Usefulness of Serum Hepatic Fibrosis Markers in the Diagnosis of Nonalcoholic Steatohepatitis (NASH)

Running titile: NASH and hepatic fibrosis markers

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Abbreviations: NASH, nonalcoholic steatohepatitis, NAFLD, nonalcoholic fatty liver diseases, FL: fatty liver, BMI, body mass index, AST, aspartate aminotransferase, ALT, alanine aminotransferase, LDH, lactate dehydrogenase, γGTP, γ-glutamyl transpeptidase, FPG, fasting plasma glucose, HbA1c, hemoglobinA1c, ALP, alkarine phosphatase, type III PIIIP, procollagen N-peptide, TyIV, type IV collagen, HA, hyaluronic acid, TNFα, tumor necrosis factor α, ELISA, enzyme-linked immunosorbent assay, ROC, receiver operating characteristic, SD, standard deviation

ABSTRACT

AIM: In the present study, we examined the usefulness of serum hepatic fibrosis markers for the diagnosis of NASH. **METHODS:** The subjects were 16 patients with NASH and 9 patients with fatty liver (FL). All were negative for serum HBsAg, HCVAb, antibodies related with autoimmune diseases, alcohol intake, and drug abuse. We measured the biochemical markers for liver function, hepatic fibrosis markers such as type III procollagen N-peptide (PIIIP), type IV collagen (TyIV), hyaluronic acid (HA) and leptin, and compared these data with histological findings of biopsy specimens. In addition, we examined the diagnostic efficiency of fibrosis markers and leptin for NASH using receiver operating characteristic (ROC) curve. Body mass index (BMI), fasting blood sugar, triglyceride, and degree of fat droplets, inflammation, iron deposition and fibrosis were significantly higher in the NASH group compared with the FL group. **RESULTS:** The diagnostic efficiencies of NASH% (cut off value) were 68% (100ng/mL) for TyIV, 68% (10ng/mL) for HA, 64% (0.62U/mL) for PIIIP and 56% (8pg/mL) for leptin. **CONCLUSIONS:** From these results, it is suggested that the serum hepatic fibrosis markers such as TyIV, in addition to liver biopsy, may be useful for the diagnosis of NASH.

INTRODUCTION

In 1979, Adler et al. reported that there are patients with liver histology similar to that of patients with alcoholic hepatitis or fibrosis[1]. In 1980, Ludwig et al.[2] described the term of nonalcoholic steatohepatitis (NASH) from the biopsy findings in patients with with steatohepatitis in the absence of significant alcohol consumption.

Estimates based on imaging and autopsy studies suggest that proximately 20~30% of adults in the United States and Western countries have excess fat accumulation in the liver. About 10 % of these individuals are eastimated to meet current diagnostic fibrosis and cirrhosis in a fraction, possibly up to one third, of those with NASH, and NASH may be a cause of cryptogenic cirrhosis[3].

The pathogenesis of NASH is multifactorial. Insulin resistance may be important causes of hepatocellular fat, whereas excess intracellular fatty acids, oxidant stress and mitochondrial dysfunction may be important causes of hepatocellular injury. From a recent study of biochemical and histological aspects in patients with NASH, one of the potential cofactors suspected to enhance oxidative stress is excessive hepatic iron accumulation[4].

TNFα is derived primarily from adipose tissue in the absence of active infections or inflammatory conditions and, under normal conditions, its plasma levels correlate with body fat mass. A major role of TNFα between adipose tissue and insulin resistance is suggested. The relative roles played by increased serum cytokines such as TNFα in mediating insulin resistance remains a major unresolved issue.

Leptin may play an important role in regulating the partitioning of fat between mitochondrial ß-oxidation and triglyceride synthesis. In the normal human liver, leptin is hardly visible. Defects in leptin signaling are associated with preferential

accumulation of fat and impaired ß-oxidation of fat in the liver. In NASH, it is suggested that leptin is necessary for the development of fibrosis[5, 6]. On the other hand, Chitturi et al. reported that circulating leptin in patients with NASH correlates with hepatic steatosis but not hepatic fibrosis[7].

The diagnostic criteria for biopsy include establishing the diagnosis and staging of the injury, but strict guidelines do not exist. Liver enzymes are insensive and cannot be used reliably to confirm the diagnosis or stage the extent of fibrosis. The research agenda for the future includes identifying better noninvasive predictors of disease, and defining effective therapy.

In the present study, we compared liver biopsy specimens with serum levels of hepatic fibrosis markers such as type III procollagen-N-peptide (PIIIP), type IV collagen (TyIV) and hyaluronic acid (HA), and investigated the diagnostic efficiency of serum fibrosis markers for diagnosing patients with NASH.

Subjects

Subjects for this study consisted of 25 patients (11 male and 14 female) with nonalcoholic fatty liver diseases (NAFLD). They were negative for hepatitis B surface antigen, hepatitis B core antigen, hepatitis C virus antibody, anti-nuclear antibody, anti-mitochondria antibody and drug abuse. In all of them, fatty liver were recognized by ultrasound of abdomen, and diagnosis was based on a liver biopsy.

Provide assurance that informed consent in writing was obtained from each patient and the study protocol conformed to the ethical guide-lines of the 1975 Declaration of Helsinki as reflected in a *priori* approval by the appropriate institutional review

committee.

Liver histology

The liver biopsies of all subjects were performed using a Silvermann needle. Histological sections were stained with hematoxylin-eosin, and Azan-Mallory stain. Biopsy specimens were evaluated by two hepatopathologists (Y. M., T. U.) without information about the background of the patients. Next, we analyzed the specimens according to the criteria described by Tetri et al.[8], and assessed the following features in each case. That is, class 1 constitutes simple steatosis, class 2 is steatosis with lobular inflammation, class 3 requires the additional presence of ballooned hepatocytes, and class 4 requires the presence of either Mallory's hyaline or fibrosis. Within this system, class 2, 3 and 4 are regarded as a NASH group, and class 1 as a FL group. From the above cliteria, 9 patients (5 male and 4 female) were diagnosed as having fatty liver (FL) and 16 patients （6 male and 10 female）had NASH. They had the history of consumption of less than 20 g per day of alcohol.

Next, we assessed the following features in each case according to the criteria described by Brunt et al.[9]:

Steatosis and inflammation

The degree of steatosis, ballooning hepatocytes, lobular inflammation, portal inflammation, lipogranuloma, nuclear vacuoles, acidophilic bodies, Mallory bodies, and neutrophil infiltration in sinusoids were scored from grade 1 to 3.

Staging for fibrosis

Stage 1 : perisinusoidal / pericellular fibrosis in Zone 3

Stage 2: perisinusoidal / pericellular fibrosis with focal or extensive fibrosis in Zones 2

and 3.

Stage 3 : perisinusoidal / pericellular fibrosis and portal fibrosis with focal or extensive bridging fibrosis.

Stage 4 : Cirrhosis.

Physical and laboratory examinations

Blood samples were collected on the day of the liver biopsy, and stored at -80℃ until measurement. Body mass index (BMI), plasma glucose, aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), γ glutamyl transpeptidase (γGTP), fasting plasma glucose (FPG), hemoglobinA1 (HbA1c), insulin, lactate dehydrogenase (LDH), alkarine phosphatase (ALP), iron, ferritin triglycedride and total cholesterol were examined. All laboratory analyses were performed by standard clinical laboratories.

In addition, type III procollagen N-peptide (PIIIP), type IV collagen (TyIV), hyaluronic acid (HA), leptin in sera were measured. That is, serum PIIIP levels were measured with a radioimmunoassay kit (Bering-Hochest, Frankfurt, Germany). Serum T4 levels were measured with a one stepsandwich enzyme immunoassay (Daiichi Chemical Phayma. Ltd. Tokyo, Japan). Serum HA levels were determined by a sandwich enzyme binding assay (Chugai Diagnostics Science Co. Ltd, Tokyo, Japan). Serum leptin levels were measured with a radioimmunoassay kit (LINCO Research Inc., ST Charles, Missouri, USA). Serum tumor necrosis factor α (TNFα) levels were measured with a enzyme-linked immunosorbent assay (ELISA) kit (Nihon Kotai Inc. Tokyo, Japan).

Study design

We compared the liver histology in the NASH group with that in the FL group. Next, to assess the ability of these serum markers for differentiating NASH from FL, we calculated the sensitivity and the specificity for each value of each test and then constructed the receiver operating characteristic (ROC) curves by plotting the sensitivity against the reverse specificity (100-specificity) at each value. The nearer a curve shifts to the top left-hand corner of the graph, the more useful test diagnosis of NASH is that which maximizes the sum of the sensitivity and specificity.

Statistical analysis

Data were expressed as mean ± SD. Statistical analysis was performed with the non-parametric Mann-Whitney U-test using the Statistics software package. Statistical significance was set at $p<0.05$. Spearman's correlation analysis was used to examine the correlations between data sets.

RESULTS

1. Clinical characteristics and blood chemistry in NASH and FL groups

In the clinical characteristics between the NASH and FL groups, BMI in the NASH group was significantly higher than that in the FL group (28±3 vs 25±4, $p<0.05$). On the other hand, there were no significances in age and gender between the two groups.

In the blood chemistry between the NASH and FL groups, serum triglyceride and total cholesterol levels in the NASH group were significantly high compared with those in the FL group (triglyceride; 186±91 vs 107±29, $p<0.05$,; FPG; 118±25 vs 92±16,

$p<0.05$). However, there were no significances in serum AST, ALT and total cholesterol levels between the two groups (Table 1).

2. Serum hepatic fibrosis markers, leptin, iron, ferittin and TNFα in NASH and FL groups

There were no significances in serum hepatic fibrosis markers, leptin, iron, ferittin and TNFα between NASH and FL groups (Table 2).

3. Correlation between liver histological findings and serum hepatic fibrosis markers in patients with NAFLD

We performed statistical analysis for the correlation between liver histological findings and serum hepatic fibrosis markers in patients with NAFLD. Serum PIIIP level was significantly correlated with the degree of steatosis ($p<0.05$). In addition, serum TyIV levels were significantly correlated with the degrees of steatosis, pericellular fibrosis and perivenular fibrosis (respectively, $p<0.05$) (Table 3).

4. Receiver-operating characteristic (ROC) curves of serum PIIIP, TyIV, HA and leptin levels predicting NASH and its efficiency in patients with NAFLD

When the ability of these serum markers to detect NASH in patients with NAFLD was assessed with a ROC curve, the best was the serum TyIV test, and second was the serum HA test (Figure 1).

At a cut-off value of 100ng/mL, serum TyIV level was 68.8% in sensitivity and 66.7% in specificity. Also, serum HA level was 75.0% in sensitivity and 55.6% in specificity at a cut-off value of 10ng/mL. For serum PIIIP level, at a cut-off value was 0.62 U/mL,

PIIIP level was 52.6% in sensitivity and 33.3% in specificity, and for serum leptin level, at a cut-off value 8 ng/mL, sensitivity was 62.5% and specificity 44.4% (Figure 1 and Table 4).

In addition, the diagnostic efficiencies predicting NASH at these cut-off values were 68% for TyIV, 68% for HA, 64% for PIIIP and 56% for leptin (Table 4). However, the cut-off values of HA, PIIIP and leptin showed normal ranges.

DISCUSSIONS

The term NASH, coined by Ludwig et al.[2] in 1980 to describe the biopsy findings in patients with steatohepatitis in the absence of significant alcohol consumption, has served the field well[10]. Recently three criteria have proposed for the diagnosis of NASH: 1) a histological picture of steatohepatitis, 2) convincing evidence of minimal or no alcohol consumption (<40g/wk), and 3) absence of serological evidence of viral hepatitis. Each of the criteria has limitations.

That is, the histological criteria for the diagnosis of steatohepatitis include macrovesicular steatosis; evidence of ballooning degeneration; Mallory bodies; scatterd predominantly lobular, inflammatory infiltrate; and perisinusoidal fibrosis. Determinaning the extent of alcohol consumption is no easy task. Direct and surrogate markers of alcohol consumption include serum γ-glutamyltransferase level, AST and ALT levels, AST/ALT ratio, mitochondrial AST and so on. The presence of non-A, non-B and non-C was originally believed to constitute an exclusion criterion for the diagnosis of NASH[11].

However, no accurate noninvasive methods can diagnose NASH because the

presence, degree, and pattern of transaminase level elevation are non-specific and do not provide a specific diagnosis. Incidentally, necroinflammation and intralobular fibrosis containing the perisinusoidal fibrosis are histological characteristics of NASH. Serum hepatic fibrosis markers such as PIIIP, TyIV and HA are increased in patients with chronic liver diseases, indicating that these serum markers are of value for dedtecting altered connective tissue metabolism in liver fibrosis. Especially, increased levels of serum PIIIP and TyIV appear to be associated with active hepatic fibrogenesis[12], and increased levels of serum HA reflects the cirrhotic liver and sinusoidal capillarization[13]. In the present study, we investigated the usefulness of hepatic fibrosis markers for the noninvasive diagnosis of NASH. In results, The diagnostic efficiencies of NASH% (cut off level) were 70% (100ng/mL) for TyIV, 68% (10ng/mL) for HA, 64% (0.62U/mL) for PIIIP and 56% (8pg/mL) for leptin. It is suggested that the hepatic fibrosis markers such as TyIV, in addition to liver biopsy, may be useful for the diagnosis of NASH.

Many issues remain unresolved regarding the diagnosis of NASH. The need for a liver biopsy to diagnose NASH in routine clinical practice is debatable. Arguments against a liver biopsy include the generally good prognosis of most patients with NASH, and risks and costs associated with biopsy. Although our results need to be repeated by another centre, we think that the number of biopsies in the diagnosis and management of NASH could be reduced if serum hepatic fibrosis markers are measured.

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FIGURE LEGENDS

Fig. 1 Receiver operating characteristic (ROC) curves showing serum PIIIP, TyIV, HA, and leptin levels in predicting NASH in patients with NAFLD

When the ability of these serum markers to detect NASH in patients with NAFLD is assessed with a receiver operating characteristic (ROC) curve.

At a cut-off value of 100ng/mL, serum TyIV level was 68.8% in sensitivity and 66.7% in specificity. Also, serum HA level was 75.0% in sensitivity and 55.6% in specificity at a cut-off value of 10ng/mL. For serum PIIIP level, at a cut-off value was 0.62 U/mL, PIIIP level was 52.6% in sensitivity and 33.3% in specificity, and for serum leptin level, at a cut-off value 8 ng/mL, sensitivity was 62.5% and specificity 44.4%.