Usefulness of Cytokeratin-18M65 in Diagnosing Non-Alcoholic Steatohepatitis in Japanese Population

Yutaka Hasegawa a  Soo Ryang Kim b  Takashi Hatae d  Mitsuhiro Ohta e  
Aya Fujinami e  Kayo Sugimoto c  Ke Ih Kim c  Susumu Imoto b  
Madoka Tohyama b  Soo Ki Kim f  Yoshihiro Ikura g  Masatoshi Kudo h

a Department of Pharmacy, Division of Medical Technology, Shinko Hospital, b Departments of Gastroenterology and Pharmacy, Kobe Asahi Hospital, c Educational Center for Clinical Pharmacy and d Medical Biochemistry, Kobe Pharmaceutical University, Kobe, e Department of Gastroenterology and Hepatology, Kyoto University, Kyoto, f Department of Pathology, Takatsuki General Hospital, Takatsuki, Osaka, and h Department of Gastroenterology and Hepatology, Kinki University, School of Medicine, Osaka-Sayama, Osaka, Japan

Key Words
Non-alcoholic steatohepatitis · Non-alcoholic fatty liver disease · CK-18M65 · Serum biomarker · Japanese population

Abstract
Objective: The aim of this study was to evaluate cytokeratin-18M65 (CK-18M65) for distinguishing between simple steatosis (SS) and non-alcoholic steatohepatitis (NASH) against healthy individuals (HIs) in Japanese population. Methods: The serum from 24 HIs, 21 patients with SS and 20 patients with NASH were examined. Serum CK-18M65 was measured by enzyme-linked immunosorbent assay. Results: Aspartate aminotransferase was significantly different between NASH patients and HIs with p < 0.0001 (SS patients and HIs: p < 0.0001), as was alanine aminotransferase between NASH patients and HIs with p < 0.0001 (SS patients and HIs: p < 0.0001). Serum CK-18M65 increased in a stepwise fashion in HIs and also in SS and NASH patients. Multivariate logistic regression analysis revealed that NASH could be diagnosed with the use of CK-18M65 alone (p = 0.0285, OR 1.0038, 95% CI 1.0004–1.0073). At the optimal cut-off level of 548 U/l, CK-18M65 had an AUC value of 0.7369, 60.00% sensitivity and 85.70% specificity. In patients with NASH, no significant difference was observed between low fibrosis (Stage 0–1, 794.30 ± 454.41, n = 10) and high fibrosis (Stage 2–3, 809.70 ± 641.43, n = 10; p = 0.5967) and between slight steatosis (<33%, 512.89 ± 229.65, n = 9) and moderate steatosis (≥33%, 655.13 ± 480.78, n = 32) in patients with non-alcoholic fatty liver disease (NAFLD; p = 0.7647) with the use of CK-18M65. Conclusion: Serum CK-18M65 distinguished NASH from SS, but could not assess the severity of steatosis in NAFLD patients or the grade of fibrosis in NASH patients in Japanese population.

Introduction

Non-alcoholic fatty liver disease (NAFLD), one of the most common and chronic liver diseases worldwide [1], comprises simple steatosis (SS) and non-alcoholic steatohepatitis (NASH); the latter is considered the hepatic manifestation of metabolic syndrome.
Among individuals with NAFLD, SS is a benign condition, but NASH can progress to fibrosis, cirrhosis and ultimately hepatocellular carcinoma [2–6]. The precise mechanism of NASH is poorly understood [7], although its etiology is attributed to insulin resistance and oxidative stress. Although used in the detection of NAFLD, imaging techniques such as CT and ultrasonography are not adequate for distinguishing between SS and NASH [7, 8]. Steatosis, ballooning, inflammation and fibrosis are major histopathological features of NASH [7, 9]. Liver biopsy used in the diagnosis of SS and NASH is an invasive procedure that requires hospitalization [10, 11] and may sometimes cause major complications like hemorrhage [10].

Therefore, there is an urgent need to develop simple, non-invasive serum biomarkers that can accurately distinguish between NASH and SS in patients and in healthy individuals (HIs), and to identify the grade of liver fibrosis in patients with NASH and the severity of steatosis in patients with NAFLD [7, 10].

Several serum biomarkers such as cytokeratin-18 (CK-18M65 and CK-18M30) and CD14 have been used to distinguish between SS and NASH [12–14]. CK-18M65 as a serum biomarker measuring overall cell death including cleaved and uncleaved caspase can distinguish NASH from SS patients, versus HIs in European populations, and has been useful in differentiating the severity of hepatic steatosis in patients with NAFLD [7].

The aim of this study is to assess the clinical value of CK-18M65 as a tool for distinguishing between SS and NASH, and assessing the grade of fibrosis in patients with NASH and the severity of steatosis in patients with NAFLD in Japanese population.

### Patients and Methods

NAFLD was diagnosed by abdominal ultrasonography and CT. The serum from 41 patients who underwent liver biopsy at Kobe Asahi Hospital were analyzed: 20 patients (8 men, 12 women, 58.80 ± 14.59 years) with NASH, 21 patients (15 men, 6 women 47.52 ± 14.38 years) with SS and 24 HIs (4 men, 20 women, 57.46 ± 6.88 years) who did not undergo biopsy (table 1).

Patients with evidence of excessive alcohol use (≥ 80 g/week) and other causes of liver disease (hepatitis B, hepatitis C, autoimmune liver disease and drug- or toxin-induced liver steatosis) were excluded from the study.

The study protocol was approved by the Clinical Research Ethics Committee of Kobe Asahi Hospital.

### Liver Histology

Liver biopsy specimens were fixed in formalin and embedded in paraffin, sectioned and stained with hematoxylin-eosin for routine histological analysis and evaluation by 2 expert hepatopathologists (S.R.K. and Y.I.). Fibrosis in NASH was evaluated using the Azan-Mallory stain.

Histological grading and staging of NAFLD were scored according to the system reported by Kleiner et al. [9]. Steatosis was classified by degree: F0 <5%, 5% ≤ F1 <33%, 33% ≤ F2 <66% and F3 ≥66%. Fibrosis was staged from 0 to 4: 0 = no fibrosis, 1 = perisinusoidal or periportal fibrosis, 2 = perisinusoidal and portal/periportal fibrosis, 3 = bridging fibrosis and 4 = cirrhosis.

### Clinical and Laboratory Assessments

Serum samples were examined for aspartate aminotransferase (AST; in IU/l), alanine aminotransferase (ALT; in IU/l), gamma-glutamyl transpeptidase (γ-GTP; in IU/l), total cholesterol (TC; in mg/dl), triglyceride (TG; in mg/dl), high-density lipoprotein cholesterol (HDL-C; in mg/dl), glucose (GLU; in mg/dl), hemoglobin (Hb; in g/dl) and platelets (PLT; in 10^4/μl).

The serum level of CK-18M65 was measured using the M65 ELISA kit (PEVIVA, Stockholm, Sweden) according to the manufacturers’ instructions [15] to determine the stages of fibrosis from
low (F0–F1, n = 10) to high (F2–F3, n = 10) in patients with NASH and the severity of steatosis from slight to moderate in patients with NAFLD.

**Statistical Analysis**

Variables are shown as means ± SD or percentages. Serum biomarkers in HIs as well as in patients with SS and NASH were compared using the Kruskal–Wallis and the Steel–Dwass tests.

Receiver operating characteristics (ROCs) was calculated for the cut-off value of NASH. The cut-off value was analyzed by the ROC curve. A p value of <0.05 was considered significant. Variables with a p value of <0.1 in the univariate analysis were included in stepwise multivariate logistic regression analysis. Variables with a p value of <0.05 in multivariate analysis were considered statistically significant. A multivariate logistic regression analysis was conducted to adjust for variables associated with NASH. Statistical analyses were computed with the use of Ekuseru-Toukei 2012 (Social Survey Research Information Co., Ltd., Japan).

**Results**

**Participant Characteristics and Univariate Analysis in Distinguishing between SS and NASH Patients against HIs**

The clinical and laboratory characteristics of the participants are described in table 1. Serum CK-18M65, AST, ALT, TG and GLU were high in SS and NASH patients compared with HIs. On the other hand, serum PLT was low in SS and NASH patients than in HIs. Significant differences were observed in the values of AST: NASH vs. HI (p < 0.0001) and SS vs. HI (p < 0.0001); ALT: NASH vs. HI (p < 0.0001) and SS vs. HI (p < 0.0001); γ-GTP: NASH vs. HI (p = 0.0001) and SS vs. HI (p = 0.0012); TC: SS vs. HI (p = 0.0256); TG: NASH vs. HI (p = 0.0440); HDL-C: NASH vs. HI (p = 0.0026) and SS vs. HI (p = 0.0090); GLU: NASH vs. HI (p = 0.0209); Hb: SS vs. HI (p = 0.019); PLT: NASH vs. HI (p = 0.0344). Also, CK-18M65 was significantly different among patients with NASH and SS and HIs (NASH vs. SS: p = 0.0255; NASH vs. HI: p < 0.0001; SS vs. HI: p < 0.0001).

**Diagnosis of NASH Using Serum Biomarkers in NAFLD Patients**

Multivariate logistic regression analysis revealed that NASH could be diagnosed by CK-18M65 alone (p = 0.0285, OR 1.0038, 95% CI 1.0004–1.0073; table 2).

To determine the predictive discriminating value of the serum biomarkers for the detection of NASH, ROC analysis was performed.

At the optimal cut-off level of 548 U/l, CK-18M65 demonstrated an AUC value of 0.7369, 60.00% sensitivity and 85.70% specificity in detecting NASH (fig. 1).

**Relation of CK-18 in NASH Fibrosis Grade and in NAFLD Steatosis Grade**

Fibrosis in 20 NASH patients ranged from stages 0 to 4: stage 0 (n = 0), stage 1 (n = 10), stage 3 (n = 6) and stage 4 (n = 4). No significant difference was found in the stages of fibrosis between low 0–1 (794.30 ± 454.41, n = 10) and high 2–3 (809.70 ± 641.43, n = 10; p = 0.5967; fig. 2). Also, no significant difference was observed between mild steatosis in 2 patients (<33%, 419.50 ± 12.02) and moderate steatosis in 18 patients (≥33%, 884.50 ± 555.04).

**Table 2. Multivariate logistic regression analysis associated with NASH**

<table>
<thead>
<tr>
<th>Variable</th>
<th>OR</th>
<th>95% CI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>1.0827</td>
<td>1.0126–1.1577</td>
<td>0.0200</td>
</tr>
<tr>
<td>CK-18M65</td>
<td>1.0038</td>
<td>1.0004–1.0073</td>
<td>0.0285</td>
</tr>
<tr>
<td>AST</td>
<td>1.0099</td>
<td>0.9928–1.0272</td>
<td>0.2585</td>
</tr>
<tr>
<td>TC</td>
<td>1.0224</td>
<td>0.9960–1.0495</td>
<td>0.0975</td>
</tr>
</tbody>
</table>

At a cut-off value of 548 U/l for CK-18M65, PPV and NPV in the diagnosis of NASH and SS demonstrated 80.00 and 69.23%, respectively, with a significant difference between them (p = 0.0036; fig. 1; data not shown).
Because of the small number of patients with NASH, the severity of steatosis analyzed in NAFLD patients \((n = 41)\) was mild \((<33\% \text{ in } 9 \text{ patients}, 512.89 \pm 222.65)\) and moderate \((>33\% \text{ in } 32 \text{ patients}, 655.13 \pm 480.78)\), with no significant difference between them (fig. 3).

**Discussion**

NAFLD comprises a spectrum of chronic diseases ranging from SS to NASH [16, 17].

Distinguishing between SS and NASH is crucial because the latter increases the risk of the progression of the disease to cirrhosis and hepatocellular carcinoma [18].

In distinguishing intermediate from minimal fibrosis – a prerequisite to clinical decision-making – fibrosis serum biomarkers such as collagen IV and hyaluronic acid do so only slightly, but are not useful in distinguishing NASH from SS [7].

At present, liver biopsy is the only reliable procedure for distinguishing SS from NASH and assessing the severity of liver damage and grading fibrosis [19]. It is, however, an invasive procedure that requires hospitalization.

There is, therefore, an urgent need to develop simple, non-invasive serum biomarkers that can accurately distinguish between NASH and SS vs. HIs and to identify the grade of liver fibrosis in patients with NASH and the severity of steatosis in patients with NAFLD [7, 10].

In this study, the level of serum AST was significantly higher in NASH patients than in HIs, and that of serum ALT was significantly different among HIs as well as SS and NASH patients; however, no significant difference was observed between SS and NASH. TG, AST and ALT levels increase significantly with the progression of NAFLD [20]; AST levels are significantly higher in NASH than in non-NASH patients [12]; the PLT count decreases in patients with NASH or severe fibrosis [21–23]. On the other hand, no statistical difference in platelet count has been found between non-NASH and NASH patients [24]. Nonetheless, these laboratory data are not adequate enough for differentiating NASH from SS; our results were compatible with those of Feldstein et al. [12] but not with those of Kashyap et al. [20].

Hepatocyte apoptosis has been recognized as a mechanism of liver injury that may also contribute to fibrogenesis [7]. Apoptosis is an active ATP-dependent process that contributes to the maintenance of tissue homeostasis under normal physiological conditions [25]. CK-18 is an intracellular protein mainly produced by necrosis and apoptosis of cells of epithelial origin including hepatocytes, and is a useful serum biomarker that reflects cell death [26]. Recently, serum CK-18M65 released in NAFLD patients in the process of the cellular death has been proposed as a possible serum biomarker of NASH [27, 28]. The increased serum levels of CK-18 fragments, in some clinical cohorts of obese patients or those with insulin resistance, are associated with hepatocyte injury, inflammation and fibrosis [29].

Of the 2 kinds of CK-18 (CK-18M65 and CK-18M30), CK-18M65 has been mentioned as a serum biomarker of overall cell death, including cleaved and uncleaved caspase, that can distinguish between NASH and SS patients and HIs in a European population [7] and can assess the severity of steatosis in patients with NAFLD and the grade of fibrosis in patients with NASH [7]. Nonetheless, the
clinical utility of CK-18 has been tested in 139 NAFLD patients in a European population [12], whereas there are various differences between the Japanese and the Europeans: body weight, diet, life style and genes related to disease occurrence.

Therefore, we aimed at evaluating CK-18M65 as a laboratory index for distinguishing among HIs and patients with SS and NASH and at assessing the severity of steatosis and the grade of fibrosis in patients with NASH in a Japanese population. The results demonstrated that serum CK-18M65 increased in a stepwise fashion in HIs and in patients with SS and NASH and that the value of CK-18M65 was significant (p = 0.0036) in distinguishing between SS and NASH.

By multivariate logistic regression analysis, CK-18M65 was independently associated with NASH (p = 0.0058, OR 1.00481, 95% CI 1.0014–1.0081). The ROC curve and the area under ROC curve were used to assess the utility of parameters in the diagnosis of NASH [29]. Moreover, in comparing between NASH and SS, the level of CK-18M65 detected by M65 ELISA, around 548 U/l, correctly predicted NASH with a sensitivity of 60.00%, a specificity of 85.7% and an AUC value of 0.7369 (fig. 1). These results suggest the efficacy of CK-18M65 as a serum biomarker in distinguishing patients with NASH from those with SS versus the HIs and its use as a non-invasive serum biomarker of NASH, replacing the burden of liver biopsies. It could not, however, assess the severity of steatosis and the stages of fibrosis.

The limits of this study lie in the paucity of the number of patients and the ethnic differences between the European and the Japanese populations.

Because of the limitations of our single-center study and the relatively small number of non-NASH patients, further studies in a large-scale, multicenter and validation cohort are needed to confirm our conclusions.

Acknowledgment

We are indebted to Ms Mika Matsui for assistance in the preparation of the manuscript.

Disclosure Statement

The authors have no financial conflicts of interest.

References


CK-18M65 in Diagnosing NASH

Dig Dis 2015;33:715–720

DOI: 10.1159/000439076

719


