Original Article
Serum cytokeratin 18 fragment level as a noninvasive biomarker for non-alcoholic fatty liver disease

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Abstract: Background and Aim: We evaluated the usefulness of serum cytokeratin 18 fragment (CK18-F) as a non-invasive biomarker in differentiating nonalcoholic steatohepatitis (NASH) from nonalcoholic fatty liver (NAFL) since the prognosis of the 2 diseases differ. Methods: 116 Japanese patients with nonalcoholic fatty liver disease (NAFLD) proven by liver biopsy were studied. Histological findings were classified according to the NAFLD activity score (NAS) proposed by the Nonalcoholic Steatohepatitis Clinical Research Network. The correlation between histological findings and serum CK18-F levels was investigated. Results: Serum CK18-F levels showed a positive correlation with histologic steatosis (ρ = 0.271, P = 0.0033), inflammation (ρ = 0.353, P = 0.0005), ballooning (ρ = 0.372, P = 0.0001), and the total NAS (ρ = 0.474, P = 2.68 × 10^-7). The serum CK18-F level was significantly lower for NAFL (NAS ≤ 2) than for borderline NASH (NAS of 3-4) or definite NASH (NAS ≥ 5) (P = 0.0294, P = 1.163 × 10^-5, respectively). The serum CK18-F level was significantly higher for definite NASH than for borderline NASH (P = 0.0002). The area under the receiver operating characteristic curve of serum CK18-F to predict the presence of NAFL and definite NASH was 0.762 and 0.757, respectively. The optimal cut-off point of serum CK18-F for NAFL and definite NASH was 230 and 270 U/L, respectively. The sensitivity, specificity, positive predict value, and negative predict value of serum CK18-F for NAFL were 0.89, 0.65, 0.34, and 0.97, and those for definite NASH were 0.64, 0.76, 0.72, and 0.67, respectively. Accuracies of diagnosis for both NAFL and definite NASH were 0.70. Conclusions: Serum CK18-F could be a clinically useful biomarker to discriminate between NAFL and NASH.

Keywords: Nonalcoholic fatty liver disease (NAFLD), serum cytokeratin 18 fragment (CK18-F), nonalcoholic fatty liver disease activity score (NAS), nonalcoholic fatty liver (NAFL), definite nonalcoholic steatohepatitis (NASH)

Introduction
Nonalcoholic fatty liver disease (NAFLD) is one of the most common causes of chronic liver disease and its prevalence is increasing worldwide [1, 2]. Nonalcoholic steatohepatitis (NASH), a progressive form of NAFLD, can develop into cirrhosis, hepatic failure, and hepatocellular carcinoma [3-5]. Approximately 20% of patients with NASH will develop cryptogenic cirrhosis and even end-stage liver disease [6]. In contrast, nonalcoholic fatty liver (NAFL) without inflammation is a non-progressive disease with well prognosis. Therefore, it is important to distinguish NAFL from NASH [6, 7] clinically. Liver histology is the current gold standard for the differential diagnosis and the definition of activity/fibrosis of NASH. However, many patients may not consent to a liver biopsy due to its inherent risks and for ethical reasons.

Instead of a liver biopsy, the grade of steatosis or fibrosis can be estimated using ultrasonography with a specific apparatus, magnetic resonance imaging (MRI), or a Fibroscan. However, use of these non-invasive tools is limited to specialized research centers due to their operating costs [8-11]. Although a combination of routine blood tests or specific fibrosis markers may be useful for estimating the degree of liver fibrosis caused by NASH, NASH activity is not easily determined by non-invasive methods [12-18].

The cytokeratin 18 fragment (CK18-F) which is reacted to M-30 monoclonal antibody is a sero-
logical marker of apoptosis and has been associated with severity of liver disease in patients with chronic hepatitis C (CHC) and NAFLD [19-21]. However, the ability for CK18-F to predict disease severity may differ between Japanese and Caucasian populations. Japanese populations have an elevated risk for co-morbid conditions at a lower body mass index (BMI) than Caucasian populations [22], which may be due to ethnic-specific differences in body composition profiles [23] and may influence the effectiveness of CK18-F use. Further study is needed to reevaluate the significance of CK18-F in relation to histologic findings in Japanese NAFLD patients since the previous report was relatively small scale [21].

The aim of this study was to determine whether serum CK18-F levels correlated with the NAFLD activity score (NAS) [24] and/or fibrosis in Japanese NAFLD patients. In addition, the cut-off value of CK18-F was examined for discriminating between NAFL and NASH.

### Table 1. Clinical features of 116 nonalcoholic fatty liver disease patients

<table>
<thead>
<tr>
<th>Demographic data</th>
<th>116</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>116</td>
</tr>
<tr>
<td>Sex (Male/Female)</td>
<td>41/75</td>
</tr>
<tr>
<td>Age (years)</td>
<td>61 (27-82)</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>27.2 (18.8-45.9)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Laboratory data</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>White blood cells (/μL)</td>
<td>5850 (3000-10700)</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>13.8 (10.5-17.7)</td>
</tr>
<tr>
<td>Platelets (×10⁴/μL)</td>
<td>20.2 (5.0-35.1)</td>
</tr>
<tr>
<td>Prothrombin time (%)</td>
<td>92 (55-100)</td>
</tr>
<tr>
<td>Aspartate aminotransferase (U/L)</td>
<td>42 (13-256)</td>
</tr>
<tr>
<td>Alanine aminotransferase (U/L)</td>
<td>52 (31-266)</td>
</tr>
<tr>
<td>Cholinesterase (U/L)</td>
<td>375 (169-559)</td>
</tr>
<tr>
<td>Alkaline phosphatase (U/L)</td>
<td>261 (89-573)</td>
</tr>
<tr>
<td>gamma-glutamyl-transpeptidase (U/L)</td>
<td>59 (9-263)</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>4.2 (2.5-5.1)</td>
</tr>
<tr>
<td>Fasting total cholesterol (mg/dL)</td>
<td>198 (101-343)</td>
</tr>
<tr>
<td>Fasting low density lipoprotein-cholesterol (mg/dL)</td>
<td>117 (49-234)</td>
</tr>
<tr>
<td>Fasting triglyceride (mg/dL)</td>
<td>139 (40-389)</td>
</tr>
<tr>
<td>Fasting plasma glucose (mg/dL)</td>
<td>107 (68-311)</td>
</tr>
<tr>
<td>Homeostasis model assessment-Insulin Resistance</td>
<td>2.89 (0.90-23.3)</td>
</tr>
<tr>
<td>Ferritin (ng/mL)</td>
<td>167 (7-3641)</td>
</tr>
<tr>
<td>Alpha-fetoprotein (ng/mL)</td>
<td>3 (1-18)</td>
</tr>
</tbody>
</table>

Data expressed as number of patients or median (range).

**Materials and methods**

**Patients**

A total of 116 NAFLD patients admitted to the Jikei University Katsushika Medical Center (Tokyo, Japan) between January 2010 and December 2013 for liver biopsies were enrolled. NAFLD was diagnosed using the following criteria: (1) abnormal alanine aminotransferase (ALT) levels (> 30 U/L) persisting for more than 6 months; (2) no consumption of alcohol or hepatotoxic drugs; (3) the presence of hepatic steatosis on ultrasonography [25] or cirrhosis without steatosis on a liver biopsy where steatosis was indicated in the past; (4) negative results for hepatitis B virus surface antigen, high titer of hepatitis B virus core antibody, or anti-hepatitis C virus antibody; and (5) absence of abnormal serum ceruloplasmin levels and transferrin saturation ratios. NAFLD diagnosis was confirmed by liver biopsy. Body height and weight were measured at admission and BMI was calculated.

This study was approved by the ethics committee of the Jikei University School of Medicine and the Jikei University Katsushika Medical Center and adheres to the principles of the Declaration of Helsinki. Written informed consent was obtained from all patients.

**Laboratory examination**

Fasting blood samples were obtained early in the morning on the day of the liver biopsy. Regular physical examinations, complete blood counts, and blood chemistries were carried out using standard methods. The serum low-density lipoprotein-cholesterol (LDL-C) concentrations were calculated using the Friedewald formula [26]. Remaining sera were immediately frozen and kept at -80°C until measurement of CK18-F. The homeostasis model assessment for insulin resistance (HOMA-IR) value was cal-
Cytokeratin 18 fragment as a biomarker for NAFLD

Figure 1. Relation between serum cytokeratin 18 fragment (CK18-F) level and nonalcoholic fatty liver disease activity score (NAS).

Figure 2. Serum cytokeratin 18 fragment (CK18-F) levels according to (A) nonalcoholic fatty liver disease activity score (NAS) and (B) fibrosis score.

culated as fasting glucose (mg/dL) × fasting insulin (μU/mL)/405 [27]. Serum level of CK18-F was measured using the M30-Apoptosense ELISA kit (PEVIVA AB, Bromma, Sweden).

Histopathologic examination

Ultrasonography-guided liver biopsies were performed at 2 different sites in the same lobe using a 16-gauge needle. The lengths of the sum of biopsy specimens were more than 1.8 cm. All biopsy specimens were placed in 10% neutral formalin solution for fixation and embedded in paraffin blocks, and sections were cut at 4 μm thickness stained by the hematoxylin-eosin and Masson trichrome. The median number of portal tracts found in each sample was 10 (range 7-12). Histologic findings
were assessed in a blinded fashion by an independent experienced pathologist and were scored according to the staging/grading system proposed by Brunt et al. [24]. Staging of liver fibrosis was scored as follows: stage 0: no fibrosis; stage 1: perisinusoidal or periportal fibrosis with 3 different patterns: 1A: mild, zone 3, perisinusoidal; 1B: moderate, zone 3, perisinusoidal fibrosis; stage 2: perisinusoidal and portal/periportal fibrosis; stage 3: bridging fibrosis; stage 4: cirrhosis. The grade of steatosis was scored from 0 to 3: 0: no steatosis or < 5%, 1: 5-33%, 2: 33-66%, 3: 66% < . Lobular inflammation was classified into 0 to 3: 0: no foci, 1: < 2 foci per 200 × field, 2: 2-4 foci per 200 × field, 3: 4 < foci per 200 × field. Ballooning was graded from 0 to 2: 0: none to rare, 1: few, 2: many. NAS was calculated as an un-weighted sum of the scores for steatosis (0 to 3), lobular inflammation (0 to 3) and ballooning (0 to 2), and ranged from 0 to 8. Cases with NAS of ≤ 2 were diagnosed as NAFL, while cases with NAS of 5 ≤ were diagnosed as definitive NASH. Cases with NAS of 3 and 4 were considered as borderline NASH [24].

Statistical analyses

Results were expressed as a number or as median (range). The Mann-Whitney U-test was used to analyze differences between continuous variables. Fisher’s exact tests were used to analyze differences in categorical data. Correlation coefficients were calculated using the Spearman rank correlation analysis. The serum CK18-F receiver operating characteristic (ROC) curves were plotted and the area under the ROC curves (AUROCs) calculated to represent their performance to predict NAFL or definite NASH. Statistical significance was determined by applying a two-tailed test and resulted in a P-value < 0.05. All statistical analyses were carried out using STATISTICA for Windows version 6 (StatSoft, Tulsa, OK, USA).

Results

Clinical characteristics of patients

The clinical features of 116 NAFLD patients are shown in Table 1. The median age was 61 years and median BMI was 27.2 kg/m².

Relationship between histologic findings and CK18-F levels

The median serum CK18-F levels based on steatosis grades 0, 1, 2, and 3 were 182.0, 223.0, 329.0, and 347.0 U/L, respectively. There was a positive correlation between the steatosis grade and the serum CK18-F level (ρ = 0.271, P = 0.0033). The median serum CK18-F levels based on inflammation grades 0, 1, 2, and 3 were 169.0, 235.5, 414.0, and 348.0 U/L, respectively. Serum CK18-F levels also showed positive correlation with inflammation grade (ρ = 0.353, P = 0.0005). The median serum CK18-F levels based on ballooning grades 0, 1, and 2 were 172.0, 292.0, and 445.0 U/L, respectively. Positive correlation (ρ = 0.372, P = 0.0001) was stronger for ballooning grade than steatosis and inflammation grades. In addition, there was a positive correlation between serum CK18-F levels and NAS (ρ = 0.474, P = 2.68 × 10⁻⁷) (Figure 1).

Serum CK18-F levels for differentiating between NAFL and definite NASH

The median of serum CK18-F with NAS of ≤ 2, NAS of 3 or 4 and NAS of 5 ≤ was 169.0, 244.0 and 456.0 U/L, respectively. Serum CK18-F level of NAFL (NAS ≤ 2) was significantly lower than borderline NASH (NAS 3 or 4) and definite NASH (with NAS of 5 ≤) (P = 0.0294, P = 1.163 × 10⁻⁵, respectively). In addition, serum CK18-F level of definite NASH was significantly higher than that of borderline NASH (P = 0.0002) (Figure 2A).

Relationship between serum CK18-F levels and liver fibrosis

The median serum CK18-F levels of fibrosis stage 0, 1, 2, 3, and 4 were 179.5, 264.5, 270.0, 377.5, and 400.0 U/L, respectively. The CK18-F level for fibrosis stage 0 was significantly lower than borderline NASH (NAS 3 or 4) and definite NASH (with NAS of 5 ≤) (P = 0.0294, P = 1.163 × 10⁻⁵, respectively). In addition, serum CK18-F level of definite NASH was significantly higher than that of borderline NASH (P = 0.0002) (Figure 2B).

Predict value of serum CK18-F for NAFL and definitive NASH

To assess the utility of serum CK18-F levels as a diagnostic tool in differentiating between
NAFL and definite NASH, we estimated the AUROC to be 0.762 (95% confidence interval [CI]: 0.665-0.859), and 0.757 (95% CI: 0.667-0.846) (Figure 3A, 3B), respectively. The optimal cut-off values of serum CK18-F for NAFL and definite NASH were 230 and 270 U/L, respectively. The sensitivity, specificity, positive predict value (PPV), and negative predict value (NPV) of the serum CK18-F cut-off value of 230 U/L for NAFL were 0.64, 0.76, 0.72 and 0.67, respectively, and of the serum CK18-F cut-off value of 270 U/L for definite NASH were 0.64, 0.76, 0.72 and 0.67, respectively. Diagnostic accuracies for both NAFL and definite NASH were 0.70 (Table 2).

Discussion

NAFL is a relatively benign form of NAFLD, while NASH commonly causes cryptogenic cirrhosis and may even result in hepatocellular carcinoma [3-5]. The gold standard for the differential diagnosis of NASH or NAFL is liver histology, but many patients do not consent to a liver biopsy.

Serum CK18-F has been reported as a noninvasive biomarker for differentiating NASH from NAFL or predicting the activity of NASH [21, 28-30]. CK18-F is only expressed during apoptosis, while total CK18 could be released from damaged cells during loss of cell membrane integrity [31]. Since CK18-F is generated mainly by caspase 3, which is reportedly activated in a NASH liver, it is conceivable that CK18-F is increased in the sera of NASH patients [32]. It has been reported that serum CK18-F levels were higher in NASH than that in non-NASH patients [21, 28, 29]. However, in the report from Belgium, the value of CK18-F is limited to predict development of NASH. Therefore, the significance of CK18-F on differential diagnosis of NASH has been controversial [33]. There have been only a few reports on CK18-F levels with regard to NAFLD in Asian countries [21, 34]. With respect to the development of NASH, the difference in genetic and nongenetic backgrounds of general populations between Asian and Western countries have been suggested [2]; therefore, further studies on the significance of CK18-F levels for NAFLD are required.

Figure 3. A. Receiver operating characteristic (ROC) plot for cytokeratin 18 fragment (CK18-F) in differentiating non-alcoholic fatty liver disease activity score (NAS) ≤ 2 in the total cohort (n = 116). CK18-F had an area under the receiver-operator curve (AUROC) of 0.762 (95% confidence interval [CI]: 0.665-0.859). B. ROC plot for CK18-F in differentiating NAS ≥ 5 in the total cohort. CK18-F had an AUROC of 0.757. (95% CI: 0.667-0.846).

Table 2. Performance of CK 18-F to predict NAS ≤ 2 in those with determinant value

<table>
<thead>
<tr>
<th>CK18 fragment</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
<th>Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>270 (for NAS ≤)</td>
<td>0.64</td>
<td>0.76</td>
<td>0.72</td>
<td>0.67</td>
<td>0.70</td>
</tr>
<tr>
<td>&lt; 230 (for NAS ≤ 2)</td>
<td>0.89</td>
<td>0.65</td>
<td>0.34</td>
<td>0.97</td>
<td>0.70</td>
</tr>
</tbody>
</table>

CK 18-F, cytokeratin 18 fragment; NAS, non-alcoholic fatty liver disease activity score NPV, negative predictive value; PPV, positive value.
In the present study, similar to previous studies, serum CK18-F levels were closely correlated with individual disease components (steatosis, inflammation, and ballooning) and with the overall NAS. Among the individual disease components, ballooning had the strongest correlation with serum CK18-F level. Brunt et al. reported that ballooning is the most significant histological feature in determining a diagnosis of NASH [24]. Since there was a high correlation between the serum CK18-F level and ballooning, CK18-F may be useful for differentiating between NAFL and NASH. However, similar to the previous report [21], our findings indicated that the serum CK18-F level did not associate with advanced fibrosis in NASH.

We tried to determine the serum CK18-F cut-off value for diagnosis of definite NASH compared to NAFL since a value did not previously exist [35]. The optimal cut-off points of serum CK18-F for NAFL and definite NASH were 230 and 270 U/L, respectively. Serum CK18-F (< 230 U/L) performed well as a screening test for NAFL given its high sensitivity (0.89) and high NPV (0.97), while it had low specificity (0.65) and low PPV (0.34). The serum CK18-F (> 270 U/L) did not perform well as a screening test for definite NASH given its low sensitivity (0.64) and NPV (0.67), but did have a relatively higher specificity (0.76) and PPV (0.72). Accuracies of diagnosing both NAFL and definite NASH were comparative.

Although previous reports showed a limited value of using serum CK18-F levels as a biomarker for NASH and fibrosis in patients with NAFLD [36], our findings suggest that serum CK18-F level may be useful for differentiating between NAFL and NASH.

In conclusion, this study established the use of serum CK18-F as a noninvasive biomarker for differentiating between NAFL and NASH. By combining the serum CK18-F level with other noninvasive markers, higher-precision prognosis prediction for NAFLD may be attained.

Disclosure of conflict of interest

None.

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References

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