Research Article

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An apoptosis panel for nonalcoholic steatohepatitis diagnosis

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Background & Aims: The extrinsic death receptor-mediated pathway of apoptosis is involved in nonalcoholic steatohepatitis (NASH) development. Our aims were to create and validate a noninvasive prediction model for NASH diagnosis based on specific circulating markers of apoptosis.

Methods: Our initial cohort consisted of 95 consecutive patients undergoing a liver biopsy for clinically suspected NASH. Blood was obtained from each patient at the time of liver biopsy. Plasma caspase 3 generated cytokeratin-18 fragments (CK-18), soluble Fas (sFas), and soluble Fas ligand (sFasL) were measured. Histology was assessed by an experienced hepatopathologist. The validation cohort consisted of 82 consecutive patients that underwent liver biopsy at the time of bariatric surgery.

Results: Patients with NASH had significantly higher levels of CK-18 and sFas than patients in the "not NASH" group [median (25th, 75th percentile): 508 (280, 846) U/L versus 176 (131, 224) U/L (p <0.001), and 11.8 (7.8, 12.5) ng/ml versus 5.9 (4.8, 8.3) ng/ml (p <0.001), respectively]. A significant positive correlation was revealed between the apoptosis markers and liver histopathology independent of other metabolic factors. A prediction model was generated including CK-18 fragments and sFas levels that showed an AUC of 0.93 and 0.79 in the initial and validation cohorts, respectively. A cutoff value using this model predicted NASH with a sensitivity and specificity of 88% and 89%, respectively.

Conclusions: Quantification of circulating levels of two apoptotic markers accurately predicts the presence of NASH, supporting the potential usefulness of these markers in clinical practice for noninvasive diagnosis of NASH.

Keywords: Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Apoptosis; Noninvasive diagnosis.

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Abbreviations: NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; TNFR, tumor necrosis factor receptor; CK-18, cytokeratin-18; NAS, NAFLD Activity Score; sFas, soluble Fas; sFasL, soluble Fas ligand; ELISA, enzyme-linked immunosorbent assay; ROC, receiver operating characteristic; AUC, area under the receiver operating characteristic curve; BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HOMA, homeostatic model assessment; HDL, high-density lipoprotein; LDL, low-density lipoprotein; CI, confidence interval; OR, odds ratio; FFA, free fatty acids.

Introduction

Nonalcoholic fatty liver disease (NAFLD) has become the most common form of chronic liver disease, currently affecting 20–30% of adults and 10% of children in the United States [1]. The spectrum of NAFLD is wide ranging from hepatic steatosis to nonalcoholic steatohepatitis (NASH) and cirrhosis [2]. Patients with hepatic steatosis appear to have a non-progressive course with more benign prognosis. Patients with NASH may progress to cirrhosis in as many as 25% of cases and suffer from its complications including portal hypertension, liver failure, and hepatocellular carcinoma [3,4]. Liver biopsy remains the gold standard for differentiating between hepatic steatosis and NASH in addition to providing information regarding the degree of steatosis, severity of inflammatory activity, and stage of fibrosis [5]. However, liver biopsy is an invasive procedure that carries possible significant risks. There are several clinical trials investigating therapies for NASH; the results of which will hopefully provide physicians with treatment options for this condition. This underscores the importance of a screening test that identifies NASH in patients with NAFLD. Such a screening test should be simple, noninvasive, reproducible, and accurately differentiate NASH from hepatic steatosis.

Hepatocyte apoptosis plays a critical role in liver injury and NASH development [6–8]. Increase in hepatocyte apoptosis is typically present in humans as well as animal models of NASH but absent in those with hepatic steatosis [8]. Increasing evidence suggests a role for both the so called extrinsic (death receptor mediated) pathway and the intrinsic (organelle-initiated) pathway of apoptosis. Fas, a death receptor member of the TNFR family, appears to have a prominent role. Fas protein expression is increased in liver samples from NASH patients [6]. Expression of this receptor increases in experimental models of NASH and results in increased sensitivity to Fas mediated apoptosis [9]. Accumulation of free fatty acids in liver cells results in upregulation of Fas in the cell surface [9]. Although the relative importance of the two main apoptotic pathways in human NASH remains to be elucidated, in hepatocytes, both pathways tend to converge at the level of the mitochondria resulting in permeabilization of the mitochondrial outer membrane and release of

multiple proteins from the mitochondrial inter-membrane space into the cytosol [10]. This results in activation of effector caspases (mainly caspase 3) which will then cleave a number of different substrates inside the cell including cytokeratin 18 (CK-18), the major intermediate filament protein in the liver, resulting in the characteristic morphologic changes of apoptosis [10]. We have previously demonstrated that caspase generated CK-18 fragments are significantly elevated in NASH patients [7]. Since the initial report, we and others have confirmed the utility of quantification of this marker for NASH diagnosis [11–14]. The aim of the present study was to test a panel of circulating apoptotic markers for diagnosis of NASH.

Patients and methods

Patients characteristics

The study was approved by the Cleveland Clinic Institutional Review Board, and all patients gave written informed consent prior to participation. Our initial cohort consisted of 95 consecutive patients undergoing a baseline liver biopsy for clinical suspicion of NASH by their treating hepatologists. Up to date, there are no established guidelines for performing a liver biopsy in patients with suspected NAFLD. Thus, the decision to perform the biopsy was individualized, and mostly performed due to persistently abnormal liver enzymes (mainly serum ALT) in a patient with clinically suspected NASH. The validation cohort included 82 consecutive patients who underwent liver biopsy at the time of bariatric surgery as part of their standard of care. Demographic, clinical, and laboratory data were collected. The absence of past or current excessive alcohol use was defined by an average daily consumption of alcohol of <20 g/day for men and <10 g/day for women. Absence of other liver diseases was confirmed via serological testing, imaging studies, or histological findings. Blood was obtained from each patient at the time of liver biopsy. Patients were subsequently divided into two groups according to their histological diagnosis (see 'Liver Histology'): "NASH" and "not NASH".

Liver histology

The histological diagnosis was established using hematoxylin–eosin and Masson trichrome stains of formalin-fixed paraffin-embedded liver and graded by an experienced hepatopathologist (L.Y.) who was blinded to the clinical characteristics of the patients including levels of serum biomarkers. The hepatopathologist provided an overall diagnostic interpretation based on the criteria reported by Brunt et al. [15] and also reported a NAFLD Activity Score (NAS) for each liver biopsy based on the NAFLD scoring system recently proposed by the National Institute of Diabetes and Digestive and Kidney Diseases NASH Clinical Research Network [16]. According to this scoring system, the degree of steatosis and inflammatory activity is measured using a zero to eight scale (steatosis = 0–3; lobular inflammation = 0–3; ballooning = 0–2). The NAS is the unweighted sum of steatosis, lobular inflammation, and hepatocellular ballooning scores. The stage of fibrosis was assessed using a zero to four scale (0 = no fibrosis; 1 = mild/moderate zone three perisinusoidal fibrosis, or portal/periportal fibrosis only; 2 = perisinusoidal and portal/periportal fibrosis; 3 = bridging fibrosis; 4 = cirrhosis). Patients were divided into two groups according to their histological diagnosis (hepatopathologist's overall diagnostic interpretation based on Brunt's criteria): "NASH" and "not NASH".

Measurement of caspase-generated CK-18 fragments, soluble Fas, and soluble Fas ligand in the blood

Blood samples obtained from patients at the time of their liver biopsies were initially processed to plasma then stored frozen at −80 °C. Plasma caspase 3 generated CK-18 fragments, soluble Fas (sFas), and soluble Fas ligand (sFasL) were quantitatively measured using specific sandwich ELISA based immunoassays for each. The M30-Apoptosense ELISA kit (PEVIVA; Alexis, Grünwald, Germany) was used for quantitative measurement of CK-18 fragments by selectively recognizing the caspase cleavage-generated neoepitope in the C-terminal domain of CK-18. The Human Fas/TNFRSF6 Quantikine ELISA Kit (R&D systems, Minneapolis, MN) was used for quantitative measurement of sFas and the Human Fas Ligand/TNFSF6 Quantikine ELISA Kit (R&D systems, Minneapolis, MN) was used for quantitative measurement of sFasL. All assays were performed in duplicate, and the absorbance was determined by using a microplate reader (Molecular Devices M2, Sunnyvale, CA).

Statistical analysis

Descriptive statistics were computed for all factors. These included means, standard deviations, and percentiles for continuous variables and frequencies for categorical factors. Univariable analysis was done to compare subjects with NASH to those without. Student's *t*-tests and Wilcoxon rank sum tests were used to compare continuous variables and Pearson's chi-square tests were used for categorical variables. Receiver operating characteristic (ROC) analysis was performed to assess the role of CK-18 fragments, sFas and sFasL levels in the diagnosis of NASH. The area under the ROC curves (AUC) and corresponding 95% confidence intervals were estimated. In addition, multivariable logistic regression analysis was performed to assess the combinations of the three hepatocyte apoptotic biomarkers and whether addition of age, BMI, gender, race, ALT, AST, insulin, glucose, HOMA, presence of diabetes, hypertension, metabolic syndrome, or hyperlipidemia improved NASH prediction; DeLong's method [17] was used to compare AUCs. A p <0.05 was considered statistically significant. SAS version 9.2 software (The SAS Institute, Cary, NC) and R version 2.4.1 software (The R Foundation for Statistical Computing, Vienna, Austria) were used to perform all analyses.

Results

Patient characteristics

The main clinical and serological characteristics of the initial cohort of patients are described in Table 1. The mean age of patients was 50 (± 11.6) years. The patients' gender (50.5% male) and race (83.2% Caucasian) did not statistically differ between the two histologic groups. Patients with NASH were significantly older and had significantly higher body mass index. They also had significantly higher prevalence of hypertension, clinical diabetes and metabolic syndrome, and significantly higher serum ALT and AST levels and HOMA index compared with patients in the "not NASH" group (all p <0.05). Triglyceride level was significantly higher and HDL level was lower in patients with NASH, though did not reach statistically significant difference. Table 2 lists the histologic characteristics of the liver biopsies of the initial cohort.

Cytokeratin-18 fragments and sFas are markedly increased in patients with NASH

Plasma levels of CK-18 fragments ranged from 60 to 2306 U/L [median (25th, 75th percentile): 224 (151, 431) U/L]. Patients with NASH had significantly higher levels of CK-18 fragments than patients with "not NASH" [median (25th, 75th percentile): 508 (280, 846) U/L versus 176 (131, 224) U/L; p <0.001]. Plasma levels of sFas ranged from 3.3 to 17.5 ng/ml [median (25th, 75th percentile): 7.6 (5.4, 10.3) ng/ml] and plasma levels of sFasL ranged from 5 to 200 pg/ml [median (25th, 75th percentile): 78 (65, 91) pg/ml]. Plasma sFas levels were significantly higher in patients with NASH compared with those with "not NASH" [median (25th, 75th percentile): 11.8 (7.8, 12.5) ng/ml versus 5.9 (4.8, 8.3) ng/ml; p <0.001]. Patients with NASH had higher levels of sFasL than patients with "not NASH", but did not reach statistically significant difference [median (25th, 75th percentile): 80 (66, 92) pg/ml versus 76 (65, 85) pg/ml, p = 0.2].

Both CK-18 fragment levels and sFas levels in the plasma showed a significant positive correlation with the liver histopathology characteristics, mainly with the presence of lobular