)
Q.11) Sweets (≥0.5 times per week)	59/73 (81%)	14/20 (70%)	35/46 (76%)	24/32 (75%)
Q.12) Cakes (≥once per week)	22/73 (30%)	5/20 (25%)	16/46 (35%)	11/32 (34%)
Q.13) No regular exercise	59/73 (81%)	14/20 (70%)	35/46 (76%)	24/32 (75%)
Q.14) Elevator/escalator (prefer to use)	52/73 (71%)	14/20 (70%)	33/46 (72%)	22/32 (69%)
Q.15) Commuting method (percentage of cars)	9/73 (12%)	1/20 (5%)	1/46 (2%)	1/32 (3%)

Q, question; *p<0.05, **p<0.01.

Table 4. The comparison among non-obese groups

	Male			Female		
	Non-obese NASH	Non-obese NAFL	Non-obese control	Non-obese NASH	Non-obese NAFL	Non-obese control
Number	20	5	19	32	4	27
Age (average)	53	47	46	60	65	53.3
Ethanol (g/week) (average)	15	79.4	66.1	6	0	54.8
Brinkman index (average)	126	80	37.9	0	0	4.4
DM	6/20 (30%)	0	0	13/32 (41%)**	0	0
HT	3/20 (15%)	2/5 (40%)	2/19 (11%)	16/32 (50%)	3/4 (75%)	0
Dyslipidemia	5/20 (25%)	3/5 (60%)△	2/19 (11%)	14/32 (44%)**	2/4 (50%)	3/27 (11%)
10 kg BW gain (compare to their 20's)	7/20 (35%)*	2/5 (40%)△	1/19 (5%)	9/32 (28%)*	1/4 (25%)	1/27 (4%)
Q.1) Having breakfast everyday	11/20 (55%)	4/5 (80%)	12/19 (63%)	26/32 (81%)	4/4(100%)	22/27 (81%)
Q.2) Time required for dinner (<30 min)	12/20 (60%)	3/5 (60%)	8/19 (42%)	16/32 (50%)	2/4 (50%)	11/27 (41%)
Q.3) Rice at dinner (>1 bowl of rice)	7/20 (35%)	3/5 (60%)	4/19 (21%)	4/32 (13%)	0	0
Q.4) Meat dishes (≥3 times per week)	13/20 (65%)	3/5 (60%)	6/19 (32%)	14/32 (44%)	2/4 (50%)	20/27 (74%)
Q.5) Fries (≥3 times per week)	9/20 (45%)**	1/5 (20%)△	0	9/32 (28%)	0	4/27 (15%)
Q.6) Chinese noodles (≥3 times per week)	10/20 (50%)*	1/5 (20%)	3/19 (16%)	13/32 (41%)	1/4 (25%)	10/27 (37%)
Q.7) Eating out (≥3 times per week)	11/20 (55%)	2/5 (40%)	6/19 (32%)	10/32 (31%)	0	4/27 (15%)
Q.8) Instant food (≥2 times per week)	15/20 (75%)**	2/5 (40%)	1/19 (5%)	4/32 (13%)	0	5/27 (19%)
Q.9) Fast food (≥once per week)	8/20 (40%)	2/5 (40%)	3/19 (16%)	13/32 (41%)	0	6/27 (22%)
Q.10) Canned coffee or juice (≥3 bottles per week)	3/20 (15%)	0	2/19 (11%)	0	0	0
Q.11) Sweets (≥0.5 times per week)	14/20 (70%)	2/5 (40%)	8/19 (42%)	24/32 (75%)	2/4 (50%)	25/27 (93%)
Q.12) Cakes (≥once per week)	5/20 (25%)	0	5/19 (26%)	11/32 (34%)	2/4 (50%)	7/27 (26%)
Q.13) No regular exercise	14/20 (70%)	3/5 (60%)	9/19 (47%)	24/32 (75%)	3/4 (75%)	15/27 (56%)
Q.14) Elevator/escalator (prefer to use)	14/20 (70%)	2/5 (40%)	9/19 (47%)	22/32 (69%)	3/4(75%)	17/27 (63%)
Q.15) Commuting method (percentage of cars)	1/20 (5%)	3/6 (50%)	8/19 (42%)	1/32 (3%)	0	6/27 (22%)

Q, question; NASH vs Control *p<0.05, **p<0.01; NAFL vs Control △p<0.05, △△p<0.01. There is no difference between NASH and NAFL.

least once a week, there is a benefit for NAFLD patients.

Frequency of a proclivity for eating cakes was higher in both male and female NASH patients with severe fibrosis than in patients with mild fibrosis. The contents of dairy products, sugar, and high fat in cakes might be risk factors promoting liver fibrosis.

In this study, we compared the lifestyle of NASH to that of NAFL. The lifestyle tendencies of NAFL were almost the same as those of NASH. There are possible reasons why we could not detect a difference between NASH and NAFL. One is that some other factors, such as genomic background, might be involved. In

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fact, Tokushige[22] and Kawaguchi[17] have reported an influence of genomic background in the difference between NASH and NAFL. Another reason might be that the sample size of NAFL was too small.

In the comparison between obese NASH and non-obese NASH, no clear lifestyle differences were found. Further, Table 4 showed that even in male non-obese NASH, lifestyle was different from that in male non-obese subjects, suggesting that lifestyle plays an important role even in non-obese groups.

Samaha *et al.*[23] reported that a low-carbohydrate diet is effective against obesity and metabolic syndrome. Several studies have discussed which is more effective against obesity, a low-carbohydrate diet or a low-fat diet.[24] Although a low-carbohydrate diet induces oxidative stress, representative of the second hit of NASH, some articles have reported that a low-carbohydrate diet was effective against NASH.[25,26] It is not clear whether fats or carbohydrates are more important for the pathogenesis of NASH. Multivariate analysis showed that intake of rice, meat, and elevator use were important factors. We could not decide which, carbohydrate or fat, was more important.

When we care for NASH patients, investigation of the lifestyle of each patient is expected to suggest which points to emphasize. For example, we should recommend some or all of the following to male NASH patients: 1) When you eat dinner, take more than 30 min. 2) Limit yourself to one bowl of rice at dinner. 3) Eat only two meat dishes per week. 4) Eat fries twice or less per week. 5) Eat Chinese noodles a maximum of twice a week. 6) Eat instant food only once a week. 7) Eat as few sweets as possible. 8) Exercise regularly. 9) Use stairs as much as possible.

Toshimitsu and Cortez-Pinto[27,28] showed daily total carbohydrates, total fat and total calories in NASH patients. Although these data are scientific, the difficulty lies in the fact that patients had to guess the quantity of each nutrient from their meals. Our data are concrete, and the volume of rice and the frequencies of eating out, meat dishes, Chinese noodles, and fries are useful for the guidance and education of NASH patients. We expect that our data can be linked to therapeutic approaches, especially in male patients with NASH.

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Conflict of Interest

No potential conflicts of interest were disclosed.

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Efficient Induction of Apoptosis by Wee1 Kinase Inhibition in Hepatocellular Carcinoma Cells

Tomomi Kogiso[1]*, Hikaru Nagahara[2], Etsuko Hashimoto[1], Shunichi Ariizumi[3], Masakazu Yamamoto[3], Keiko Shiratori[1]

1 Institute of Gastroenterology, Department of Internal Medicine, Tokyo Women's Medical University, Tokyo, Japan, **2** Aoyama hospital, Tokyo Women's Medical University, Tokyo, Japan, **3** Institute of Gastroenterology, Department of Surgery, Tokyo Women's Medical University, Tokyo, Japan

Abstract

Transforming growth factor-β1 (TGF-β1) potently inhibits human hepatocellular carcinoma (HCC) cell growth. Here we demonstrated that TGF-β1-induced apoptosis is mediated by decreased phosphorylation of cdc2 at Tyr15 accompanied by down-regulation of Wee1 kinase expression. As expected from these results, a Wee1 kinase inhibitor efficiently induced apoptosis in HCC cells in the absence of TGF-β1 treatment. In surgically resected samples, Wee1 kinase was expressed in moderately to poorly differentiated HCC, whereas no Wee1 kinase expression was observed in non-cancerous tissue, including cirrhotic tissue. Our results suggest that Wee1 kinase inhibitors may be a practical novel therapeutic option against advanced HCC.

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* Email: kogiso@ige.twmu.ac.jp

Introduction

Human hepatocellular carcinoma (HCC) is a widespread disease brought about by long-term liver inflammation [1,2]. Although surgical and percutaneous radiofrequency ablation improve patient survival, new therapeutic options are needed to achieve effective treatment of advanced HCC [3–6]. Apoptotic induction by chemotherapy and low molecular weight material delivery are both promising options [6,7].

Previous studies showed that transforming growth factor-β1 (TGF-β1) stimulates cell proliferation in non-epithelial cells, such as fibroblasts and stellate cells, whereas it suppresses cell growth in both rodent and human HCC cells by inducing apoptosis or cell cycle arrest [8,9]. However, the main molecules involved in TGF-β1-induced apoptosis in HCC cells are largely unknown.

In this study, we evaluated human HCC cell lines to elucidate TGF-β1-induced apoptotic mechanisms. Our results demonstrated that we efficiently induced apoptosis in HCC cell lines using a Wee1 kinase inhibitor. These results may lead to development of novel therapeutic options against human HCC.

Materials and Methods

Cell culture

The human hepatocellular carcinoma cell line, HuH7, was purchased from the Riken Cell Bank (Wako, Saitama, Japan). The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% heat-inactivated fetal calf serum (FCS). TGF-β1-induced apoptosis-resistant HuH7 cells (HuH7R) were established from the HuH7 line by maintaining the cells with low-dose TGF-β1 (0.2 μg/mL; R&D Systems, Minneapolis, MN, USA) for 1-2 months. TGF-β1-induced apoptotic resistance was confirmed by flow cytometry following TGF-β1 treatment.

Flow cytometric analyses

Cells were trypsinized, harvested, and fixed with 70% ethanol at −20°C for 1 h. After washing with phosphate buffered saline (PBS), the cells were stained with propidium iodide (PI), and the cell cycle was analyzed using a FACScan (Becton Dickinson, Franklin Lakes, NJ, USA).

Cell treatment

For TGF-β1 treatment, cells were incubated in medium containing 0.1% FCS and 2 μg/mL TGF-β1. To counteract TGF-β1-mediated apoptosis, 20 μM roscovitine (Calbiochem, Nottingham, UK) was added to the medium 1 h prior to TGF-β1.

PD166285, a Wee1 kinase inhibitor, was provided by Pfizer (Ann Arbor, MI, USA) and used at a concentration of 200 nM. Roscovitine (20 μM) was also added 1 h prior to PD166285 administration to inhibit cdc2 activity resulting from the inhibition of PD166285-mediated apoptosis.

Immunoprecipitation and immunoblotting

Cells were lysed using 0.4 mL E1A lysis buffer [ELB: 50 mM HEPES (pH 7.2) 250 mM NaCl, 2 mM EDTA, 0.1% Nonidet P-40, 1 mM DTT, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 50 μg/mL phenylmethylsulfonyl fluoride, 0.5 mM NaP_2O_7, 0.1 mM

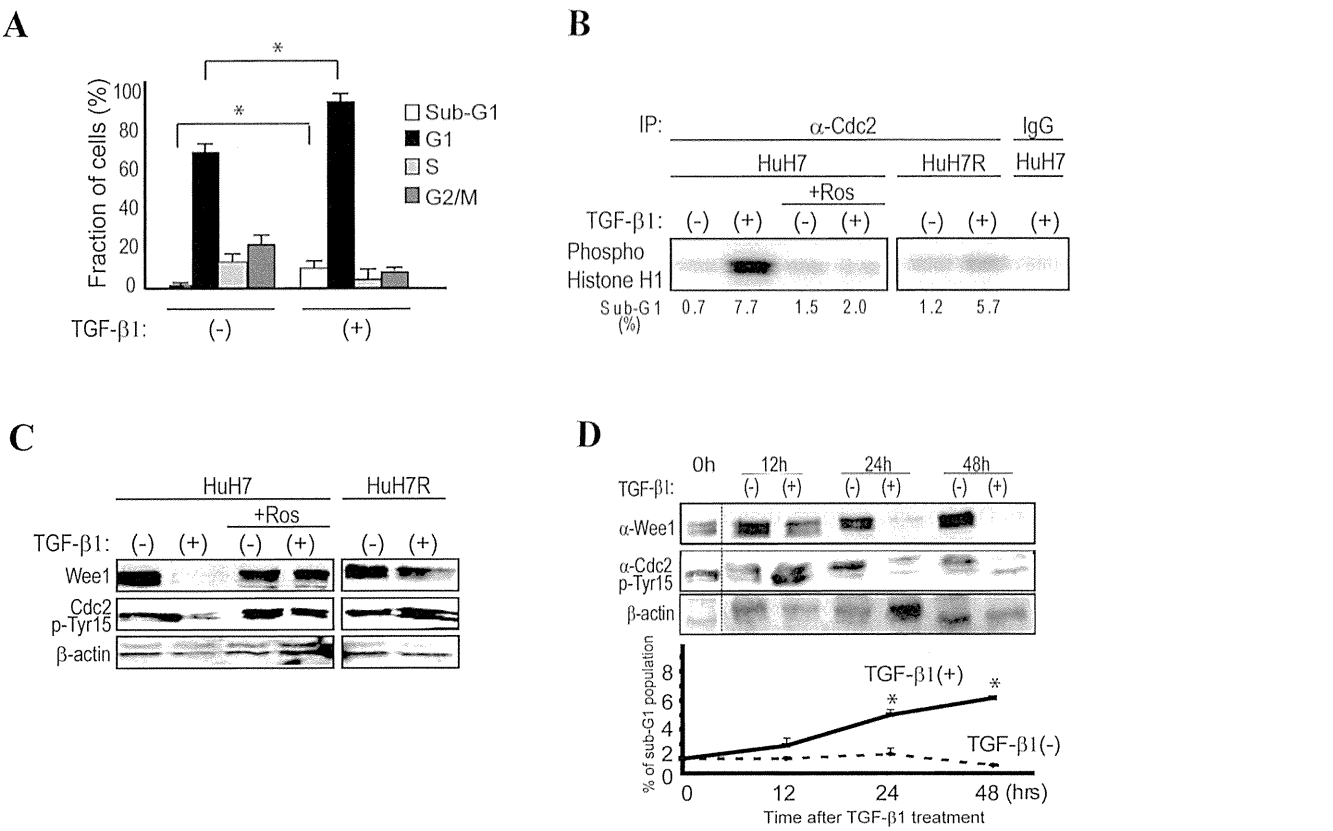

Figure 1. TGF-B1 induces apoptosis through cdc2 activation. A, FACS analyses showed that the sub-G1 phase HuH7 cell population increased from approximately 0.7% to 7.7%, and cells in the G1 phase increased from approximately 69% to 87% 48 h after TGF-β1 treatment. Data represent the means ± S.D. of three experiments. * indicates significant differences between each group ($P<0.05$). B, Cdc2 kinase activity was determined based on the level of phosphorylated histone H1 using histone H1 as a substrate. Cdc2 was activated 48 h after TGF-β1 treatment in HuH7 cells. However, cdc2 was not activated in HuH7R cells, which were isolated as an apoptosis-resistant clone from TGF-β1-treated HuH7 cells. Roscovitine (Ros)-pretreated HuH7 cells did not show cdc2 activation. A representative image from three independent experiments is shown. **C,** After TGF-β1 treatment (48 h), we observed cdc2 Tyr15 dephosphorylation in association with Wee1 kinase down-regulation in apoptotic cells. Pretreatment with 20 μM roscovitine completely abolished apoptosis and restored Wee1 kinase expression. TGF-β1 treatment induced G1 cell cycle arrest in HuH7R cells; however, Wee1 kinase expression and non-phosphorylated cdc2 Tyr 15 were similar to those of roscovitine-pretreated HuH7 cells. A representative image of three experiments is shown. **D,** Wee1 down-regulation and cdc2 Tyr15 dephosphorylation commenced approximately 24 h after TGF-β1 treatment, which was similar to thea initiation of apoptosis. A representative Western blot image is shown in the upper panels. The results in the lower graphs represent the means ± S.D. of three experiments. * indicates significant differences between each group ($P<0.05$).
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$NaVO_4$, and 5.0 mM NaF] (all reagents were purchased from Sigma-Aldrich, St Louis, MO, USA). The lysed cell solution was centrifuged, protein G or cdc2 antibodies were added to the supernatant, and the mixture was incubated at 4°C overnight. Immunoblots were prepared as described previously [10] and probed with anti-Wee1, anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or anti-cdc2-phospho-Tyr15 (Cell Signaling Technology, Danvers, MA, USA).

Kinase assay

TGF-β1-treated or untreated whole cell extracts were incubated with an anti-cdc2 antibody overnight at 4°C. The precipitates were washed and incubated with α-^{32}P-ATP for 20 min at 30°C in a kinase solution [10] containing histone H1 (Sigma-Aldrich), as a substrate of cdc2, and then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The incorporation rate of ^{32}P into histone H1 was measured using a PhosphoImager (BioRad Laboratories Hercules, CA, USA).

Transfection of short interfering RNA (siRNA)

siRNAs against Wee1 kinase were synthesized and cloned into a piGENEPURhU6 vector (Toyobo, Tokyo, Japan) and named psiWee1. psiWee1 was transfected into HuH7 cells using Optifect Reagent (Invitrogen, Carlsbad, CA, USA). The target sequences against Wee1 kinase were GGCAGAAGATGATCATATG (18-2) and GGCAGAAGCTGATCTTCTC (18-3).

Immunohistostaining

HCC tissue samples (n = 26) were obtained by surgical resection and authorized for immunohistochemical analysis after receiving written informed consent from each patient. This study was approved by the ethics committee of Tokyo Women's Medical University Hospital (Tokyo, Japan). The tissue sections were placed into 10 mM EDTA (pH 9.0), heated at 90–95°C for 40 minutes, and then incubated with normal rabbit serum and reacted with an anti-Wee1 antibody (1:200; Santa Cruz Biotechnology). We used DAKO Envision+System (DAKO, Glostrup, Denmark) horseradish peroxidase (HRP) as a secondary antibody

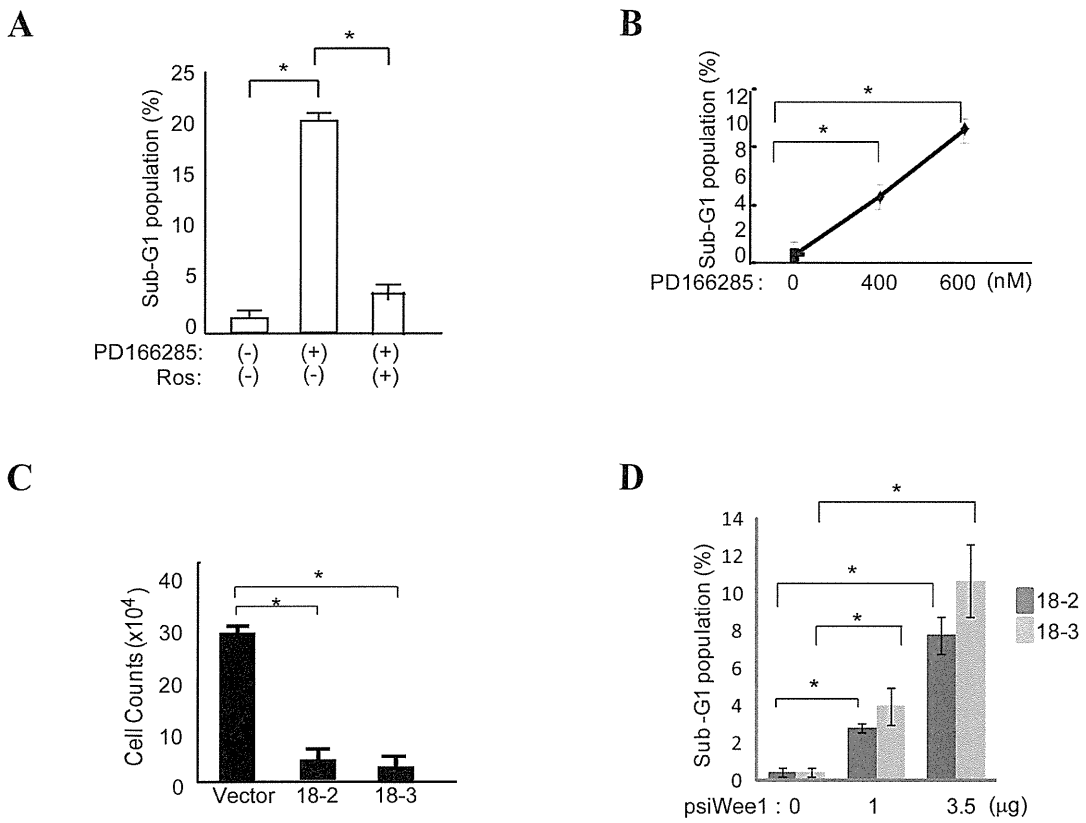

Figure 2. Targeting Wee1 kinase induces apoptosis in HCC cells. A, The number of sub-G1 HuH7 cells significantly increased after PD166285 treatment (200 nM). PD166285-mediated apoptosis of HuH7 cells was completely inhibited by pretreatment with 20 μM roscovitine (Ros). * indicates significant differences between each group ($P<0.05$). The results are presented as means ± S.D. of three experiments. **B,** PD166285 decreased the number of HuH7 cells in a concentration-dependent manner after 72 h. * indicates significant differences between each group ($P<0.05$). The representative results of three independent experiments are shown. **C** and D, Wee1 kinase-specific siRNAs (18-2 or 18-3) significantly reduced the total number of cells and increased the sub-G1 cell population 48 h after transfection in a concentration-dependent manner compared with control-vector transfectants. * indicates significant differences between each group ($P<0.05$).
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according to the manufacturer's instructions and detected signals using 3,3′ diaminobenzidine (DAB) as a substrate.

Statistical analyses

Significant differences between groups were determined using a Student's *t*-test or a chi-square test. $P<0.05$ was considered to be statistically significant.

Results

TGF-β1 induces apoptosis in HCC cells by cdc2 activation

TGF-β1 administration resulted in an increased proportion of the HuH7 cell population in the sub-G1 (demonstrative of apoptosis) and G1 phases (demonstrative of cell cycle arrest) (Figure 1A). FACS analyses revealed that the sub-G1 phase cell population increased from approximately 0.7% to 7.7%, and HuH7 cells in the G1 phase increased from approximately 69% to 87% after 48 h of TGF-β1 treatment (Figure 1A and B).

Consistent with previous reports [11], cdc2 was activated after TGF-β1 treatment in HuH7 cells (Figure 1B). We then examined whether cdc2 activity was necessary for the induction of apoptosis. We established an apoptosis-resistant HuH7 clone (HuH7R) that we isolated after long-term culture under low TGF-β1 concentration conditions. In these HuH7R cells, cdc2 was not activated, even after TGF-β1 treatment (Figure 1B). To further validate the role of cdc2 activation in the induction of apoptosis after TGF-β1 stimulation, we pre-treated HuH7 cells with roscovitine, a chemical inhibitor of cdc2. Roscovitine pretreatment markedly decreased the sub-G1 cell population and the cdc2 activity even after TGF-β1 treatment (Figure 1B). These results suggest that cdc2 activation is important for TGF-β1-induced apoptosis.

Cdc2 activity is regulated by its phosphorylation at Tyr15 [12–16]; thus, we examined Wee1 kinase expression and cdc2 Tyr15 phosphorylation after TGF-β1 treatment. As predicted, Wee1 kinase expression was down-regulated, and cdc2 Tyr15 was dephosphorylated in TGF-β1-treated cells (Figure 1C). Apoptosis-resistant HuH7R cells also preserved Wee1 kinase expression and Tyr15 phosphorylation of cdc2 even after TGF-β1 stimulation (Figure 1C). In a time course experiment, down-regulation of Wee1 expression and Tyr15 dephosphorylation of cdc2 commenced approximately 24 h after TGF-β1 treatment and was consistent with the initiation of apoptosis (Figure 1D).

Targeting Wee1 kinase induces apoptosis in HCC cells

Given the above findings, we tested whether apoptosis could be induced in HCC cells by inhibiting Wee1 kinase using a specific Wee1 kinase inhibitor, PD166285 [17,18], or siRNA against Wee1 kinase in the absence of TGF-β1 stimulation. Treatment with PD166285 alone increased the sub-G1 population of HuH7 cells in a concentration-dependent manner (Figure 2A and B).

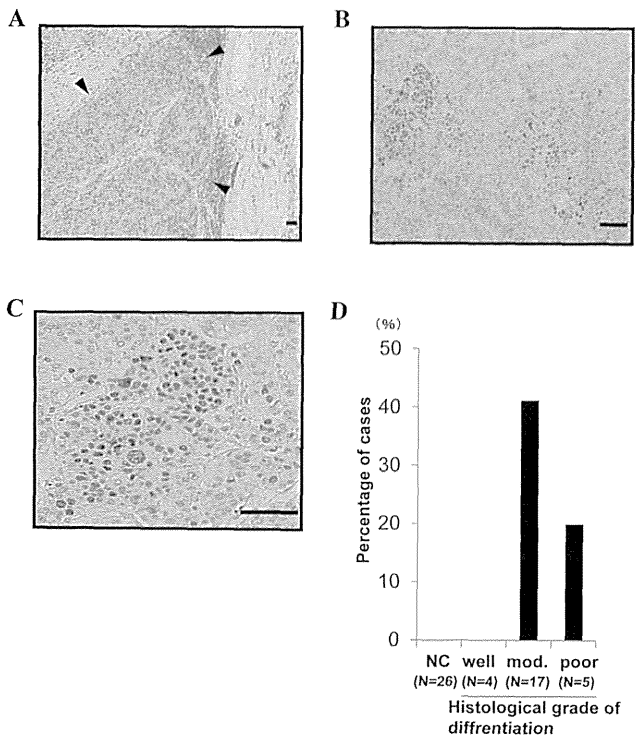

Figure 3. Wee1 kinase is expressed in moderately to poorly differentiated HCC. A-C, Wee1 kinase was stained with anti-Wee1 antibodies in surgically resected HCC samples (▼ HCC lesion). Anti-Wee1 antibody staining revealed mostly nuclear localization in Wee1 kinase-positive cells. The magnification is 40 x (A), 100 x (B), or 200 x (C). Scale bars = 200 μm. **D,** Wee1 kinase is expressed in moderately to poorly differentiated HCC. A chi-square test revealed significant differences ($p = 0.0026$). (NC; Non-cancerous lesion, Mod.; Moderately).
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Next we investigated the effect of roscovitine treatment on PD166285-mediated apoptosis, since roscovitine can inhibit TGF-β1-mediated apoptosis (Figure 1B). As expected, roscovitine exclusively inhibited apoptosis mediated by the Wee1 kinase inhibitor (Figure 2A), suggesting that this apoptotic pathway is mediated by an active form of cdc2. Although these are possible molecular mechanisms for the induction of apoptosis by the Wee1 kinase inhibitor, we do not fully exclude other possibilities.

We subsequently used two different siRNAs (18-2 and 18-3) targeting separate sites of Wee1 kinase mRNA to inhibit Wee1 kinase expression. HuH7 cells were transfected with these Wee1 siRNAs, and the resulting cell number and sub-G1 population of the transfectants were analyzed (Figure 2C and D). Both siRNAs induced apoptosis efficiently, compared with vector alone (Figure 2C), in a concentration-dependent manner (Figure 2D).

Wee1 kinase is expressed in moderately to poorly differentiated HCC but not in non-cancerous lesions

To determine the expression of Wee1 kinase in human HCC, we obtained tissue samples from surgically resected HCC and performed immunohistochemistry. Wee1 kinase immunohistochemistry revealed positive nuclear staining in cells located at the margins of HCC and non-cancerous lesions (Figure 3A–C). Wee1 kinase expression was detected in 7/17 (41.2%) moderately differentiated HCC tissues and 1/5 (20%) poorly differentiated HCC tissues, while there were no apparent positive cells in four well-differentiated HCC tissues and the corresponding 26 non-cancerous lesions (Figure 3D, $p = 0.0026$).

Discussion

TGF-β potently inhibits HCC cell growth by inducing cell cycle arrest and/or apoptosis [19–22]. Here we showed that the induction of apoptosis by TGF-β1 in HCC cells is mediated by de-phosphorylation of cdc2 Tyr15 (i.e., the active form of cdc2). This cdc2 activation was induced by Wee1 kinase down-regulation. In addition, we induced apoptosis in HCC cells by inhibiting Wee1 kinase using a specific inhibitor or siRNA.

Previous studies have identified several molecules that may play key roles in the induction or inhibition of apoptosis by TGF-β1 [23,24]. We determined here that cdc2 activation due to decreased Tyr15 phosphorylation may be crucial for TGF-β1-induced apoptosis in HCC cells. We observed decreased Wee1 kinase expression after TGF-β1 treatment, and cdc2 phosphorylation is regulated by Wee1 kinase. Therefore, it is possible that TGF-β1-mediated apoptosis was induced by a Wee1/cdc2 axis. However, the molecular mechanisms underlying the TGF-β1-mediated downregulation of Wee1 kinase are unclear.

In surgically resected samples, Wee1 kinase was over-expressed in HCC, particularly in moderately to poorly differentiated or advanced HCC. Wee1 kinase is a negative regulator of cdc2; thus, questions remain regarding the role of increased Wee1 kinase in HCC.

Based on our results, we focused on Wee1 kinase as a possible therapeutic target. As expected, a kinase inhibitor and siRNAs against Wee1 kinase efficiently induced apoptosis in HCC cells in the absence of TGF-β1. Because TGF-β1 is a multifunctional cytokine, it may not be a practical therapeutic option.

Overexpression of Wee1 kinase has been reported in other tumor types, including brain tumors and leukemia, and the usefulness of Wee1 kinase inhibitor has been demonstrated [25,26]. In brain tumors, the expression of Wee1 kinase was upregulated only in tumor cells, not in normal cerebellum, and the upregulated kinase had an important role in cancer cell survival [25]. Wee1 kinase negatively regulates G2/M phase by inhibiting the initiation of mitosis before DNA damage is repaired [27–29]. Thus, it is thought that Wee1 kinase abrogates premature mitotic entry and subsequent cell death [28,29]. Therefore, the inhibition of Wee1 kinase may be a promising new therapeutic strategy in Wee1 kinase-overexpressing carcinomas [25].

Wee1 kinase was expressed focally in the carcinoma tissues. Thus, for clinical applications, there would be some limitations on effectiveness. However, a Phase II trial using a Wee1 kinase inhibitor for the treatment of advanced solid cancers is currently underway [29,30]; thus, the use of this type of inhibitor may be a realistic therapeutic option against advanced HCC.

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Author Contributions

Conceived and designed the experiments: TK HN EH KS. Performed the experiments: TK HN. Analyzed the data: TK HN. Contributed reagents/materials/analysis tools: TK HN SA MY. Contributed to the writing of the manuscript: TK HN.

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INTERNAL MEDICINE

□ ORIGINAL ARTICLE □

Investigation of Ornithine Carbamoyltransferase as a Biomarker of Liver Cirrhosis

Noriko Matsushita[1,2], Etsuko Hashimoto[1], Katsutoshi Tokushige[1], Kazuhisa Kodama[1], Maki Tobari[1], Tomomi Kogiso[1], Nobuyuki Torii[1], Makiko Taniai[1], Keiko Shiratori[1] and Hiroshi Murayama[3]

Abstract

Objective Ornithine carbamoyltransferase (OCT) is a liver-specific mitochondrial matrix enzyme and potential biomarker of liver fibrosis. This study investigated the OCT levels in patients with chronic liver disease with or without cirrhosis in order to assess the usefulness of OCT as a biomarker of cirrhosis.

Methods The subjects included 440 Japanese patients with chronic liver disease and 80 control subjects. The patients were divided into two groups, those with and without cirrhosis, both of which were further stratified into high-OCT and low-OCT subgroups.

Results In the non-cirrhosis group, the patients with non-alcoholic steatohepatitis (NASH), alcoholic liver disease, primary biliary cirrhosis and primary sclerosing cholangitis (PSC) comprised the high-OCT subgroup, while the patients with hepatitis B, hepatitis C and autoimmune hepatitis formed the low-OCT subgroup. There were significant differences in the OCT levels, OCT/aspartate aminotransferase ratios and OCT/alanine transaminase (ALT) ratios between these two subgroups ($p<0.001$). The same findings were observed in the cirrhosis group. The OCT levels were markedly higher in the cirrhosis group than in the non-cirrhosis group, particularly among the patients with PSC ($p<0.001$). The most useful biomarker for predicting cirrhosis was the OCT/ALT ratio in the patients with hepatitis C and NASH and the OCT level in patients with PSC.

Conclusion The OCT level differs among patients with different chronic liver diseases. The role of OCT should be further evaluated in order to improve our understanding of the pathogenesis of these diseases. The OCT level is a useful surrogate marker of cirrhosis, particularly in PSC patients.

Key words: ornithine carbamoyltransferase (OCT), biomarker, liver cirrhosis, non-alcoholic steatohepatitis (NASH), primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC)

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Introduction

Ornithine carbamoyltransferase (OCT) is a mitochondrial matrix enzyme that catalyzes the second step of urea synthesis. It is almost exclusively expressed in the mitochondria of the liver and is not found in other organs apart, from a low level of expression in the small intestine (1).

The serum level of OCT exhibits a strong correlation with the levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Both of these transaminases are largely derived from the cytosol of hepatocytes, while OCT is released following mitochondrial injury; thus, the OCT/AST and OCT/ALT ratios indicate the degree of leakage of hepatic mitochondrial matrix enzymes relative to that of cytosolic enzymes (2).

The serum concentrations of these enzymes are influenced by the rates of leakage, degradation and clearance from the

[1]Department of Medicine and Gastroenterology, Tokyo Women's Medical University, Japan, [2]Support Center for Women Health Care Professionals and Researchers, Tokyo Women's Medical University, Japan and [3]Diagnostics Department, YAMASA Corporation, Japan
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Correspondence to Dr. Etsuko Hashimoto, drs-hashimoto@mti.biglobe.ne.jp

blood. Previous studies have demonstrated that the serum OCT level and OCT/AST and OCT/ALT ratios are significantly elevated in patients with chronic liver disease, especially those with alcoholic liver disease (ALD), followed by hepatocellular carcinoma (HCC) and liver cirrhosis (LC) (3-6).

Despite the discovery of OCT over 50 years ago, measurement of the OCT level has not become a common clinical test, although it is a highly liver-specific and abundant protein with a half-life of several hours (6).

A sensitive and reliable enzyme-linked immunosorbent assay (ELISA) for OCT was recently developed (7). Since then, we have been investigating this old biomarker of liver disease and have previously reported that the serum OCT level and OCT/ALT ratio both increase in parallel with the stage of fibrosis in patients with non-alcoholic steatohepatitis (NASH), with both parameters showing marked elevation in NASH patients with HCC (8). Such findings suggested to us that OCT is a potential serum marker of liver fibrosis.

Accordingly, the aims of the present study were to determine whether the serum OCT level differs among patients with various liver diseases (with or without LC) and to assess the usefulness of OCT as a biomarker for predicting the presence of LC.

Materials and Methods

Patients

Four hundred and forty Japanese patients who attended Tokyo Women's Medical University Hospital between January 2010 and December 2011 were included in the present case-control study. These patients had chronic liver disease caused by infection with hepatitis B virus (HBV) (hepatitis B, n=31) or hepatitis C virus (HCV) (hepatitis C, n=60), as well as autoimmune hepatitis (AIH, n=33), NASH (n=182), ALD (n=26), primary biliary cirrhosis (PBC, n=68) or primary sclerosing cholangitis (PSC, n=40). Patients with HCC or other malignancies were excluded. The control subjects (n=80) were Japanese adults without a history of chronic liver disease who underwent liver function tests and ultrasonography and were confirmed to have normal laboratory data with no evidence of fatty liver. Informed consent was obtained from all of patients and controls before entry into the study. This study conformed to the ethical guidelines of the Declaration of Helsinki (2008 revision), and the protocol was approved by our institutional research ethics committee.

Diagnosis of chronic hepatitis

Hepatitis B was diagnosed in patients with chronic hepatitis who were positive for HBV surface antigens, while hepatitis C was diagnosed in patients with anti-HCV antibodies and HCV-RNA. Patients who had received interferon or nucleoside analogs to treat HBV/HCV infection were excluded. AIH was diagnosed according to the criteria of the International Autoimmune Hepatitis Group (9). NASH was diagnosed based on the following criteria: (a) the detection of steatohepatitis on a histologic examination and (b) the exclusion of all secondary causes of hepatic fat accumulation, such as significant alcohol consumption (10). The diagnosis of ALD was made based on a history of chronic excessive alcohol intake (>70 g daily for more than five years), clinical evidence of liver disease with typical laboratory abnormalities and the exclusion of other causes of chronic liver disease. Ethanol intake was assessed by interviewing the patients and their family members (11). PBC was diagnosed in patients who met any two of the following three criteria: chronic cholestatic liver disease, positivity for anti-mitochondrial antibodies or anti-M2 antibodies or diagnostic liver histology (granulomatous cholangitis, etc.) (12). PSC was diagnosed using endoscopic retrograde cholangiopancreatography, and all patients with PSC met the criteria of the American Association for the Study of Liver Diseases (13).

The presence of cirrhosis was determined based on the results of a histopathological examination or unequivocal clinical and laboratory evidence of cirrhosis, such as ultrasound and/or computed tomography findings indicating cirrhosis (an irregular liver surface, splenomegaly, etc.) and the detection of signs/symptoms consistent with decompensated cirrhosis (jaundice, varices due to portal hypertension, ascites or hepatic coma). Fibrosis was staged as follows: stage 1 included portal fibrosis or zone 3 fibrosis in patients with ALD or NASH, stage 2 included periportal fibrosis, stage 3 included bridging fibrosis and stage 4 included LC. The patients were divided into groups, those with and without LC (non-LC group and LC group, respectively), then category of chronic liver disease was stratified into high-OCT and low-OCT subgroups based on a median OCT level of 50 ng/mL in the non-LC group and 70 ng/mL in the LC group. It has been reported that the OCT levels differ among patients with various chronic liver diseases (6). We hypothesized that the OCT levels in patients with steatohepatitis (NASH and ALD) and cholestatic hepatitis (PBC and PSC) would be higher than those observed in patients with viral hepatitis or hepatocyte injury (hepatitis B, hepatitis C and AIH). Because the pathogenesis of each chronic liver disease is quite different, we attempted to validate this hypothesis by investigating the subgroups. We set the normal range of the OCT level (mean ± 1.96 SD in 80 control=43 ng/mL) based on data obtained from 80 healthy control subjects (8).

Methods

All patients underwent various laboratory tests, including measurement of the AST, ALT, total billirubin (T-Bil), gamma-glutamyl transferase (GGT), albumin, platelet (Plt), immunoglobulin G and immunoglobulin M levels, as well as serology for HBV, HCV and the titers of autoantibodies. All patients also underwent ultrasonography.

The serum OCT levels were measured using ELISA, as previously reported (5, 6). In brief, 50μL of the horseradish peroxidase-conjugated F (ab') fragment of a monoclonal

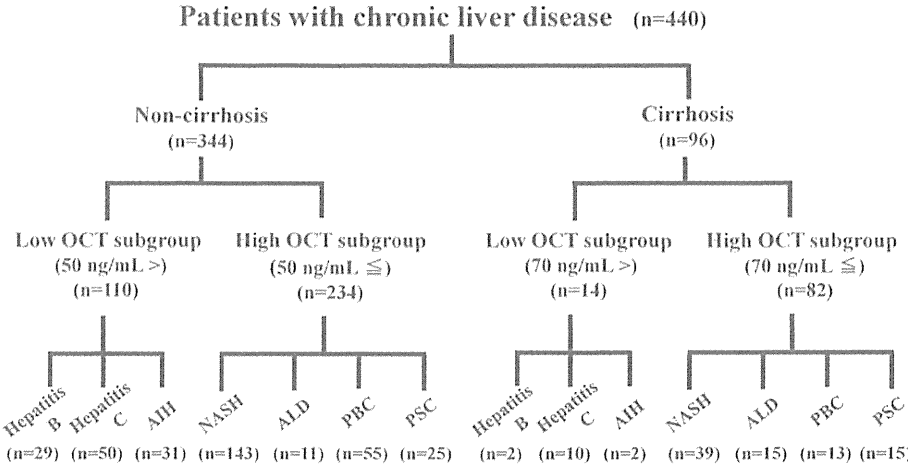

Figure 1. Diagram of the groups created in this study. Abbreviations: hepatitis B: chronic hepatitis B, hepatitis C: chronic hepatitis C, AIH: autoimmune hepatitis, NASH: non-alcoholic steatohepatitis, ALD: alcoholic liver disease, PBC: primary biliary cirrhosis, PSC: primary sclerosing cholangitis. Patients in the cirrhosis group with hepatitis B (n=2) or AIH (n=2) were excluded from the analysis due to their small numbers.

anti-OCT IgG antibody (secondary antibody: Mo5B11) and 50μL of standard solution or sample diluted 10-fold with assay buffer (250 mmol/L glycine buffer (pH 9.4) containing 0.1% bovine serum albumin, 50 mmol/L NaCl and 0.1% ProClin950) were added to the wells of an antibody-coated dish (primary antibody: Mo3B11). After mixing, incubation was performed for two hours, and the dish was washed with washing buffer (10 mmol/L phosphate buffer (pH 7.4) containing 0.1% BSA, 150 mmol/L NaCl and 0.1% ProClin 950). Then, a substrate solution (200μg/mL 3, 3', 5, 5'-teramethylbenzidine with 0.001% H_2O_2) was added for the coloring reaction. After 20 minutes, the reaction was terminated by adding a stop solution (0.5 mol/L H_2SO_4), and the absorbance at 450 nm was measured using a microplate reader.

Statistical analysis

The statistical analysis was performed using the IBM SPSS Version 20.0 (IBM SPSS Statistics, Armonk, USA) and Stat Light Yukms ver2.00 (Yukms Co., Ltd., Tokyo, Japan) software programs. The data are expressed as medians, with 25th and 27th percentiles. Statistical comparisons between the different categories of chronic liver disease were made using the Steel-Dwass test, while comparisons between the non-LC and LC groups or the high- and low-OCT subgroups were made using the Mann-Whitney U-test. The Kruskal-Wallis test was used to assess whether there were significant differences between any of the eight groups. The chi-square test was employed for categorical factors. If there were significant differences in the OCT levels or OCT/AST and OCT/ALT ratios between the non-LC and LC groups, a receiver operating characteristics (ROC) analysis was performed to assess the predictive value for LC. The area under the ROC curve (AUROC) was calculated to estimate cut-off values predicting LC with the optimum sensitivity and specificity. Spearman's rank correlation analysis was performed to assess the associations between the Plt and OCT levels and the OCT/AST and OCT/ALT ratios among the patients with each type of chronic liver disease.

Results

Characteristics of the non-LC group

Fig. 1 shows the method used to group the patients in the present study. The characteristics of the non-LC group (n=344) are listed in Table 1. The median ages of the patients with hepatitis C, AIH, ALD, PBC and PSC were in the 60's, whereas those of the patients with hepatitis B and NASH were in the 40's. Men accounted for approximately 50% of the patients with hepatitis B, hepatitis C, NASH and PSC. Although only approximately 10% of the patients with PBC and AIH were men, all of the ALD patients were men.

The median OCT levels were ranked in the following order: ALD, PSC, NASH, PBC, hepatitis C, AIH and hepatitis B. The differences were significant according to the Kruskal-Wallis test ($p<0.001$), and the median OCT level of the patients with each disease was also significantly higher than that of the control subjects according to the Kruskal-Wallis test ($p<0.001$).

In the non-LC group, the patients with NASH, ALD, PBC and PSC were classified into the high-OCT subgroup, while those with hepatitis B, hepatitis C and AIH formed the low-OCT subgroup. The differences in the OCT levels between the high-OCT subgroup (54.4 ng/mL) and the low-OCT subgroup (32.2 ng/mL) were statistically significant ($p<0.001$). The OCT/AST ratios in the high-OCT subgroup were significantly higher than those observed in the low-