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Original contribution

Costaining for keratins 8/18 plus ubiquitin improves detection of hepatocyte injury in nonalcoholic fatty liver disease☆

Cynthia D. Guy MD [a], Ayako Suzuki MD, PhD [b], James L. Burchette HT [a], Elizabeth M. Brunt MD [c], Manal F. Abdelmalek MD, MPH [b], Diana Cardona MD [a], Shannon J. McCall MD [a], Aynur Ünalp MD, PhD [d], Patricia Belt BS [d], Linda D. Ferrell MD [e], Anna Mae Diehl MD [b,*], Nonalcoholic Steatohepatitis Clinical Research Network

[a]*Department of Pathology, Duke University Medical Center, Durham, NC 27710, USA*
[b]*Division of Gastroenterology, Duke University Medical Center, Durham, NC 27710, USA*
[c]*Department of Pathology and Immunology, Washington University School of Medicine, Saint Louis, MO 63110, USA*
[d]*NASH CRN Data Coordinating Center, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD 21205, USA*
[e]*Department of Pathology, University of California at San Francisco, San Francisco, CA 94143, USA*

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Summary Nonalcoholic fatty liver disease is a global health dilemma. The gold standard for diagnosis is liver biopsy. Ballooned hepatocytes are histologic manifestations of hepatocellular injury and are characteristic of steatohepatitis, the more severe form of nonalcoholic fatty liver disease. Definitive histologic identification of ballooned hepatocytes on routine stains, however, can be difficult. Immunohistochemical evidence for loss of the normal hepatocytic keratin 8/18 can serve as an objective marker of ballooned hepatocytes. We sought to explore the utility of a keratin 8/18 plus ubiquitin double immunohistochemical stain for the histologic evaluation of adult nonalcoholic fatty liver disease. Double immunohistochemical staining for keratin 8/18 and ubiquitin was analyzed using 40 adult human nonalcoholic fatty liver disease core liver biopsies. Ballooned hepatocytes lack keratin 8/18 staining as previously shown by others, but normal-size hepatocytes with keratin loss are approximately 5 times greater in number than keratin-negative ballooned hepatocytes. Keratin-negative ballooned hepatocytes, normal-size hepatocytes with keratin loss, and ubiquitin deposits show a zonal distribution, are positively associated with each other, and are frequently found adjacent to or intermixed with fibrous matrix. All 3 lesions correlate with fibrosis stage and the hematoxylin and eosin diagnosis of

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* Corresponding author. Chief, Division of Gastroenterology, Duke University School of Medicine, Durham, NC 27710, USA.
E-mail address: diehl004@mc.duke.edu (A. M. Diehl).

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steatohepatitis (all $P < .05$). Compared with hematoxylin and eosin staining, immunohistochemical staining improves the receiver operating characteristics curve for advanced fibrosis (0.77 versus 0.83, 0.89, and 0.89 for keratin-negative ballooned hepatocytes, normal-size hepatocytes with keratin loss, and ubiquitin, respectively) because immunohistochemistry is more sensitive and specific for fibrogenic hepatocellular injury than hematoxylin and eosin staining. Keratin 8/18 plus ubiquitin double immunohistochemical stain improves detection of hepatocyte injury in nonalcoholic fatty liver disease. Thus, it may help differentiate nonalcoholic steatohepatitis from nonalcoholic fatty liver.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is composed of 2 major histologic subtypes: nonalcoholic fatty liver (NAFL) and nonalcoholic steatohepatitis (NASH). Lipid accumulates within hepatocytes in both NAFL and NASH. The 2 entities differ, however, with regard to the prevalence and severity of hepatocyte injury and death [1]. NASH is a more severe form of liver injury than NAFL and, consequently, has a significantly greater likelihood of eventual cirrhosis and hepatocellular carcinoma [2-6]. Because the prognosis of NAFL and NASH are quite different, accurate diagnosis is important so that patients may be offered appropriate follow-up and treatment.

At present, histologic diagnosis is the gold standard for distinguishing NASH from NAFL [1,7,8]. Pathologists review hematoxylin and eosin–stained (H&E) liver biopsies for features indicative of ongoing hepatocyte injury. The latter includes apoptotic bodies, inflammatory cell infiltration, and hepatocytes with aberrant morphology, such as cellular ballooning and accumulation of ubiquitinated cytokeratins (called Mallory-Denk bodies [MDB]) [9]. Because the diagnosis of NASH rests upon histologic evidence of hepatocyte injury, hepatocellular ballooning is one of the major diagnostic criteria for NASH. However, such ballooned hepatocytes (BH) can sometimes be difficult to appreciate on standard H&E-stained sections. A recent study by Professor Denk's group suggested that immunohistochemical (IHC) staining for ubiquitin (Ub) and keratin 8/18 (K8/18) facilitates identification of BH, which are typically Ub positive but K8/18 negative [10]. These staining characteristics are consistent with experimental data, which demonstrate that pathways that normally mediate turnover of intracellular proteins (eg, ubiquitination and proteasomal degradation) become disrupted in NASH, leading to endoplasmic reticulum (ER) stress that contributes to hepatocyte injury and increases the risk of fibrosis [11]. Extension of this logic suggests that BH may be the extreme morphological manifestations of dysfunctional protein turnover and that improved methods to demonstrate ubiquitinated proteins and loss of K8/18 would improve sensitivity and/or specificity for detecting hepatocyte injury and thereby diagnosing NASH. Improved ability to distinguish NASH from NAFL, in turn, is predicted to increase the accuracy of predicting risk for subsequent liver fibrosis.

In this study, we performed double IHC staining for K8/18 and Ub on a relatively large group of adult liver biopsy samples from the Nonalcoholic Steatohepatitis Clinical Research Network (NASH CRN) Database Study [12]. All of the biopsies had been reviewed previously by the NASH CRN Pathology Committee. During that process, various histologic features were assigned scores according to NASH CRN criteria [7,13], and each case was given a final diagnosis of definite NASH, definitely not NASH or suspicious for NASH based upon the group's consensus. The main objective of the current study was to determine if K8/18 plus Ub IHC staining improved detection of BH. Secondary aims were to characterize the relationship between loss of K8/18 staining, Ub accumulation, and other histologic features of liver damage, particularly fibrosis. We also examined correlations between the new markers of disrupted protein turnover and previously identified clinical predictors of fibrosis progression, such as age, body mass index (BMI), and insulin resistance. Our results confirm that IHC staining improves detection of BH and support the concept that BH are the extreme manifestation of a more widespread defect in protein turnover, the severity of which strongly correlates with both the level of insulin resistance and fibrosis stage. The data also demonstrate that IHC staining reduces ambiguity in NAFLD diagnosis, thereby facilitating more accurate classification of NAFLD cases as either NAFL or NASH.

2. Materials and methods

2.1. Study population and slide selection criteria

We evaluated 1 unstained section from 40 liver biopsies of clinically and histologically well-characterized adult patients with NAFLD enrolled in the NASH CRN Database Study. Detailed description of the study design and operational structure of the NASH CRN and its associated clinical trials has been reported [12]. All available liver biopsies from the enrolled patients were stained with H&E and Masson trichrome and reviewed and scored centrally by the NASH CRN Pathology Committee according to a validated scoring system [7]. Briefly, consensus scoring is performed by the NASH CRN Pathology Committee to establish: steatosis grade (0, <5% steatosis; 1, 5%-33% steatosis; 2, 34%-66% steatosis; and 3, >66% steatosis),