The Use of Protein Tyrosine Phosphatase 1B and Insulin Receptor Immunostains to Differentiate Nonalcoholic From Alcoholic Steatohepatitis in Liver Biopsy Specimens

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Key Words: Nonalcoholic steatohepatitis; NASH; Obesity; Insulin resistance; Immunohistochemistry; Cirrhosis

Abstract
Nonalcoholic steatohepatitis (NASH) and alcoholic steatohepatitis (ASH) typically are indistinguishable histologically. The diagnosis relies on reporting of alcohol consumption. The metabolic syndrome involving insulin resistance is associated with nonalcoholic fatty liver disease (NAFLD). Protein tyrosine phosphatase 1B (PTP1B) negatively regulates the insulin receptor (IR). Increased PTP1B expression is seen in obesity and possibly is responsible for the insulin resistance seen in the metabolic syndrome. The study objective was to determine whether biopsy specimens with steatohepatitis could be classified accurately as alcoholic or nonalcoholic by immunohistochemical stains. We selected 241 cases of steatohepatitis, comprising 53 and 188 cases of alcoholic and NAFLD, respectively. Specimens were stained with PTP1B and IR (β subunit) and classified as NASH or ASH. The staining pattern predicted 60 cases of ASH and 181 cases of NASH. Results correlated with clinical diagnoses in 70% and 88% of ASH and NASH cases, respectively (odds ratio, 16.6; 95% confidence interval, 8.2-35.4).

Steatohepatitis is a morphologic pattern of liver tissue injury with various causes. Two diagnoses commonly considered during the microscopic examination are alcoholic steatohepatitis (ASH) and nonalcoholic steatohepatitis (NASH).1 While prominent cholestasis and excessive Mallory hyaline are suggestive of ASH, the 2 conditions typically are indistinguishable by morphologic features alone. Accurate diagnosis requires clinicopathologic correlation rooted in a thorough clinical history that emphasizes truthful subjective reporting of the level of alcohol consumption. Even with careful and gentle clinical interactions, many cases remain unresolved because of persistent doubt in the truthfulness of the patient’s reporting.

Although steatosis represents a common morphologic manifestation of reversible liver cell injury, the mechanisms of lipid accumulation within the hepatocyte vary. In alcoholic liver disease, the metabolism of ethanol to acetate leads to an excess of reduced nicotinamide adenine dinucleotide. This excess alters the normal pathway of fatty acid β oxidation and leads to the accumulation of free fatty acids, then the formation of triacylglycerol, to yield steatosis. In contrast with ASH, in which the toxic effects of alcohol metabolism lead to steatosis, the accumulation of fat in NASH seems to represent the initial step in the pathway of liver injury. The excess free fatty acids presented to the liver are thought to be due to increased mobilization from abundant body stores.2 The metabolism of free fatty acids leads to the formation of reactive oxygen species and contributes to lipid peroxidation and hepatocyte injury.3

The metabolic syndrome associated with insulin resistance, in the setting of obesity, has been associated with nonalcoholic fatty liver disease.4 The insulin receptor (IR)
is acted on by a unique group of regulatory proteins, including protein tyrosine phosphatase 1B (PTP1B), which negatively regulates the IR through dephosphorylation. Increased expression of PTP1B is seen in obesity and possibly is responsible for a portion of the insulin resistance seen in obese patients.

The objective of this study was to determine whether seemingly morphologically identical cases of steatohepatitis could be distinguished and classified as nonalcoholic or alcoholic on the basis of differing expressions of PTP1B and IR, as determined by immunohistochemical staining.

Materials and Methods

We identified 427 liver biopsy specimens with steatohepatitis by searching the pathology database for the period January 1, 1994, through December 31, 2003. Medical records were reviewed, and cases with alternative primary or confounding liver disease (ie, viral hepatitis, hereditary hemochromatosis, Wilson disease, or known hepatotoxic medications) were excluded. All cases in which the clinical diagnosis was not clearly established and supported by current ancillary studies also were excluded, as were those in which the paraffin-embedded tissue sample was determined to be insufficient for further immunohistochemical study. The original diagnostic slides were reviewed (by S.O.S.) without knowledge of the clinical diagnosis and evaluated for the necroinflammatory grade and fibrotic stage of steatohepatitis, according to Brunt et al.

Paraffin blocks were cut at 5 µm, and tissue sections were stained with antibodies for PTP1B, clone H-135 (Santa Cruz Biotechnology, Santa Cruz, CA) and the IR β subunit, clone CT-3 (NeoMarkers, distributed by LabVision, Fremont, CA) using standard immunohistochemical techniques. Briefly, antigen retrieval was performed by treating the slides with a 1-nmol/L concentration of EDTA (pH 8.0) for 30 minutes in a steamer. The PTP1B antibody was used in a 1:50 dilution. The IR antibody was used in a 1:100 dilution for 30 minutes in a steamer. The PTP1B antibody was used for 15 minutes of incubation (EnVision+ polymer, horseradish peroxidase, DakoCytomation). DAB+ [diaminobenzidine] horseradish peroxidase was used as the chromogen (DakoCytomation).

Control tissue samples, as suggested by the commercial vendors, showed the expected positive staining results. In the liver samples, the cellular localization of the 2 monoclonal antibodies was distinct, with PTP1B localizing in the hepatocyte cytoplasm, had heterogeneous distribution within the liver acini, and IR staining was absent or patchy and weak. NASH also was predicted when there was only weakly positive staining for PTP1B as long as the stain was coarse and/or heterogeneous and associated with absent staining for IR.

Statistical analysis (JMP software, release 5.0.1.2, SAS Institute, Cary, NC) involved computing the odds ratios (ORs) with 95% confidence intervals (CIs), SD, and P values. The Mayo Clinic Institutional Review Board (Rochester, MN) approved the study.

Results

We identified 241 cases in which adequate tissue remained for immunohistochemical staining. In all cases, a diagnosis of alcohol-induced liver disease or nonalcoholic fatty liver disease was stated clinically and documented in the medical record. The presence of steatohepatitis was confirmed histologically and Image 1A, Image 1B, Image 2A, and Image 2B. The sex distribution was 51.0% female, overall. The clinical diagnosis in 53 cases (22.0%) was ASH and in 188 cases (78.0%) was NASH. There were significant differences in body mass index (BMI) values, inflammatory grade, and fibrotic stage between the 2 groups Table 1. Of all patients, 42 (17.4%) had cirrhosis (14 with ASH and 28 with NASH).

The determination of cause based on staining profile was made by dependent comparison of the PTP1B and IR stains Table 2. NASH was chosen if the PTP1B was positive, coarse, and heterogeneous Image 1C and the IR was negative Image 1D. ASH was chosen if the PTP1B was absent to weakly positive, fine, and homogeneous Image 2C and the IR was positive and continuous Image 2D. The staining profile and the clinical diagnosis correlated in 37 (70%) of 53 cases of ASH and in 87.8% of cases of NASH (OR, 16.6; 95% CI, 8.2-35.4). In the cirrhosis cases, the
staining profile correlated with the clinical diagnosis of ASH in 11 (79%) of 14 cases and NASH in 24 (86%) of 28 cases (OR, 22; 95% CI, 4.7-138) Table 3.

There were several cases in which the staining profile did not correlate with the clinical diagnosis Table 4. Biopsy specimens from 16 patients with a clinical diagnosis of alcoholic disease yielded a staining profile typical for NASH (PTP1B, strongly positive; IR, weakly positive to absent). This group was, on average, overweight (BMI, 28.4 kg/m²; SD, 4.0). In 23 biopsy specimens from patients with a clinical diagnosis of nonalcoholic disease, a staining profile typical for ASH was found (PTP1B, negative to weakly positive; IR, strongly positive). The average BMI for this group was in the obese range (BMI, 30.0 kg/m²; SD, 5.9). The liver biopsy specimens from patients with a clinical diagnosis of ASH showed high levels of PTP1B expression (mean intensity, 2.6), low-level expression of IR (mean intensity, 0.6), mild to moderate necroinflammatory activity (mean grade, 1.7), and perportal fibrosis (mean stage, 2.4) (data not shown). The liver biopsy specimens from patients with a clinical diagnosis of NASH showed modest levels of PTP1B and IR staining (mean intensity, 2.0 for each), with only mild necroinflammatory activity and mild fibrosis (mean grade, 1.2; mean stage, 1.3) (data not shown).
The results of our study demonstrate unique staining patterns of PTP1B and IR that permitted discrimination between alcoholic and nonalcoholic causes of steatohepatitis in a selected study population. Liver biopsy specimens from patients with NASH showed increased expression of PTP1B and decreased expression of IR, whereas specimens from patients with ASH tended to have low-level expression of PTP1B and normal expression of IR.

The increasing rate of obesity in the Western world has led to an increase in the clinical awareness of liver disease encompassing nonalcoholic fatty liver disease and NASH. NASH, as a specific entity, was described by Ludwig et al in 1980. The initial difficulty in establishing NASH as a stand-alone clinical entity came from the somewhat intricate and often overlapping morphologic features of alcoholic- and non-alcoholic-induced disease within the steatohepatitis spectrum. These features include steatosis, ballooning degeneration, Mallory hyaline, lobular inflammation, perisinusoidal fibrosis, zone 3 fibrosis, and a component of portal tract and...
lobular inflammation with a minor but significant component of neutrophils. Occasionally, the morphologic features are more typical of one cause than the other, but often the distinction cannot be made from the conventional evaluation of the liver biopsy specimen.1,9,10 This could lead to clinical speculation about the social habits of the patient with steatohepatitis, and, occasionally, there might be doubt about the credibility of the information embedded in the medical history.

The National Cholesterol Education Program (Adult Treatment Panel III) and the World Health Organization have proposed definitions for the clinical determination of the metabolic syndrome.11,12 Although the specific diagnostic guidelines put forth recognize slightly different criteria, these definitions revolve around several common findings, including abdominal obesity, dyslipidemia, hypertension, and elevated fasting glucose levels or hyperinsulinemia. The metabolic syndrome encompasses the interrelated pathophysiologic complex of steatosis, hyperlipidemia, diabetes mellitus type 2, and insulin resistance.12 The association between obesity and steatohepatitis was described by Wanless and Lentz13 in their autopsy study of the prevalence of fatty liver hepatitis in obese individuals. In a risk-assessment study, Marchesini et al4 showed, by logistic regression analysis, that the presence of multiple metabolic disorders is associated significantly with a high risk of NASH in patients with nonalcoholic fatty liver disease. The pathogenesis of the metabolic syndrome and the ultimate development of NASH seem to be centered on the phenomenon of insulin resistance.14,15

Our study findings suggest that the pathogenesis of the metabolic syndrome provides a potential morphologic link to the etiologic assessment of steatohepatitis. We attempted to use direct immunostaining for PTP1B and IR as markers of the metabolic syndrome. In this regard, we expected to find that in a patient with NASH there would be some indication that the normal pathway of IR signaling had been altered. The highly selected nature of the studied cases allowed for the possible immunohistochemical determination of the metabolic syndrome. Failure to identify this staining profile was the indication that the metabolic syndrome was not present, and the case, by design of the highly selected biopsy specimens, was considered to be ASH, not NASH. The finding of reduced levels of IR staining in our cases of NASH provides an intuitive explanation for insulin resistance, but the exact mechanism that might help explain this finding is unknown. In addition, it also is unclear how PTP1B functions to alter the expression of IR. A study by Lam et al16 found that leptin administration in \textit{ob/ob} obese diabetic mice resulted in

### Table 1
Clinical and Histologic Information for Study Cases

<table>
<thead>
<tr>
<th></th>
<th>Alcoholic Steatohepatitis (n = 53)</th>
<th>Nonalcoholic Steatohepatitis (n = 188)</th>
<th>P</th>
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<tr>
<td>Male/female ratio</td>
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<td>1:1.3</td>
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<td>Age (y)</td>
<td>51.4 (11.8)</td>
<td>49.8 (12.7)</td>
<td>.4</td>
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<td>Body mass index (kg/m²)</td>
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<td>33.3 (7.4)</td>
<td>&lt;.001</td>
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<tr>
<td>Grade</td>
<td>1.8 (0.9)</td>
<td>1.3 (0.5)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Stage</td>
<td>2.4 (1.3)</td>
<td>1.7 (1.4)</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

* Data are given as mean (SD) unless otherwise indicated.

### Table 2
Comparison of Clinical Diagnoses With Staining Profile Predictions for All Study Cases

<table>
<thead>
<tr>
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<th>Staining Profile Prediction</th>
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<tbody>
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<td>Clinical Diagnosis</td>
<td>Alcoholic Steatohepatitis</td>
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<tr>
<td>Alcoholic steatohepatitis</td>
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</tr>
<tr>
<td>Nonalcoholic steatohepatitis</td>
<td>23</td>
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</tbody>
</table>

### Table 3
Comparison of Clinical Diagnoses With Staining Profile Predictions in Cases of Cirrhosis

<table>
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<th>Staining Profile Prediction</th>
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<tr>
<td>Clinical Diagnosis</td>
<td>Alcoholic Steatohepatitis</td>
</tr>
<tr>
<td>Alcoholic steatohepatitis</td>
<td>11</td>
</tr>
<tr>
<td>Nonalcoholic steatohepatitis</td>
<td>4</td>
</tr>
</tbody>
</table>

### Table 4
Comparison of Cases in Which Clinical Diagnosis and Staining Profile Prediction Did Not Correlate

<table>
<thead>
<tr>
<th></th>
<th>Clinical Diagnosis/</th>
<th>Staining Profile Prediction</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASH/NASH (n = 16)</td>
<td>NASH/ASH (n = 23)</td>
<td>P</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>28.4 (4.0)</td>
<td>30.0 (5.9)</td>
</tr>
<tr>
<td>Grade</td>
<td>1.7 (0.8)</td>
<td>1.2 (0.5)</td>
</tr>
<tr>
<td>Stage</td>
<td>2.4 (1.2)</td>
<td>1.3 (1.5)</td>
</tr>
</tbody>
</table>

ASH, alcoholic steatohepatitis; NASH, nonalcoholic steatohepatitis.

Data are given as mean (SD).
improved fasting glucose homeostasis, probably related to short-term improvement in insulin sensitivity, but the PTP1B levels in these mice later increased, suggesting a possible role as an inhibitory mechanism against the actions of insulin.

We chose to stain for the β subunit of IR because it is the portion of the heterotetrameric glycoprotein that spans the cell membrane and contains the tyrosine kinase domain. This domain undergoes autophosphorylation after insulin binds to the extracellular α subunit of IR. The target of the IR antibody used (C-terminus) should not be sensitive to the state of receptor autophosphorylation. It is the autophosphorylation that is targeted by the action of PTP1B. Our data suggest a relationship between overexpression of PTP1B in the cytoplasm and diminished expression of IR in the cellular membrane of hepatocytes. We have not determined whether the lack of IR staining in our NASH cases correlated directly with the clinical determination of insulin resistance or whether it merely represented an immunophenotypic clue to the cause of the underlying liver disease. It is possible, although unproven, that overexpression of PTP1B and the consequent absent to low-level expression of IR could be reversed when the metabolic syndrome subsides, such as following body weight reduction. Kral et al demonstrated improvements in indicators of the metabolic syndrome in patients who underwent gastrointestinal surgery for obesity.

The cases of steatohepatitis in which the staining profile and the clinical diagnosis did not correlate are potentially instructive. The patients in cases diagnosed clinically as ASH but designated NASH by staining profile were borderline obese (mean BMI, 28.4), and at least 1 patient, who was lean, had documented hyperlipidemia. Although the staining profiles for this group were typical of NASH (high PTP1B expression; low-level IR expression), the degree of necroinflammatory activity and fibrosis were more fitting with the general trend seen in patients with ASH. Given the finding of increased PTP1B expression and reduced IR expression, one might postulate that the injury pattern associated with the metabolic syndrome is at least partly to blame for the liver injury in patients with alcohol-related disease. There is no reason that ASH and NASH have to be mutually exclusive.

The cases in which patients were diagnosed clinically as having NASH but that did not demonstrate a staining profile consistent with NASH are difficult to understand. This group showed the typical clinical obese phenotype and had mild degrees of necroinflammatory activity and lower stages of fibrosis characteristic of the NASH group overall. Levels of PTP1B expression were modest, and levels of IR expression were higher. A possible explanation lies within the pathophysiological spectrum of the metabolic syndrome. It seems that the system in which PTP1B overexpression is used by the liver as an inhibitory mechanism against the effects of IR signaling is not activated in these cases. It remains to be explained whether there exists an early phase of insulin resistance that has yet to produce the minimum level of PTP1B overexpression required to negatively affect IR expression or whether additional unknown factors are operating in these cases.

The staining profile predicted the cause of ASH or NASH in several liver biopsy specimens showing established cirrhosis. This ability to elucidate a cause for “poststeatohepatitic cirrhosis” might prove valuable in the treatment of patients with end-stage steatohepatitis and provide prognostic information. It has been shown that NASH represents the underlying causative agent in many cases of cryptogenic cirrhosis. Propst et al studied a large cohort of patients with chronic liver disease through a 15-year follow-up period and retrospectively calculated life expectancy. The calculated 5- and 10-year survival probabilities for alcoholic cirrhosis were 23% and 7%, respectively. The 5- and 10-year survival probabilities for cryptogenic cirrhosis were calculated as 33% and 20%, respectively. In addition, their group calculated the survival probability for patients with steatosis and found a statistically significant difference in the 5-year survival between patients with alcoholic and nonalcoholic steatosis, with the 5-year survival of alcohol-induced liver disease as 38% (P = .0001).

The staining profile of PTP1B and IR might help to reliably distinguish cases of steatohepatitis in which the clinical diagnosis of NASH or ASH remains unresolved. The identification of a staining profile that indicates the metabolic syndrome might offer useful information for appropriate therapy and overall prognosis. Future studies that explore beyond this unique phenomenon of liver staining with PTP1B and IR are indicated. Studies that correlate the degrees of insulin resistance, diabetes mellitus treatment, severity of obesity, and possible staining reversal after weight loss might provide insight into the complex hepatic manifestations of the metabolic syndrome.

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Presented in part at the annual meeting of the United States and Canadian Academy of Pathology, Vancouver, Canada, March 6-12, 2004.

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References


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