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Soluble forms of extracellular cytokeratin 18 may differentiate simple steatosis from nonalcoholic steatohepatitis

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Abstract

AIM: To investigate whether serum levels of two soluble forms of extracellular cytokeratin 18 (M30-antigen and M65-antigen) may differentiate nonalcoholic steatohepatitis (NASH) from simple steatosis in patients with nonalcoholic fatty liver disease (NAFLD).

METHODS: A total of 83 patients with suspected NAFLD and 49 healthy volunteers were investigated. Patients with suspected NAFLD were classified according to their liver histology into four groups: definitive NASH (n = 45), borderline NASH (n = 24), simple fatty liver (n = 9), and normal tissue (n = 5). Serum levels of caspase-3 generated cytokeratin-18 fragments (M30-antigen) and total cytokeratin-18 (M65-antigen) were determined by ELISA.

RESULTS: Levels of M30-antigen and M65-antigen were significantly higher in patients with definitive NASH compared to the other groups. An abnormal value (> 121.60 IU/L) of M30-antigen yielded a 60.0% sensitivity and a 97.4% specificity for the diagnosis of NASH. Sensitivity and specificity of an abnormal M65-antigen level (> 243.82 IU/L) for the diagnosis of NASH were 68.9% and 81.6%, respectively. Among patients with NAFLD, M30-antigen and M65-antigen levels distinguished between advanced fibrosis and early-stage fibrosis with a sensitivity of 64.7% and 70.6%, and a specificity of 77.3% and 71.2%, respectively.

CONCLUSION: Serum levels of M30-antigen and M65-antigen may be of clinical usefulness to identify patients with NASH. Further studies are mandatory to better assess the role of these apoptonecrotic biomarkers in NAFLD pathophysiology.

Key words: Steatosis; Steatohepatitis; Cytokeratin 18; M30-antigen; M65-antigen

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a common condition comprising a wide spectrum of liver damage associated with metabolic disorders, including central obesity, dyslipidemia, hypertension, and hyperglycemia. The spectrum of disease is variable, ranging from simple steatosis with benign prognosis, to nonalcoholic steatohepatitis (NASH), advanced fibrosis and cirrhosis, conferring increase in morbidity and mortality[1,2].

NASH could be present in one third of NAFLD cases and appears to have a higher likelihood of progression to cirrhosis. Furthermore, an increased risk of hepatocellular carcinoma and end-stage liver disease has been reported among patients with NASH[3,4]. It follows that early recognition of subjects with NASH is crucial to prevent the development of severer forms of liver disease and improve the clinical outcome. Differentiation of simple steatosis from NASH requires histopathologic evaluation[5]. In this regard, liver biopsy remains the most sensitive and specific means of providing important diagnostic and prognostic information[6,7]. Nonetheless, given that liver biopsy is an expensive procedure, with occasional complications and poor patient acceptance[8-11], the search for surrogate markers to replace liver biopsy is highly recommended.

A growing body of evidence has recently suggested that dysregulation of hepatocyte apoptosis could play an important role in the progression of NAFLD to

NASH[12-14]. During apoptosis, a number of intracellular proteins are cleaved by caspases. A neoepitope in cytokeratin 18 (CK18), termed M30-antigen, becomes available at an early caspase cleavage event during apoptosis and is not detectable in vital or necrotic cells[15-18]. A monoclonal antibody, M30, specifically recognizes a fragment of CK18 cleaved at Asp396 (M30-antigen)[19]. By contrast, the cytosolic pool of uncleaved CK18 (also termed M65-antigen) is released from cells during necrosis[19]. These findings implicate that assessments of different forms of CK18 in patient sera (M30-antigen for apoptosis and M65-antigen for necrosis) could be used to examine different cell death modes *in vivo*. Two robust immunoassays are currently available to measure the levels of M30-antigen and M65-antigen[20-22].

In this study, we have measured different forms of CK18 (M30-antigen and M65-antigen) in serum from NAFLD patients as an approach for differentiating simple steatosis from NASH. Specifically, we wanted to establish a reliable diagnostic model by using these non-invasive biomarkers.

MATERIALS AND METHODS

Study participants

Between November 2005 and October 2006, we enrolled a total of 83 patients (38 females and 45 males, age range: 25-76 years) with suspected NAFLD. By the ATP Ⅲ Expert Panel of the U.S. National Cholesterol Education Program criteria[23], approximately one third of participants had the metabolic syndrome. Patients with viral hepatitis, hemochromatosis, Wilson's disease, autoimmune hepatitis, primary biliary cirrhosis, sclerosing cholangitis, biliary obstruction, alpha-1 antitrypsin deficiency, or malignancies were excluded from the present study. None of the subjects was using any medications, including estrogens, amiodarone, steroids, tamoxifen, or herbal supplements. Furthermore we excluded patients with daily alcohol intake exceeding 20 g/d or previous abdominal surgery. For control purposes, 49 healthy age- and gender-matched volunteers were recruited. All controls were judged to be in good health, with normal results on liver function tests and confirmed as having normal liver by ultrasound. Subjects with a consumption of alcohol > 20 g/d or who were taking any medication were not included in the control group.

A written informed consent was obtained from all participants. The study protocol was reviewed and approved by the Ethics Committee of the Uludag University Medical School.

Clinical assessment

All subjects underwent physical examination, anthropometric measurements and biochemical screening. Liver ultrasound (US) scanning was performed to assess the degree of steatosis. All US procedures were performed by the same operator. Liver steatosis was assessed semiquantitatively on a scale of 0 to 3: 0, absent; 1, mild; 2, moderate; and 3, severe. Computed tomography (CT) evaluation of liver parenchyma was performed in all NAFLD patients. An experienced pathologist examined liver histology according to the NIDDK NASH Clinical Research Network scoring system[24]. Patients with suspected NAFLD were classified according to their liver histology into four groups: definitive NASH (n = 45), borderline NASH (n = 24), simple fatty liver (n = 9), and normal tissue (n = 5). In a secondary analysis, patients with borderline NASH, simple fatty liver, and normal tissue were grouped together and analyzed as a single group which is named as no-NASH.

Histological analysis

Ultrasonography-guided liver biopsies were performed under conscious sedation using a 16-gauge Klatskin needle. The length of histological specimens was not smaller than 2.5 cm. All biopsy specimens were placed in formalin solution for fixation and embedded in paraffin blocks. Serial sections (sectioned at 4 μm intervals) were stained with hematoxylin-eosin, Masson's trichrome. An experienced pathologist blinded to clinical data scored the liver biopsies according to the NIDDK NASH Clinical Research Network scoring system[24]. Steatosis was scored from 0 to 3 with a four grades scoring system from S0 to S3: S0: no steatosis or less than 5%, S1: 5%-33%, S2: 33%-66%, S3: > 66%. Lobular inflammation was graded as follows: stage 0, no foci; stage 1: < 2 foci per 200 × field; stage 2: 2-4 foci per 200 × field; stage 3: > 4 foci per 200 × field. Fibrosis was staged 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, and 1C portal/periportal fibrosis; stage 2: perisinusoidal and portal/periportal fibrosis; stage 3: bridging fibrosis; stage 4: cirrhosis. The histological NASH score was defined as the unweighted sum of the scores for steatosis (0-3), lobular inflammation (0-3), and ballooning (0-2); thus ranging from 0 to 8. Cases with scores of 0 to 2 were considered as having simple steatosis; on the other hand, cases with scores of 5 or greater were diagnosed as definitive NASH[24]. Cases with activity scores of 3 and 4 were considered as borderline (probable) NASH.

ELISA assays

Blood samples were centrifuged at 2500 g for 10 min, and serum aliquots were stored at -80℃ until immediately before analysis. All samples were analyzed in duplicate and in a blinded fashion. Serum levels of M30-antigen and M65-antigen were determined by commercially available immunoassays (M30-Apoptosense ELISA kit and M65 ELISA kit, Peviva AB, Bromma, Sweden) according to the manufacturer's instructions. The M65-ELISA assay measures native soluble CK18 (M65-antigen), whereas the M30-Apoptosense ELISA kit measures the levels of the CK18-Asp396 neo-epitope (M30-antigen). Briefly, samples were placed into wells coated with a mouse monoclonal antibody as a catcher. After washing, a horseradish peroxidase conjugated antibody (M30 or M65) was used for detection. Reference concentrations of M30-antigen or M65-antigen were used to prepare assay calibration. The absorbance was determined with an ELISA reader at 450 nm.

Table 1 General and biochemical characteristics of patients with suspected NAFLD

Characteristic	Entire cohort (n = 83)	Definitive NASH (n = 45)	Borderline NASH (n = 24)	Simple steatosis (n = 9)	Normal tissue (n = 5)
Male gender	54.2%	62.2%	50.0%	44.4%	20.0%
Age (yr)	48.9 ± 9.1	47.8 ± 8.8	48.7 ± 6.2	53.1 ± 10.1	52.4 ± 19.2
Body mass index (kg/m^2)	30.3 ± 4.8	30.2 ± 4.1	31.1 ± 6.5	29.5 ± 3.7	28.0 ± 3.0
Waist circumference (cm)	99 ± 9	101 ± 8	100 ± 9	94 ± 10	93 ± 11
Waist/hip ratio	0.93 (0.01-1.08)	0.95 (0.09-1.08)	0.93 (0.09-1.02)	0.8 (0.74-1.05)	0.86 (0.01-0.87)
Hypertension	33.7%	28.9%	50%	22.2%	20%
Systolic blood pressure (mmHg)	122 ± 13	124 ± 13	121 ± 13	121 ± 16	115 ± 11
Diastolic blood pressure (mmHg)	77 ± 10	80 ± 9	76 ± 11	71 ± 10	72 ± 9
Diabetes mellitus	14.5%	13.3%	16.7%	22.2%	0%
Fasting glucose (mg/dL)	103 ± 22	102 ± 21	105 ± 25	109 ± 25	93 ± 7
Fasting insulin (μU/mL)[1]	24 (1-298)	34 (3-298)	21 (1-281)	20 (1-110)	11 (1-16)
HOMA index	3.0 (0.2-16.5)	3.1 (0.3-16.5)	2.9 (0.3-8.1)	2.6 (0.2-4.9)	2.3 (1.6-3.8)
Hyperlipidemia history	83.1%	80.0%	91.7%	77.8%	80%
Total cholesterol (mg/dL)	213 ± 40	213 ± 44	224 ± 31	190 ± 27	199 ± 49
Triglycerides (mg/dL)[1]	149 (38-424)	151 (38-424)	164 (90-398)	102 (49-286)	149 (44-182)
Metabolic syndrome	34.9%	37.8%	33.3%	33.3%	20.0%
Lp(a) (mg/dL)[1]	10 (2-157)	9 (2-98)	20 (2-157)	6 (2-68)	19 (5-32)
Microalbuminuria (μg/mL)[1]	1.0 (0.2-48.4)	4.2 (0.2-48.4)	0.7 (0.2-3.8)	0.65 (0.3-8.9)	0.4 (0.2-1.0)
AST (IU/L)[1]	42 (16-102)	44 (18-95)	33 (16-102)	32 (17-65)	28 (19-91)
ALT (IU/L)[1]	60 (10-184)	68 (23-184)	47 (12-146)	47 (19-84)	35 (10-149)
AST/ALT ratio	0.68 (0.34-1.90)	0.65 (0.34-0.95)	0.71 (0.45-1.42)	0.77 (0.59-1.21)	0.8 (0.6-1.9)
ALP (UI/L)[1]	82 (19-154)	84 (44-152)	85 (43-132)	94 (19-154)	76 (61-131)
GGT (UI/L)[1]	47 (15-526)	52 (16-526)	29 (15-199)	65 (22-164)	73 (17-124)
LDH (UI/L)[1]	196 (132-379)	198 (143-375)	193 (132-295)	184 (163-379)	171 (152-340)
Total Bilirubin (mg/dL)[1]	0.7 (0.1-2.5)	0.7 (0.1-2.5)	0.6 (0.1-1.7)	0.5 (0.1-0.8)	0.8 (0.4-2.1)
HbA1c	5.6 (4.6-9.9)%	5.7 (4.7-9.9)%	5.5 (4.6-7.7)%	5.6 (5.1-7.4)%	5.5 (5.4-6.2)%
Transferrin saturation	19 (2-48)%	20 (2-48)%	17 (5-29)%	23.5 (11-44)%	18 (8-28)%
Ferritin (ng/mL)[1]	77 (6-406)	93 (6-399)	69 (12-297)	96 (10-406)	23 (9-61)
Positive ANA	21.6%	23.9%	20.8%	0%	40.0%
Positive AMA	1.2%	2.1%	0%	0%	0%
Positive ASMA	7.2%	4.3%	4.4%	0%	20.0%
Positive LKM-1	0%	0%	0%	0%	0%

[1]Data expressed as median (minimum-maximum); HOMA: homeostasis model assessment; HDL: high-density lipoprotein; Lp(a): lipoprotein(a); AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase; GGT: gamma glutamyl transpeptidase; LDH: lactate dehydrogenase; HbA1c: glycated hemoglobin; ANA: antinuclear antibodies; AMA: antimitochondrial antibodies; ASMA: anti-smooth muscle antibody; LKM-1, liver-kidney microsomal antigen.

Statistical analysis

Variables are presented as counts and percentages or means ± SD, unless otherwise indicated. Pearson's χ^2 test was used for comparison of categorical variables. Between group mean differences were tested by the Mann-Whitney U test or the Kruskal-Wallis test, as appropriate. The sensitivity, specificity, positive predictive value, and negative predictive value of the serum biomarkers in predicting definitive NASH were calculated using receiver operating characteristic (ROC) analyses. Diagnostic properties were expressed as percentages, with 95% confidence intervals (95% CIs). Cut-off values for serum biomarkers were determined using the MedCalc demo statistical software (Mariakerke, Belgium). Univariate (simple) and multivariate (forward stepwise) regression models were used to assess the independent predictors of definitive NASH in patients with suspected NAFLD. A $P < 0.05$ was retained for statistical significance. All computations were made using SPSS 10.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Patients Characteristics

The control (n = 49) and NAFLD (n = 83) groups were well balanced for age and gender (data not shown). Table 1 shows the clinical and biochemical characteristics of the NAFLD patients stratified by their liver histology into four groups: definitive NASH (n = 45), borderline NASH (n = 24), simple fatty liver (n = 9), and normal tissue (n = 5).

There was a statistically significant difference in microalbuminuria ($P < 0.05$), ALT levels ($P < 0.05$) and AST/ALT ratio ($P < 0.05$) between the four groups. By contrast, there were no statistically significant differences between the groups in age, gender, BMI, waist circumference, history of hypertension, hyperlipidemia, diabetes, or prevalence of the metabolic syndrome. Values of alkaline phosphatase, gamma-glutamyl transferase, lactic acid dehydrogenase, ferritin, and homeostasis model assessment-estimated insulin resistance (HOMA) were not significantly different across the four patient groups. 27% of patients with suspected NAFLD had positive serological testing for autoantibodies.

Histopathology

The histological features of patients with suspected NAFLD are presented in Table 2. Of 83 subjects, 5 had normal biopsies with < 5% steatosis. Nine subjects had steatosis alone. In the present study, 24 patients with suspected NAFLD had histological borderline NASH.

Table 2 Histological findings in 83 patients with suspected NAFLD

Variable	Definition	Score	Entire cohort (n = 83) (%)	Definitive NASH (n = 45) (%)	Borderline NASH (n = 24) (%)	Simple steatosis (n = 9) (%)	Normal tissue (n = 5) (%)
Steatosis	< 5%	0	8.40	0	8.30	0	100
	5%-33%	1	39.80	13.30	75.00	100	0
	> 33%-66%	2	30.10	46.70	16.70	0	0
	> 66%	3	21.70	40.00	0	0	0
Lobular inflammation (foci per 200 × field)	No foci	0	15.70	0	0	88.90	100
	< 2 foci	1	26.50	8.90	70.80	11.10	0
	2-4 foci	2	43.40	64.40	29.20	0	0
	> 4 foci	3	14.50	26.70	0	0	0
Ballooning	None	0	12	0	0	55.60	100
	Few balloon cells	1	24.10	6.70	70.90	44.40	0
	Many cells/prominent ballooning	2	63.90	93.30	29.10	0	0
Fibrosis stage	None	0	54.20	33.30	66.70	100	100
	Mild, zone 3, perisinusoidal	1A	8.40	11.10	8.30	0	0
	Moderate, zone 3, perisinusoidal	1B	3.60	6.70	0	0	0
	Portal/periportal	1C	13.30	15.60	16.60	0	0
	Perisinusoidal and portal/periportal	2	13.30	22.20	4.20	0	0
	Bridging fibrosis	3	7.20	11.10	4.20	0	0
	Cirrhosis	4	0	0	0	0	0

Table 3 Imaging findings in 83 patients with suspected NAFLD

Steatosis		Entire cohort (n = 83)	Definitive NASH (n = 45)	Borderline NASH (n = 24)	Simple steatosis (n = 9)	Normal tissue (n = 5)
Sonographic grade of hepatosteatosis	Mild	44.00%	27.50%	50.00%	77.80%	100.00%
	Moderate	40.00%	55%	31.80%	11.10%	0%
	Severe	16.00%	17.50%	18.20%	11.10%	0%
Liver attenuation on CT (HU)		43.8 ± 14.6	36.9 ± 11.9	49.5 ± 15.1	55.2 ± 6.2	64.1 ± 3.3

Criteria for definitive NASH, as proposed by the NASH Clinical Research Network[24], were fulfilled by 45 patients. Among patients with definitive NASH, liver biopsy showed that steatosis was mild in 13.3%, moderate in 46.7%, and severe in 40.0%. Lobular inflammation and ballooning were present in all biopsy specimens from definitive NASH patients in varying amounts. 77% of definitive NASH patients exhibited fibrosis. No liver biopsy demonstrated cirrhosis.

Imaging

Ultrasound and CT findings are summarized in Table 3. Among the 83 subjects with suspected NAFLD, ultrasonographic examination revealed mild steatosis in 44%, moderate steatosis in 40% and severe steatosis in 16% of cases. By CT scans, the mean (SD) absolute liver density value of the entire study cohort was 43.8 ± 14.6 Hounsfield units (HU).

Serum biomarkers

As shown in Table 4, the serum levels of M-30 antigen were markedly higher in the definitive NASH group than in the control ($P < 0.001$), simple fatty liver ($P < 0.01$) and borderline NASH ($P < 0.001$) groups. Serum levels of M-30 antigen did not differ between patients with definitive NASH and subjects with normal biopsy.

Serum concentrations of M-65 antigen were higher in the patients with definitive NASH than in those with borderline NASH ($P < 0.001$), simple fatty liver ($P < 0.01$), normal tissue ($P < 0.05$), or in healthy individuals ($P < 0.001$). Serum levels of both biomarkers did not differ between the possible NASH, simple steatosis, and normal biopsy groups compared with the control group.

Subjects with possible NASH, simple fatty liver, or normal tissue were then grouped together (no-NASH group, n = 38) for a secondary analysis. The results of this analysis showed that the serum levels of both biomarkers were markedly higher in the definitive NASH group than in the no-NASH group ($P < 0.001$ for both biomarkers).

Diagnostic value of serum biomarkers for the prediction of definitive NASH

Table 5 lists the sensitivity, specificity, positive predictive value, and negative predictive value of the M30-antigen and the M65-antigen in predicting definitive NASH. In addition, the area under the ROC curve (ROC AUC) for both biomarkers was determined. The ROC AUC provides a measure of the overall discriminative ability of a test. In general, ROC AUC $\geqslant$ 7 indicates acceptable discrimination, ROC AUC $\geqslant$ 8 excellent discrimination

Table 4 Concentrations of serum M30-antigen and M65-antigen in the study groups

Biomarker	Definitive NASH (n = 45)	Borderline NASH (n = 24)	Simple steatosis (n = 9)	Normal tissue (n = 5)	Healthy volunteers (n = 49)	No-NASH (n = 38)	[1]P
M30-antigen (IU/L)	200.4 ± 183.1	69.8 ± 37.1	60.1 ± 36.8	81.0 ± 17.5	43.3 ± 45.9	69.0 ± 34.9	< 0.001
[2]P	Reference category	< 0.001	< 0.01	NS	< 0.001	< 0.001	
M65-antigen (IU/L)	362.8 ± 178.7	210.6 ± 59.6	215.3 ± 78.2	207.0 ± 47.4	150.9 ± 44.1	211.2 ± 61.5	< 0.001
[2]P	Reference category	< 0.001	< 0.01	< 0.05	< 0.001	< 0.001	

[1]P value calculated using Kruskal-Wallis test. [2]Comparisons between definitive NASH patients and other patient groups were performed by Mann-Whitney U Test.

Table 5 Diagnostic performance of serum M30-antigen and M65-antigen levels for the diagnosis of definitive NASH

Patient category	Serum biomarker	Cut-off value[1] (IU/L)	Sensitivity (95% CI)	Specificity (95% CI)	Positive predictive value (%)	Negative predictive value (%)	Negative likelihood ratio	Area under the ROC curve (95% CI)
Borderline NASH	M30-antigen	121.6	60 (44.3-74.3)	95.8 (78.8-99.3)	96.40	56.10	0.42	0.783 (0.668-0.783)
	M65-antigen	241.34	68.9 (53.3-81.8)	83.3 (62.6-95.2)	88.60	58.80	0.37	0.809 (0.697-0.894)
Simple steatosis	M30-antigen	109.64	62.2 (46.5-76.2)	100 (66.2-100.0)	100.00	34.60	0.38	0.83 (0.703-0.918)
	M65-antigen	186.77	93.3 (81.7-98.5)	66.7 (30.1-92.1)	93.30	66.70	0.10	0.807 (0.677-0.902)
Normal tissue	M30-antigen	96.5	64.4 (48.8-78.1)	100 (48.0-100.0)	100.00	23.80	0.36	0.724 (0.508-0.841)
	M65-antigen	227.18	73.3 (58.1-85.4)	80 (28.8-96.7)	97.10	25.00	0.33	0.809 (0.673-0.906)
Healthy controls	M30-antigen	82.52	75.6 (60.5-87.1)	85.7 (72.7-94.0)	82.90	79.20	0.29	0.881 (0.798-0.938)
	M65-antigen	189.64	91.4 (77.6-98.6)	68.1 (36.1-94.2)	92.00	68.20	0.18	0.94 (0.839-0.980)
No-NASH	M30-antigen	121.6	60 (44.3-74.3)	97.4 (86.1-99.6)	96.40	67.30	0.41	0.787 (0.683-0.869)
	M65-antigen	243.82	68.9 (53.3-81.8)	81.6 (65.7-92.2)	81.60	68.90	0.38	0.809 (0.708-0.887)

[1]Cut-off value corresponding to the highest accuracy value (minimal false negative and false positive results).

and ROC AUC $\geqslant$ 9 outstanding discrimination (very unusual). As shown in Table 5, both biomarkers revealed good discriminative ability for predicting definitive NASH. Notably, M30-antigen and M65-antigen gave similar diagnostic abilities.

Relationship between liver fibrosis and serum biomarkers

Table 6 shows the sensitivity, specificity, positive predictive value, and negative predictive value of M30-antigen and M65-antigen in differentiating early liver fibrosis from advanced fibrosis. Serum levels of both M30-antigen ($P < 0.01$) and M65-antigen ($P < 0.01$) were higher in patients with advanced fibrosis compared to those with early fibrosis. Both biomarkers appeared to have similar diagnostic ability.

Relationship between transaminases and serum biomarkers

We found a weak, albeit significant, positive correlation between M30-antigen levels with both AST ($r = 0.441$, $P < 0.001$) and ALT values ($r = 0.425$, $P < 0.001$). Similarly, concentrations of M65-antigen were positively related to both AST ($r = 0.490$, $P < 0.001$) and ALT ($r = 0.473$, $P < 0.001$).

Multivariate analysis

To assess the independent contribution of M30-antigen, M60-antigen as well as CT findings in identifying subjects with definitive NASH, simple and multiple linear regression analyses were performed (Table 7). Simple regression analysis showed that AST/ALT ratio, microalbuminuria, waist/hip ratio, CT density as well as levels of M30-antigen and M-65 antigen were all significantly associated with the presence of definitive NASH (data not shown). Variables associated with definitive NASH on univariate analysis were then entered into a multivariate model. The model showed that the addition of M-30 antigen to computed tomography yielded a 86.1% sensitivity and a 83.3% specificity for NASH. Similarly, the addition of M-60 antigen to computed tomography yielded a 83.3% sensitivity and a 83.3% specificity for NASH.

DISCUSSION

The results of our study show that different forms of CK18 in patient sera (M30-antigen and M65-antigen) may be of clinical usefulness in discriminating NASH from simple fatty liver or NAFLD patients with severe fibrosis from subjects with early fibrosis.

NAFLD is increasingly recognized as one of the most common causes of liver disease worldwide[25,26]. In this regard, in view of the epidemic of the metabolic syndrome, the prevalence of NAFLD is expected to continue to increase in future years[25]. A liver biopsy remains the gold standard for the evaluation of liver histology and is therefore a key test used to establish the diagnosis of NAFLD[1]. Unfortunately, it is an invasive procedure associated with discomfort and some risk[8,9]. It is therefore not suitable for the evaluation of all individuals with suspected NAFLD. The limitations of a liver biopsy have led to considerable interest and attempts to diagnose this condition with laboratory tests and imaging modalities.

Although simple fatty liver has been considered a benign disease, NASH appears to be a common cause of cryptogenic cirrhosis and may even result in hepatocellular carcinoma[26-28]. To date, the pathophysiological pathways

Table 6 Diagnostic performance of serum M30-antigen and M65-antigen levels in discriminating NAFLD patients with severe fibrosis from subjects with early fibrosis

	Cut-off value[1] (IU/L)	Sensitivity (95% CI) (%)	Specificity (95% CI) (%)	Positive predictive value (%)	Negative predictive value (%)	Negative likelihood ratio	Area under the curve (95% CI)
M30-antigen	121.6	64.7 (38.4-85.7)	77.3 (65.3-86.7)	42.30	89.50	0.46	0.733 (0.624-0.824)
M65-antigen	243.82	70.6 (44.1-89.6)	71.2 (58.7-81.7)	38.70	90.40	0.41	0.742 (0.635-0.832)

[1]Cut-off value corresponding to the highest accuracy value (minimal false negative and false positive results).

Table 7 Diagnostic value of serum biomarkers in combination with CT scans for the diagnosis of definitive NASH according to multivariate regression analysis

		P	Odds ratio	95% CI	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Accuracy (%)
Combination	CT	0.001	0.891	0.831-0.955	86.10	83.30	86.10	83.30	84.80
	M30-antigen	0.002	1.029	1.011-1.048					
Combination	CT	0.003	0.916	0.865-0.971	83.30	83.30	85.70	86.60	83.30
	M65-antigen	0.003	1.015	1.005-1.025					

involved in liver damage and in the progression of pure fatty liver to NASH remain largely unknown. Currently, the most accepted theory to explain the pathogenesis of NAFLD is the so-called "two-hit" hypothesis[29]. According to this model, the development of hepatic steatosis constitutes the first hit, and cellular events leading to hepatic inflammation constitute the second hit.

Experimental and clinical data have suggested a role for hepatocyte apoptosis in liver inflammation and tissue damage, regeneration of parenchyma, and fibrosis[30-33]. In this regard, a reduction in hepatocyte apoptosis has been shown to result in decreased liver fibrosis in animal models of cholestasis[32]. Of interest is also the observation that fibrosis has been suggested to rely on induction of hepatic stellate cells apoptosis[32]. In the clinical setting, Bantel *et al*[34] have previously shown that sera from patients with HCV infection had a markedly higher caspase activation than controls. In addition, measurements of caspase activity in serum were found to be related to the extent of steatosis or presence of fibrosis.

Recently, there has been a growing interest in measuring different CK18 forms in peripheral blood as a means to examine different cell death modes. Intriguingly, a recent report by Wieckowska *et al*[35] has shown that measurement of serum M30-antigen levels may allow discrimination of definitive NASH patients from simple fatty liver with high sensitivity and specificity. However, the sample size in this study was small, and no attempt was made to study the concentrations of M-30 antigen in patients with possible NASH.

Our present results confirm and expand previous findings[35] on the potential clinical usefulness of different forms of CK18 (M30-antigen and M65-antigen) as biochemical assays to accurately distinguish definitive NASH from simple fatty liver. Notably, we are also able to investigate a group of patients with possible NASH and to establish reliable cutoffs for the differential diagnosis of possible NASH from definitive NASH. Appropriate cut-off values for serum M-30 antigen and M-65 antigen were 121.60 IU/L (sensitivity, 60.0%; specificity, 95.8%; positive predictive value, 96.4%; negative predictive value, 56.1%) and 241.34 IU/L (sensitivity, 68.9%; specificity, 83.3%; positive predictive value, 88.6%; negative predictive value, 58.8%), respectively. Altogether, these biochemical findings seem to indicate that possible NASH more closely resembles the clinical and pathophysiological features of simple fatty liver than those of definitive NASH.

Importantly, it remains a clinically crucial issue to distinguish definitive NASH from more benign forms of NAFLD[1]. The appropriate cut-off values for serum M-30 antigen and M-65 antigen that distinguish between NASH and no-NASH (borderline NASH, simple fatty liver, normal tissue) were 121.60 IU/L (sensitivity, 60.0%; specificity, 97.4%; positive predictive value, 96.4%; negative predictive value, 67.3%) and 243.82 IU/L (sensitivity, 68.9%; specificity, 81.6%; positive predictive value, 81.6%; negative predictive value, 68.9%), respectively. Of great interest, multivariate regression analysis showed that the combination of M-30 antigen and CT increased the sensitivity and specificity for definitive NASH to 86.1% and 83.3%, respectively. Similarly, the combination of M-65 antigen and CT increased the sensitivity and specificity for NASH to 83.3% and 83.3%, respectively. Altogether, our data suggest that measurement of different forms of CK18 in combination with CT has greater diagnostic utility for the identification of patients with definitive NASH than the use of either test alone.

A weak, albeit significant, correlation between serum forms of CK18 (both M30-antigen and M65-antigen) and levels of hepatic transaminases was found. Although it has been suggested that aminotransferases are released during necrosis at a higher rate than in apoptosis[36], we found that hepatic transaminases were significantly correlated with levels of both M-30 antigen (a marker of apoptosis) and M-65 antigen (a marker of necrosis). To quantify the rates of apoptosis and necrosis in patients with definitive NASH, we also determined the M30:M65 ratio[22] as a means to investigate mode of cell death of hepatocytes

(data not shown). Of note, differently from other forms of NAFLD, the mode of such cellular death was apoptosis dominant in definitive NASH individuals compared to other NAFLD patient groups.

In summary, the results of the present study suggest that noninvasive monitoring of different forms of CK18 (M30-antigen and M65-antigen) in sera of patients with suspected NAFLD may represent a reliable tool to differentiate definitive NASH from simple fatty liver. Additionally, these biomarkers may be useful for identifying NAFLD patients with more severe liver fibrosis. Further validation studies in larger groups of patients are needed to confirm our findings.

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COMMENTS

Background

Liver biopsy remains the gold standard for assessing histologic lesions of non-alcoholic fatty liver disease (NAFLD). Unfortunately, it is an invasive procedure associated with discomfort and some risk. The limitations of a liver biopsy have led to considerable interest and attempts to diagnose this condition with laboratory tests and imaging modalities.

Research frontiers

As liver biopsy is impossible to perform as a diagnostic mass-screening tool, great effort is currently being spent in the validation of simple non-invasive markers of liver injury in patients with NAFLD. Specifically, the early identification of patients with nonalcoholic steatohepatitis (NASH) is of clinical importance because of the prognostic implications.

Innovations and breakthroughs

We demonstrated that noninvasive monitoring of different forms of cytokeratin-18 (M30-antigen and M65-antigen) in sera of patients with suspected NAFLD may represent a reliable tool to differentiate definitive NASH from simple fatty liver. Additionally, these biomarkers may be useful for identifying NAFLD patients with severer liver fibrosis.

Applications

In patients with non-alcoholic fatty liver disease, measurement of different forms of cytokeratin-18 in patient sera, a simple and non-invasive biomarker reliably predicts the presence or absence of NASH.

Terminology

Cytokeratin-18: a cytoskeletal protein found primarily in epithelial cells; M30-antigen: a fragment of cytokeratin-18 cleaved at Asp396 by caspases (a marker of apoptosis); M65-antigen: full-length soluble cytokeratin-18 (a marker of necrosis).

Peer review

This manuscript is pertinent, timely, with a fairly rigorous statistical approach to an important clinical question: what would be the best biological markers in human NAFLD.

REFERENCES

1 **Brunt EM**. Nonalcoholic steatohepatitis. *Semin Liver Dis* 2004; **24**: 3-20
2 **Sheth SG**, Gordon FD, Chopra S. Nonalcoholic steatohepatitis. *Ann Intern Med* 1997; **126**: 137-145
3 **Bugianesi E**, Leone N, Vanni E, Marchesini G, Brunello F, Carucci P, Musso A, De Paolis P, Capussotti L, Salizzoni M, Rizzetto M. Expanding the natural history of nonalcoholic steatohepatitis: from cryptogenic cirrhosis to hepatocellular carcinoma. *Gastroenterology* 2002; **123**: 134-140
4 **Marrero JA**, Fontana RJ, Su GL, Conjeevaram HS, Emick DM, Lok AS. NAFLD may be a common underlying liver disease in patients with hepatocellular carcinoma in the United States. *Hepatology* 2002; **36**: 1349-1354
5 **Angulo P**. Nonalcoholic fatty liver disease. *N Engl J Med* 2002; **346**: 1221-1231
6 **Van Ness MM**, Diehl AM. Is liver biopsy useful in the evaluation of patients with chronically elevated liver enzymes? *Ann Intern Med* 1989; **111**: 473-478
7 **Sorbi D**, Boynton J, Lindor KD. The ratio of aspartate aminotransferase to alanine aminotransferase: potential value in differentiating nonalcoholic steatohepatitis from alcoholic liver disease. *Am J Gastroenterol* 1999; **94**: 1018-1022
8 **McGill DB**, Rakela J, Zinsmeister AR, Ott BJ. A 21-year experience with major hemorrhage after percutaneous liver biopsy. *Gastroenterology* 1990; **99**: 1396-1400
9 **Poynard T**, Ratziu V, Bedossa P. Appropriateness of liver biopsy. *Can J Gastroenterol* 2000; **14**: 543-548
10 **Ratziu V**, Charlotte F, Heurtier A, Gombert S, Giral P, Bruckert E, Grimaldi A, Capron F, Poynard T. Sampling variability of liver biopsy in nonalcoholic fatty liver disease. *Gastroenterology* 2005; **128**: 1898-1906
11 **Younossi ZM**, Gramlich T, Liu YC, Matteoni C, Petrelli M, Goldblum J, Rybicki L, McCullough AJ. Nonalcoholic fatty liver disease: assessment of variability in pathologic interpretations. *Mod Pathol* 1998; **11**: 560-565
12 **Canbay A**, Higuchi H, Bronk SF, Taniai M, Sebo TJ, Gores GJ. Fas enhances fibrogenesis in the bile duct ligated mouse: a link between apoptosis and fibrosis. *Gastroenterology* 2002; **123**: 1323-1330
13 **Takehara T**, Tatsumi T, Suzuki T, Rucker EB, Hennighausen L, Jinushi M, Miyagi T, Kanazawa Y, Hayashi N. Hepatocyte-specific disruption of Bcl-xL leads to continuous hepatocyte apoptosis and liver fibrotic responses. *Gastroenterology* 2004; **127**: 1189-1197
14 **Feldstein AE**, Canbay A, Angulo P, Taniai M, Burgart LJ, Lindor KD, Gores GJ. Hepatocyte apoptosis and fas expression are prominent features of human nonalcoholic steatohepatitis. *Gastroenterology* 2003; **125**: 437-443
15 **Ueno T**, Toi M, Linder S. Detection of epithelial cell death in the body by cytokeratin 18 measurement. *Biomed Pharmacother* 2005; **59** Suppl 2: S359-S362
16 **Caulín C**, Salvesen GS, Oshima RG. Caspase cleavage of keratin 18 and reorganization of intermediate filaments during epithelial cell apoptosis. *J Cell Biol* 1997; **138**: 1379-1394
17 **Ku NO**, Liao J, Omary MB. Apoptosis generates stable fragments of human type I keratins. *J Biol Chem* 1997; **272**: 33197-33203
18 **MacFarlane M**, Merrison W, Dinsdale D, Cohen GM. Active caspases and cleaved cytokeratins are sequestered into cytoplasmic inclusions in TRAIL-induced apoptosis. *J Cell Biol* 2000; **148**: 1239-1254
19 **Leers MP**, Kölgen W, Björklund V, Bergman T, Tribbick G, Persson B, Björklund P, Ramaekers FC, Björklund B, Nap M, Jörnvall H, Schutte B. Immunocytochemical detection and mapping of a cytokeratin 18 neo-epitope exposed during early apoptosis. *J Pathol* 1999; **187**: 567-572
20 **Ueno T**, Toi M, Bivén K, Bando H, Ogawa T, Linder S. Measurement of an apoptotic product in the sera of breast cancer patients. *Eur J Cancer* 2003; **39**: 769-774
21 **Canbay A**, Kip SN, Kahraman A, Gieseler RK, Nayci A, Gerken G. Apoptosis and fibrosis in non-alcoholic fatty liver disease. *Turk J Gastroenterol* 2005; **16**: 1-6
22 **Kramer G**, Erdal H, Mertens HJ, Nap M, Mauermann J, Steiner G, Marberger M, Bivén K, Shoshan MC, Linder S. Differentiation between cell death modes using measurements of different soluble forms of extracellular cytokeratin 18. *Cancer Res* 2004; **64**: 1751-1756

23 **Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III)**. *JAMA* 2001; **285**: 2486-2497
24 **Kleiner DE**, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, Ferrell LD, Liu YC, Torbenson MS, Unalp-Arida A, Yeh M, McCullough AJ, Sanyal AJ. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* 2005; **41**: 1313-1321
25 **Falck-Ytter Y**, Younossi ZM, Marchesini G, McCullough AJ. Clinical features and natural history of nonalcoholic steatosis syndromes. *Semin Liver Dis* 2001; **21**: 17-26
26 **Neuschwander-Tetri BA**, Caldwell SH. Nonalcoholic steatohepatitis: summary of an AASLD Single Topic Conference. *Hepatology* 2003; **37**: 1202-1219
27 **Matteoni CA**, Younossi ZM, Gramlich T, Boparai N, Liu YC, McCullough AJ. Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity. *Gastroenterology* 1999; **116**: 1413-1419
28 **Teli MR**, James OF, Burt AD, Bennett MK, Day CP. The natural history of nonalcoholic fatty liver: a follow-up study. *Hepatology* 1995; **22**: 1714-1719
29 **Day CP**, James OF. Steatohepatitis: a tale of two "hits"? *Gastroenterology* 1998; **114**: 842-845
30 **Jaeschke H**, Gores GJ, Cederbaum AI, Hinson JA, Pessayre D, Lemasters JJ. Mechanisms of hepatotoxicity. *Toxicol Sci* 2002; **65**: 166-176
31 **Rust C**, Gores GJ. Locoregional management of hepatocellular carcinoma. Surgical and ablation therapies. *Clin Liver Dis* 2001; **5**: 161-173
32 **Canbay A**, Friedman S, Gores GJ. Apoptosis: the nexus of liver injury and fibrosis. *Hepatology* 2004; **39**: 273-278
33 **Jaeschke H**, Lemasters JJ. Apoptosis versus oncotic necrosis in hepatic ischemia/reperfusion injury. *Gastroenterology* 2003; **125**: 1246-1257
34 **Bantel H**, Lügering A, Heidemann J, Volkmann X, Poremba C, Strassburg CP, Manns MP, Schulze-Osthoff K. Detection of apoptotic caspase activation in sera from patients with chronic HCV infection is associated with fibrotic liver injury. *Hepatology* 2004; **40**: 1078-1087
35 **Wieckowska A**, Zein NN, Yerian LM, Lopez AR, McCullough AJ, Feldstein AE. In vivo assessment of liver cell apoptosis as a novel biomarker of disease severity in nonalcoholic fatty liver disease. *Hepatology* 2006; **44**: 27-33
36 **Que FG**, Gores GJ. Cell death by apoptosis: basic concepts and disease relevance for the gastroenterologist. *Gastroenterology* 1996; **110**: 1238-1243

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