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Original contribution

Biopsies of hepatocellular carcinoma with no reticulin loss: an important diagnostic pitfall☆

Saba Yasir MBBS, Zongming Eric Chen MD, PhD, Samar Said MD, Tsung-Teh Wu MD, PhD, Michael Torbenson MD*

Department of Laboratory Medicine and Pathology, Mayo Clinic Rochester, MN, USA

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Summary The reticulin stain is a critical diagnostic aide used to differentiate benign hepatocellular proliferations from well differentiated hepatocellular carcinoma (HCC). Rarely, however, hepatocellular carcinomas do not show definitive loss of reticulin in liver biopsy specimens. To study this group of tumors, 11 HCC with no reticulin loss in 10 patients were collected and studied. Analysis of demographics showed a typical enrichment for men with a typical age for HCC presentation of 69 ± 7 years for adults. The background livers showed advanced fibrosis or cirrhosis in 6 of 6 cases with available information. The tumors were all well differentiated. Cytological atypia was mild and consisted of very mild nuclear atypia (8 cases), mild increase in N:C ratio (3 cases), and pseudorosette formation (4 cases). The cytological/architectural atypia was insufficient in isolation to diagnose HCC. Additional studies, however, showed an increased Ki-67 proliferative rate (N = 10/10 stained cases). The Ki-67 proliferative rate was estimated to be between 5 and 10% in all tested cases and was clearly increased from adjacent liver at low power. Glypican 3 positivity (4 tumors) and alpha fetoprotein (AFP) (1/8 stained cases) positivity also helped make the diagnosis of HCC. Morphologically, the HCC had conventional morphology with five showing steatosis/steatohepatitic features and one showing intratumoral fibrosis. A control group of macroregenerative/dysplastic nodules showed no increase in Ki-67 proliferation and no staining for glypican 3. These findings highlight an important diagnostic pitfall: rare HCC show no reticulin loss on biopsy. In these challenging cases, additional findings are useful to make a diagnosis of HCC: increased Ki-67 and positive staining for aberrant expression of proteins such as glypican 3 or AFP.
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* Corresponding author. Department of Laboratory Medicine and Pathology, Mayo Clinic, 200 First Street, SW, Rochester, MN, 55905, USA.
E-mail address: Torbenson.Michael@mayo.edu (M. Torbenson).

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1. Introduction

Distinguishing well differentiated hepatocellular carcinoma (HCC) from benign hepatocellular lesions can be challenging based on morphology alone because some well differentiated HCCs have no or minimal cytological and architectural atypia. Based on morphology, the histological differential for these well differentiated tumors in noncirrhotic livers is typically that of a well differentiated HCC versus hepatic adenoma or focal nodular hyperplasia, whereas in cirrhotic livers, the differential is typically that of a well differentiated HCC versus macroregenerative nodules or dysplastic nodules. In cases where the morphology alone does not distinguish benign from malignant lesions, the standard of care is to use immunohistochemical stains as diagnostic aides.

The most widely used histochemical stain to support a diagnosis of HCC is the reticulin stain, where loss of normal reticulin staining patterns indicates a diagnosis of HCC. Rarely, however, HCCs show normal or near normal patterns of reticulin, with insufficient changes to reliably make a diagnosis based on reticulin abnormalities. Previously, a few case reports have documented well differentiated HCCs that showed no loss of reticulin staining [1,2], but there have been no systematic studies to date. In our consult practice, we have noticed this to be a recurrent diagnostic challenge. To examine the clinical and pathological correlates, as well as to provide a diagnostic approach for these difficult cases, a cohort of these cases was collected and studied.

Other stains can be used to supplement the reticulin stain when evaluating for the possibility of HCC. For example, glypican 3 and alpha fetoprotein (AFP) are known to be negative in benign liver tissue, outside of focal glypican expression in markedly inflamed livers [3]. Beta-catenin nuclear staining and/or strong and diffuse glutamine synthetase are not seen in dysplastic nodules [4]. As a caveat, in a noncirrhotic liver, beta-catenin nuclear staining and diffuse glutamine synthetase staining can also be seen in hepatic adenomas [5], so do not distinguish HCC from hepatic adenoma. Ki-67 shows low proliferation in hepatic adenomas and in macroregenerative and low-grade dysplastic nodules [6,7]. Thus, these immunostains were also examined for their use in diagnosing well differentiated HCC when there is no definite reticulin loss.

2. Materials and methods

After IRB approval, HCC cases were collected from the pathology files of Mayo Clinic Rochester. The majority of cases were consult cases (N = 9), and they were supplemented with several in-house cases (N = 2). Clinical and imaging findings were collected when available. Macroregenerative nodules (N = 2) and low-grade dysplastic (N = 6) were selected from explanted livers to serve as controls. Macroregenerative nodules and dysplastic nodules can be difficult to reliably distinguish from each other [8]. In this study, lesions were classified as macroregenerative nodules when the cytology of the hepatocytes within the nodule was identical to that of the background liver. If there was mild cytological atypia, but more than the background liver, the lesions were classified as low-grade dysplastic nodules [9].

All cases were centrally reviewed and scored by two pathologists (S.Y., M.S.T.). Cytological atypia was assessed as none or mild (findings could be consistent with either benign or malignant proliferations) versus atypia that was moderate or greater (cytological atypia alone that would be strongly suggestive or diagnostic of malignancy). Glutamine synthetase staining was considered positive when it was strong and diffuse within the tumor cells (>90%). Positive beta-catenin staining required nuclear staining. Glypican 3 positivity was correlated with H&E morphology, to exclude nonspecific staining secondary to cross reaction with lipofuscin [10]. Ki-67 was evaluated in association with the background liver whenever possible and was estimated to the nearest 5%. This approach is in keeping with routine clinical practice, where Ki-67 proliferation is interpreted by comparison of the tumor to the adjacent liver, often best evaluated at low power, where a clear increase in the tumor over that of the nontumor liver provides evidence for a malignant process. Reticulin loss was evaluated by looking for thickening of hepatic plates (>2 cells thick) and or foci of multiple adjacent tumor cells that were not touching reticulin on any of their surfaces. To determine the frequency of this rare finding, the number of cases of HCC with no reticulin loss was identified in 100 consecutive cases of well differentiated HCC in a consult practice (M.S.T.).

3. Results

3.1. Clinical findings

Eleven HCCs with no reticulin loss were collected in 10 patients (8 biopsy specimens, 3 resection specimens). The clinicopathological features are summarized in Table 1. Nine tumors occurred in adults and 2 in a child. For the adults, the median tumor size based on imaging was 2.6 cm, range 1.7−5.6 cm. Demographic findings showed a typical enrichment for men (all were men) with an average age at presentation of 69 ± 7 years. The background livers showed advanced fibrosis or cirrhosis in 6 of 6 adult cases with available information. Underlying liver diseases were available in 5 adult cases: metabolic syndrome with obesity, hypertension, and type 2 diabetes mellitus (N = 1), alcoholic hepatitis (N = 1), chronic hepatitis C (N = 1), chronic hepatitis C and *HFE* C282Y homozygosity (N = 1), and concurrent chronic hepatitis C viral infection and steatohepatitis (N = 1). The pediatric HCC occurred in

Table 1 Clinical and morphological findings of hepatocellular carcinoma.

Case	Specimen	Age	M/F	Radiology	Liver disease	Fibrosis	Size (cm)	HCC grade/ morphology	Other findings	Immunohistochemistry
1	Liver explant	2	M	Numerous hypodense nonhyperenhancing lesions; sections of these showed macroregenerative nodules. The HCC were small and identified on gross examination only and not by imaging	Tyrosinemia	Cirrhosis	0.5, 0.4	Well		HCC 1 Ki67 increased, 5% GP3 positive GS positive diffuse but weak AFP negative Retic focal equivocal loss HCC 2 Ki67 increased, 5% GP3 positive very focal GS negative AFP positive Retic focal equivocal loss
2	Biopsy	66	M	Ill-defined area of diminished attenuation. Nonspecific	HCV SH	Bridging fibrosis	2.6	Well Mild steatosis	Pseudoglands	GP3 positive AFP negative
3	Biopsy	84	M	Not available	Unavailable	Probable cirrhosis based on bx	Unavailable	Well	Pseudoglands	GP3 negative AFP negative Bcat negative GS negative Ki-67 increased, 10%
4	Biopsy	73	M	Not available	Unavailable	Insufficient background liver to evaluate	Unavailable	Well	Focal necrosis Occasional mitoses	GP3 negative Ki-67 increased, 10%
5	Biopsy	63	M	Indeterminate, mass suspicious for neoplasm	Unavailable But mild steatosis in background liver	Insufficient background liver to evaluate	5.6 (biopsy target); 3.9 (not biopsied)	Well		GP3 negative AFP negative Bcat negative GS negative Ki-67 increased, ~5%
6	Biopsy	65	M	Arterial enhancement, and other features diagnostic of HCC	Unavailable	Ccirrhosis	3.4	Well Steatohepatitic	Rare pseudoglands Rare mitoses Possible portal tract invasion	GP3 negative AFP negative Bcat negative GS negative Ki-67 increased, ~5%
7	Biopsy	61	M	Two hyperenhancing lesions, suspicious for HCC	ETOH	Cirrhosis	2.3, also one smaller lesion, size not specified and not	Well Mild steatosis		GP3 negative AFP negative Bcat negative GS positive diffuse but weak Ki-67 increased, ~10%

8	Liver explant	68	M	Enhancement characteristics of HCC	HCV HFE	Cirrhosis	0.9 (of viable tumor)	Well Status post treatment; 10% viable	Mild	GP3 negative AFP negative Bcat positive, nuclear staining GS diffuse positive Ki-67 increased, ~5%
9	Biopsy	63	M	Not available	HCV	Cirrhosis	Unavailable	Well Mild steatosis	Rare pseudoglands	GP3 positive Ki-67 increased, ~10%
10	Biopsy	74	M	Radiology differential of HCC versus abscess	Metabolic syndrome	Insufficient background liver to evaluate	5.2	Well steatohepatitic	Abundant intra-tumoral fibrosis	GP3 negative Ki-67 increased, ~10%

GP3, glypican 3; HCC, hepatocellular carcinoma; AFP, alpha fetoprotein; Bcat, beta-catenin nuclear staining; GS, glutamine synthetase; ETOH, alcohol liver disease; SH, steatohepatitis; HFE, hereditary hemochromatosis.

the liver of a young boy with a history of cirrhosis from tyrosinemia.

Imaging findings were available in 7 patients. Of those, 3 patients (case #1, #2, and #5) showed indeterminate and nonspecific findings. In 2 patients, radiology findings were consistent with HCC by imaging criteria, but clinical uncertainty led to a biopsy. Two patients had imaging features that were suspicious but not diagnostic of HCC. Clinicopathological features of the control group of macroregenerative/dysplastic nodules are shown in Table 2.

3.2. Histological findings

All of the tumors were well differentiated, with mild cytological atypia, manifesting primarily as mild nuclear atypia (N = 8), subtly increased nuclear to cytoplasmic (N:C) ratios compared with adjacent non-neoplastic hepatocytes (N = 3 cases) and pseudoglands (N = 4 cases) (Figs. 1–3). The mild cytological atypia was considered insufficient in isolation to diagnose HCC in all of the cases, by both the referring pathologists (in consult cases) and by the consultant pathologists. All of the tumors had conventional morphology with trabecular or solid growth patterns. Five cases showed intratumoral steatosis/steatohepatitic features and one showed intratumoral fibrosis. Rare mitotic figures were identified in two cases. A single case showed possible portal tract invasion.

3.3. Immunohistochemical findings

In nine cases, reticulin stains were essentially normal (Figs. 2B and 3C), with no plate thickening and no loss of reticulin, whereas in two cases, there was very focal and equivocal reduction in reticulin (Fig. 1B). Ki-67 stain showed increased proliferation in all of 10 stained cases, with the Ki-67 proliferation rate estimated to be between 5 and 10% (Figs. 1C and 3D). In all cases with adjacent nontumor liver, the proliferation was clearly increased from adjacent liver at low power examination. AFP was positive in 1 of 8 stained HCCs (Fig. 1D). Four HCCs were also glypican 3 positive (Fig. 2D); two separate nodules of HCC were identified in case #1 and both were glypican 3 positive. Beta-catenin showed nuclear positive staining in 1 of the 5 stained cases. Glutamine synthetase showed diffuse positive staining in 3 of the 7 studied cases.

In the control group, there was one dysplastic nodule where focal equivocal disruption of reticulin was noted (case 8; Table 2), but the morphology and other immunostaining profile did not reach the level of HCC. All other dysplastic and macroregenerative nodules in the control group showed intact reticulin meshwork. All dysplastic nodules in the control group showed a low Ki-67 staining that was indistinguishable from the background liver and were negative for glypican 3 and AFP.

Table 2 Clinical and histological findings of dysplastic and macroregenerative nodules.

Case	Specimen	Age	M/F	Liver disease	Fibrosis	Size (cm)	Diagnosis	Immunohistochemistry
1	Liver explant	58	M	Cryptogenic	Cirrhosis	0.9	Macroregenerative nodule	Ki67, less than 1% GP3 negative GS negative AFP negative Retic intact Bcat negative
2	Liver explant	20	M	PSC-AIH overlap	Bridging fibrosis to cirrhosis	0.5	Dysplastic nodule, low grade	Ki67, 1% GP3 negative GS negative AFP negative Retic intact Bcat negative
3	Liver explant	20	M	PSC-AIH overlap	Bridging fibrosis to cirrhosis	0.4	Dysplastic nodule, low grade	Ki67, 1% GP3 negative GS negative AFP negative Retic intact Bcat negative
4	Liver explant	20	M	PSC-AIH overlap	Bridging fibrosis to cirrhosis	0.5	Dysplastic nodule, low grade	Ki67, 1% GP3 negative GS negative AFP negative Retic intact Bcat negative
5	Liver explant	62	M	Chronic hepatitis C and a focus of well diff HCC	Cirrhosis	0.8	Macroregenerative nodule	Ki67, 1% GP3 negative GS negative AFP negative Retic intact Bcat negative
6	Liver explant	64	M	Steatohepatitis and multifocal HCC	Cirrhosis	0.9	Dysplastic nodule, low grade	Ki67, 1% GP3 negative GS negative AFP negative Retic intact Bcat negative
7	Liver explant	64	M	Steatohepatitis and multifocal HCC	Cirrhosis	0.5	Dysplastic nodule, low grade	Ki67, 1% GP3 negative GS negative AFP negative Retic focal equivocal loss Bcat negative
8	Liver explant	64	M	Steatohepatitis and multifocal HCC	Cirrhosis	0.6	Dysplastic nodule, low grade	Ki67, 1% GP3 negative GS negative AFP negative Retic focal equivocal loss Bcat negative

GP3, glypican 3; AFP, alpha fetoprotein; Bcat, beta-catenin nuclear staining; GS, glutamine synthetase; retic: reticulin; PSC, primary sclerosing cholangitis; AIH, autoimmune hepatitis; HCC, hepatocellular carcinoma.

3.4. Estimation of frequency

The frequency of HCC without reticulin loss was estimated by determining the number of HCCs without reticulin loss in 100 consecutive HCC diagnoses made in a consult practice. Four cases were identified (4%), with the caveat that this approach likely overestimates the true frequency because consult cases tend to be enriched for challenging specimens [11].

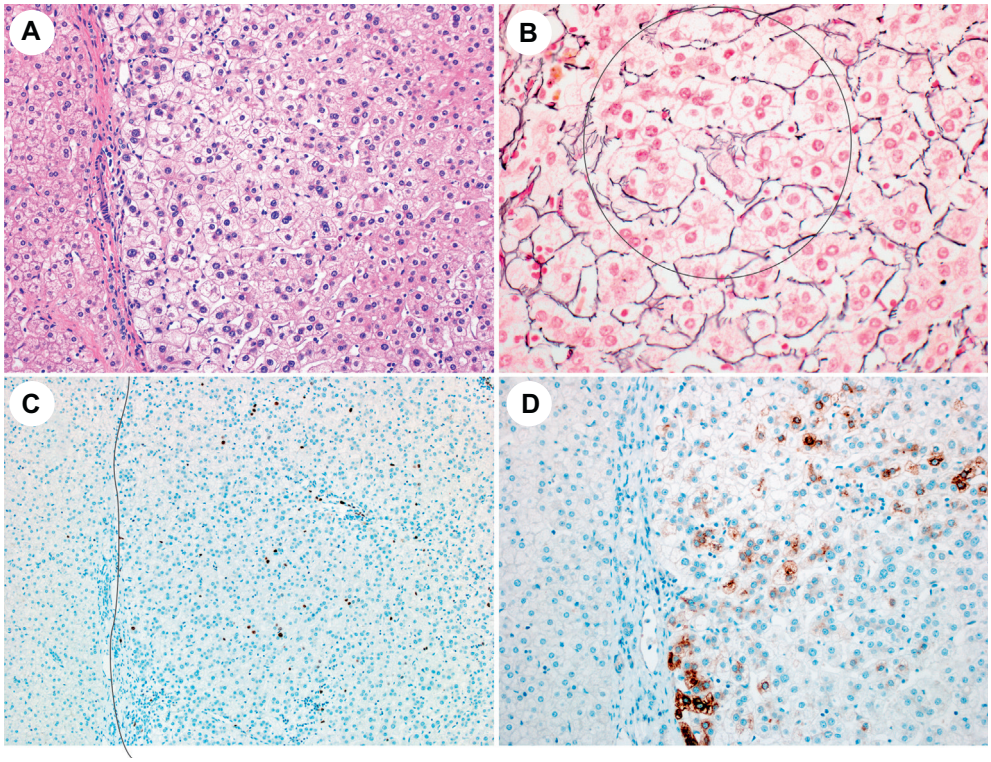

Fig. 1 **Case 1 is illustrated.** Panel 1A. There is mild atypia; tumor is on the right side of the image and the non-neoplastic liver is on the left. Panel 1B. There is focal equivocal reticulin loss (circled); because reticulin loss was limited to this single, small focus, it was not considered diagnostic in isolation. Panel 1C. The Ki67 is definitely increased in the tumor compared with nontumor (left side of image). Panel 1D. AFP is focally positive. AFP, alpha fetoprotein.

4. Discussion

The use of reticulin as an aide for diagnosing HCC represents a major advance in the diagnostic approach for HCC, as before this time the diagnosis was based exclusively on morphology, including cytological atypia, angiolymphatic invasion, and/or metastatic disease [12]. Reticulin loss in HCC was mentioned in a 1973 article [13], but its potential was specifically highlighted in a 1977 study of HCC in cirrhotic livers [14]. Subsequently, this observation was extended to neoplasms in noncirrhotic livers [15] and reticulin loss was fully incorporated into standardized criteria for diagnosing HCC [16], including fine needle aspiration specimens [15]. Since then, the use of reticulin for diagnosing HCC has been confirmed by decades of experience from centers across the world in all types of underlying liver disease.

Reticulin loss is defined essentially by two different approaches, both of which work well [9]. In the benign liver, hepatic cords are one to two cells in width, but the hepatic cords become consistently thickened (>2 cells in thickness) in HCC. The other method of assessing the reticulin stain is based on the observation that each hepatocyte in the benign liver is touching reticulin on one of its borders, whereas with HCC, tumor cells are readily found that show no contact with reticulin. Although the identification of reticulin loss is a very robust diagnostic tool, it needs to be interpreted in association with the morphology and with common sense. For example, focal equivocal reticulin loss is considered insufficient in isolation for a diagnosis of HCC [5,9]. This point bears additional emphasis, being a diagnostic pitfall in its own right, where over interpretation of reticulin changes can lead to an incorrect diagnosis of malignancy. Known diagnostic pitfalls include poor quality stains and fatty liver disease, which can have physiological loss or fragmentation of the reticulin in areas of steatosis [17]. When evaluating tumors with fatty change, the best approach is to evaluate those areas of the tumor that have no or minimal steatosis. Rapidly regenerating benign liver can also show patchy mild thickening of the hepatic trabecule [5]. Even outside these settings, the reticulin framework often has minor nonspecific changes that can be over interpreted as carcinoma. In one study of consult material [11], 15% of all submitted diagnoses of HCC were not confirmed on review, with the majority of diagnoses being changed from HCC to benign liver.

Previously, a few case reports have documented well differentiated HCCs that showed no loss of reticulin staining on biopsy specimens [1,2], but this study is the first to systematically examine this important diagnostic pitfall. The cases in this study were all men, reflecting the general

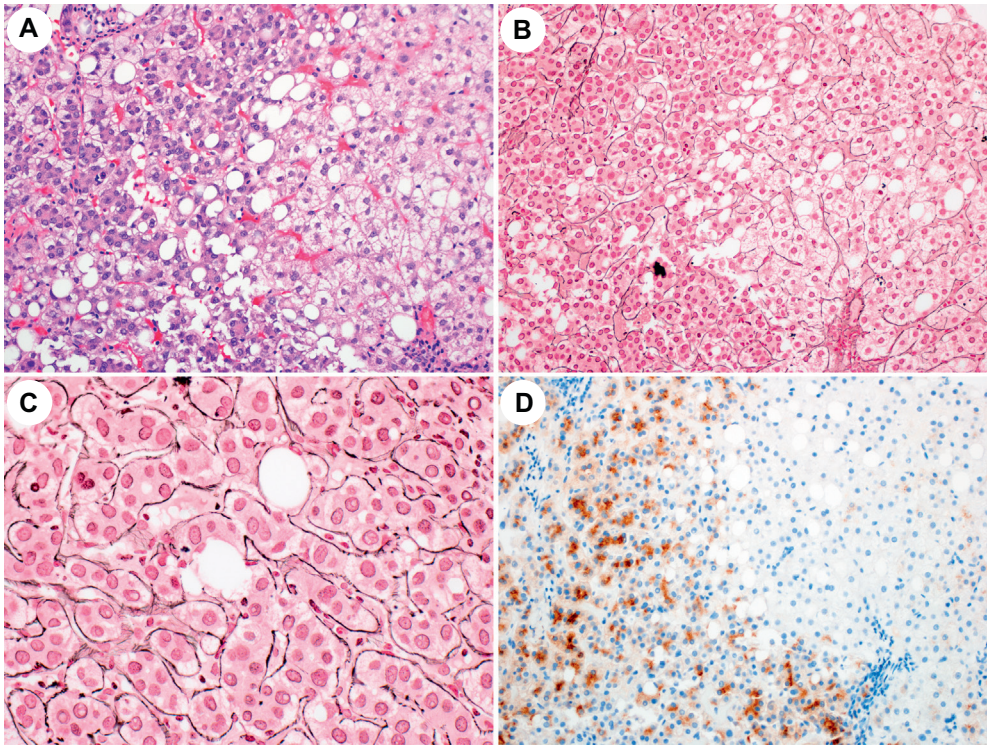

Fig. 2 **Case 2 is illustrated.** Panel 2A. There is mild atypia in the left side of the image. Mild fatty change is also present. Panel 2B. There is mild reticulin fragmentation in the area of fatty change, but no definite reticulin loss. Panel 2C. A higher magnification, confirming that there is no reticulin loss in areas without fat. Panel 2D. Glypican 3 is positive.

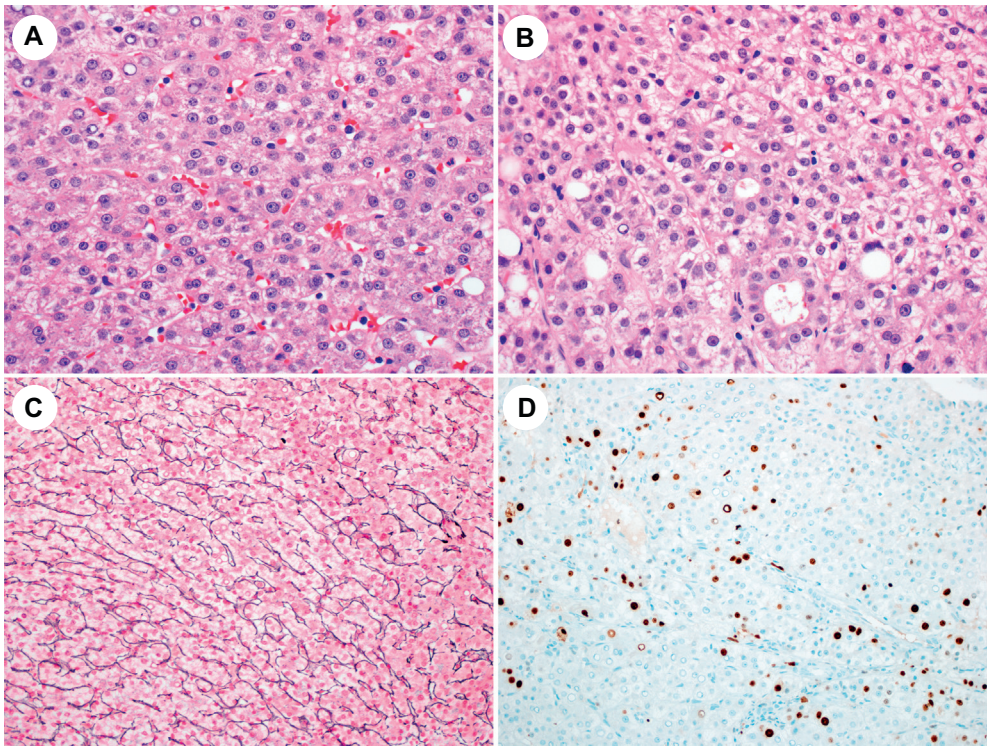

Fig. 3 **Case 3 is illustrated.** Panel 3A. There is no significant nuclear atypia, with an equivocal increase in the N:C ratio. Panel 3B. Focally, pseudoglands are present. Panel 3C. There is no reticulin loss. Panel 3D. A Ki-67 shows an increased proliferative rate.

demographics of HCC. There are no reasons to suspect that the results do not extend to women, but the relatively small numbers and the lack of women are limitations of this study. Eight of the cases in this study were on biopsy material, so it seems likely that many or even all of these tumors may have reticulin loss in the resected specimen. Three of the cases were fully resected, but small, HCCs found in cirrhotic livers, suggesting that early low-grade HCCs can sometimes show intact reticulin expression.

A rational, systematic approach is useful for these difficult biopsy specimens. The findings in this study support the following method for making a diagnosis of HCC when there is no definite reticulin loss: (1) at least mild cytological atypia should be present. In addition, there should be at least one of the following two features; both do not need to be present, although the diagnosis is strengthened when both are present: (2) Ki-67 proliferation that is clearly increased above the background liver; (3) strong expression of abnormal oncofetoproteins, such as Glypican 3 or AFP. These latter two stains are helpful when positive but are noninformative when negative. Although not used in this study, EZH2 [18] and HSP70 [4,19] are other useful immunostains to identify malignancy and could fulfill criteria number 3. In the present study, 1 of the 5 studied cases showed aberrant nuclear expression of beta-catenin and 3 of the 7 cases showed diffuse glutamine synthetase expression; these findings would also support a diagnosis of HCC when present in a nodule in a cirrhotic liver, fulfilling criteria 3. This approach will not solve every difficult case that has only mild cytological atypia but will help with many of them. For cases that do not reach these criteria, then rebiopsy is likely the best course to establish a tissue diagnosis for a radiographically concerning lesion.

The reticulin stain is thought to identify mostly type III collagen [20] but also other extracellular proteins such as type IV collagen [21] and laminin [22]. Previous studies have shown a general correlation between the grade of HCC and reduced laminin and or type IV collagen staining by immunohistochemistry [23,24]. The precise biological mechanism is unclear, however, for the reduced extracellular matrix accumulation. One possibility is the reduced reticulin results from increased extracellular matrix remodeling within the HCC, through the expression of various matrix metalloproteinases [25].

For biopsies showing neoplastic/lesional tissue with loss of portal tracts, the histological differential in non-cirrhotic livers is mostly that of hepatic adenomas and focal nodular hyperplasia. The diagnosis of focal nodular hyperplasia can sometimes be challenging [11], but the diagnosis is reliably established using the typical histological features of focal nodular hyperplasia along with glutamine synthetase stains. Hepatic adenomas should not have increased Ki-67 proliferation compared with the non-neoplastic livers and lack expression of glypican 3 and AFP. Other findings such as portal tract invasion and mitotic figures would also help exclude a hepatic adenoma. Immunostains used to subtype hepatic adenomas (Liver fatty acid binding protein, LFABP; C-reactive protein, CRP; serum amyloid A, SAA; beta-catenin; glutamine synthetase) are not useful to differentiate a hepatic adenoma from a well differentiated HCC [26].

In cirrhotic livers, the histological differential shifts to macroregenerative nodules and dysplastic nodules. Here, we studied 8 cases of macroregenerative/dysplastic nodules. All cases showed intact reticulin, low Ki-67 staining (less than 1%), and negative glypican 3 and AFP. These findings are in keeping with that of others who have also found that macroregenerative/dysplastic nodules have a low proliferative rate and are negative for AFP and glypican 3 [27]. Therefore, the size of lesions (most macroregenerative nodules are <2 cm), the presence of portal tract invasion, increased proliferation by Ki-67, and the abnormal expression of glypican 3 and AFP can help establish a diagnosis of HCC.

In summary, rare HCCs show no loss of reticulin or have only focal equivocal disruption of the reticulin meshwork, at least on biopsy specimens, and pose diagnostic challenges. These HCCs occur in the same clinical setting of typical HCC, are well differentiated, and the diagnosis can be approached using a combination of histological and immunostain findings.

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