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Recent Advances in the Immunohistochemistry-Aided Differential Diagnosis of Benign Versus Malignant Hepatocellular Lesions

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1. Introduction

Rather than obliterating the need for histopathologic examination, the availability of modern imaging modalities and efficient antitumor therapies heighten the importance of timely and accurate histologic diagnosis of hepatocellular carcinoma (HCC). More than ever before, there is now real hope of curative intervention for HCC discovered in an early stage. Thus, a key diagnostic role is assigned to histopathology whenever radiologic findings are inconclusive. While classical micromorphologic (cytologic and architectural) features are still at the heart of histopathologic evaluation, there is ongoing effort to complement histologic findings with the analysis of characteristic immunohistochemical (IHC) markers. In all modern pathology centers, IHC has become a cornerstone of the diagnostic process. The aid provided by immunomarkers is particularly resorted to when ambiguous cases are encountered. This review focuses on the role of IHC in one of the major differential diagnostic dilemmas related to HCC: the benign versus malignant problem. The question of malignant character may arise either when evaluating biopsies of suspicious nodules obtained from high-risk patients, or when analyzing tissue from hepatocellular tumors discovered in a non-cirrhotic background. Accordingly, the review will separately discuss the problem of small nodular lesions in cirrhosis, and the issue of large tumors arising in the normal liver. In our attempt to summarize state-of-the-art expert opinion, we have largely built on two outstanding reviews (Park, 2011; Roncalli et al., 2011), and tried to provide an update on recent progress made in the field.

Since it is very challenging to propose a logical and non-overlapping classification of all IHC markers, molecules will be presented in an alphabetical order. Brief introduction of each marker at its first mentioning will be followed by evaluation of its diagnostic utility, with regard to both benefits and potential pitfalls.

2. Differentiation of HCC from small precursor lesions

2.1 Dysplastic nodules and small HCC: Definitions and the role of biopsy

It is now commonly accepted that, at least in the setting of chronic hepatitis, HCC evolves through several stages of premalignant lesions. In the current view, HCC may either evolve

directly from microscopic (< 1 mm) clusters of atypical hepatocytes called dysplastic foci, or arise within macroscopic lesions termed dysplastic nodules (DNs). The following definitions conform to the latest recommendations by the International Consensus Group for Hepatocellular Neoplasia (2009). DNs with no or minimal cytologic/architectural atypia are referred to as low-grade (LG) and are unlikely to be immediately precancerous, while those with marked atypia (that is nevertheless insufficient for the diagnosis of HCC) are termed high-grade (HG), and often harbor overtly malignant subnodules. The term large regenerative nodule (LRN) has also been in use; however, LRN cannot be confidently discriminated on a purely morphologic basis from LGDN, and due to its supposed polyclonal origin it is not considered as a distinct step in the hepatocarcinogenic process (Libbrecht et al., 2005). Finally, small HCC (sHCC) is a histologically malignant tumor below the size of 2 cm, and may present as a vaguely or distinctly demarcated nodular mass, denoted as early and progressed sHCC, respectively.

Unlike dysplastic foci that are too small to be detected by any radiological technique, DNs and sHCC may often be clearly distinguished from the surrounding parenchyma by ultrasound. Hence, ultrasound screening has become an integral part of surveillance for the high-risk populations, i.e. for hepatitis B carriers and cirrhotics with various etiologies. According to the international guidelines (Bruix & Sherman, 2005) based on the Barcelona Clinic Liver Cancer Group recommendations, nodules discovered by ultrasound, if smaller than 1 cm, are followed up strictly and monitored for enlargement; nodules between 1-2 cm are subjected to examination by two contrast-enhanced dynamic imaging modalities; and nodules greater than 2 cm are examined by one dynamic imaging technique. Being a hypervascular tumor with dominantly arterial blood supply, typical HCCs show enhancement in the arterial phase followed by washout in the portal venous phase. Nodules between 1-2 cm with characteristic imaging findings by two methods, and nodules greater than 2 cm showing typical vascular pattern by one method, are treated as HCC. On the other hand, nodules with atypical vascular pattern or inconsistent radiologic findings require confirmation of benign versus malignant character by biopsy.

2.2 Individual markers for the discrimination of dysplastic nodules vs. small HCC

alpha-Smooth muscle actin (α-SMA) is a cytoskeletal component specific to cells with smooth muscle differentiation, including vascular smooth muscle cells and activated hepatic stellate cells. Hence, α-SMA immunostaining can be used to highlight both arteries and capillarized sinusoids. Although α-SMA IHC alone is not informative enough to solve the problem in question, it may help recognize unpaired arteries (i.e., arteries not accompanied by other portal structures) characteristic of DNs and sHCC (Park et al., 1998; Roncalli et al., 1999), and reveal pericytes around capillarized blood vessels.

Agrin is a large heparan sulfate proteoglycan deposited in biliary and vascular basement membranes of the liver (Tátrai et al., 2006). Since it is missing from the sinusoids of the normal liver and cirrhotic regenerative nodules, but appears in the wall of HCC microvessels very early during malignant transformation, the presence of agrin associated to microvascular structures is suggestive of HCC. Applying semi-quantitative evaluation criteria, agrin IHC discriminated sHCC from DNs with a sensitivity of 87% and a specificity of 97% (Tátrai et al. 2009). The chief practical difficulty with the use of agrin IHC is positive

labeling of ductular reaction and, although with lesser intensity, transitional cells differentiating from ductular cells into hepatocytes. Agrin-positive basement membranes on the periphery, or occasionally in the interior, of regenerative nodules due to the presence of reactive ductules and active parenchymal regeneration may confound the untrained observer. Thus, evaluation of agrin IHC requires some caution and expertise, and agrin immunopositivity is indicative of HCC only when it colocalizes with vascular markers such as CD31 or CD34. On the other hand, positive labeling of reactive ductules may be seen as an advantage, since the presence or absence of ductular reaction in and around a nodule is a diagnostic factor *per se* (see *Cytokeratin-7 and -19* below). Agrin IHC has been tested on resected specimens but not on core biopsies; hence, its performance with small samples is as yet unknown.

Annexin A2 (ANXA2). Annexins, calcium-dependent phospholipid-binding proteins with multiple functions in the regulation of vesicular trafficking, cell division, and apoptosis, are known to be differentially expressed in many forms of human neoplasia (Mussunoor & Murray, 2008). ANXA2 is upregulated in several cancer types but silenced in others; in HCC, it is overexpressed by tumor hepatocytes, as well as by endothelial cells of tumor neovessels (Yu et al., 2007). Increased ANXA2 expression was observed in proliferating benign hepatocytes, but not in sinusoidal endothelial cells, during liver regeneration (Masaki et al., 1994). Accordingly, diffuse vascular endothelial staining of ANXA2 was seen in 28/34 (84%) of HCCs but 0/7 DNs, whereas diffuse sinusoidal CD31 staining was observed in 43% of the same lesions (Longerich et al., 2011). Thus, diffuse vascular ANXA2 staining was proposed to be specific to HCC, and was successfully applied to improve the diagnostic accuracy of the GPC3 + GS + HSP70 panel (see below).

CD31/CD34. CD31, also known as platelet endothelial cell adhesion molecule-1 or PECAM-1, is a member of the immunoglobulin superfamily, and acts as a cell adhesion and signaling receptor on hematopoietic and endothelial cells (Newman, 1997). Pathologists routinely use CD31 for the immunostaining of vascular endothelia (e.g., the vasculature of tumors), or tumors with endothelial differentiation. CD34, a glycoprotein with poorly defined functions (proposed roles include regulation of adhesion and proliferation), is expressed on endothelial cells, as well as on hematopoietic and tissue stem/progenitor cells (Nielsen & McNagny, 2008). Although in clinical practice CD34 is used as a stem cell marker for the separation of hematopoietic progenitors, and as an immunomarker it has broad applications in pathology elsewhere (e.g. in the differential diagnosis of mesenchymal neoplasms, see Ponsaing et al., 2007), in the present context it is merely regarded as a vascular endothelial marker alternative to CD31. Normal sinusoidal endothelium is nearly devoid of these markers, in contrast with capillarized sinusoids, unpaired arteries, and HCC microvessels that all exhibit CD31/CD34 immunopositivity (Couvelard et al., 1993; Park et al., 1998). Normal liver is at one end of the spectrum, with virtually no CD31/CD34 immunostaining except for portal blood vessels, and typical HCC is at the other extreme with complete and ubiquitous CD31/CD34-positive vascular pattern. Thus, CD31/CD34 IHC may in theory facilitate distinction between regenerative and dysplastic nodules, as well as between DNs and HCC. However, an abrupt jump in the number of CD31/CD34-positive capillaries was observed between LGDN and HGDN, while the transition from regenerative nodules to LGDN and from HGDN to HCC was rather smooth. CD31-positive capillary units were significantly more abundant in HGDN relative to both cirrhotic regenerative nodules and

LGDN; however, HGDN did not differ significantly from HCC in this respect (Roncalli et al., 1999). Similarly, CD34 immunostaining was shown to increase gradually from normal sinusoids through capillarized sinusoids to neovessels, but failed to discriminate between HGDN and HCC (Park et al., 1998; Tátrai et al., 2009). In conclusion, endothelial markers may confirm suspicion of malignant character, but without exact cutoff values determined for each lesion type they are hardly diagnostic on their own (Park, 2011). In addition, similar to α-SMA, endothelial markers may be suitable for the detection of unpaired arteries (see above).

Cyclase-associated protein 2 (CAP2) is the human homologue of CAP, a protein originally isolated from budding yeast and only poorly characterized in mammals; in the yeast, CAP is known to associate with both the adenylyl cyclase complex and actin cytoskeleton. CAP2 levels were found to increase gradually during the process of hepatocarcinogenesis. CAP2 was overexpressed in early HCC relative to noncancerous and precancerous lesions, and further upregulated in progressed HCC (Shibata et al., 2006). In the normal and cirrhotic liver, smooth muscle cells strongly expressed CAP2, but normal hepatocytes were negative, and only weak staining was occasionally seen on the periphery of regenerative nodules. Precancerous lesions were either negative or only focally positive (5-10% of total area immunostained), whereas all early HCCs exhibited some CAP2 positivity, and 40% of them showed rather diffuse (70-100%) CAP2 immunostaining (Sakamoto, 2009). When examining nodule-in-nodule type early HCC lesions, the more advanced component exhibited stronger CAP2 reaction. While these results are encouraging, neither sensitivity and specificity values nor data on biopsy specimens have been reported.

Cytokeratin-7 and -19 (CK7/19). In the context of liver histology, CK-7 and -19 are cholangiocytic cytokeratins that highlight bile ducts and reactive ductules. Stromal invasion, i.e., invasion of (pre)malignant hepatocytes into the portal tract or septal stroma, is one of the earliest signs of hepatocellular transformation, and the scarceness or lack of outer and inner ductular reaction (DR) due to invasive growth has been reported to sensitively reflect malignant character of hepatic nodules in both resected and biopsied specimens (Park et al., 2007). The recognition of stromal invasion may be especially challenging in biopsy specimens and in well-differentiated, vaguely nodular sHCC where invasive growth is focal and obscure. The authors propose that visualization of CK7-positive DR may help resolve this diagnostic dilemma. By scoring the intensity of DR semiquantitatively on a scale between 0 and 4+, most non-invasive lesions (diagnosed as such by trained experts) scored 3+ or 4+, while overtly invasive HCCs typically scored 0 or 1+. In our own calculation, considering 0 to +2 as "missing or scant DR" and 3+ to 4+ as "present or florid DR", the lack of strong CK7-positive DR identified histologically invasive lesions with sensitivity and specificity parameters as follows: 83% and 97% (inner DR in resected nodules); 67% and 98% (outer DR in resected nodules); 95% and 90% (biopsied nodules). As expected, well-differentiated, vaguely nodular type HCCs proved to be the most problematic because they often had significant amount of (probably residual) DR both within and around.

Enhancer of zeste homologue 2 (EZH2). As the catalytically active subunit of the polycomb repressive complex 2, EZH2 is responsible for histone methylation-mediated gene silencing, and has been reported to be upregulated in a variety of human cancers (Xiao, 2011). Overexpression of EZH2 was detected in both HCC cell lines and tissue samples, and siRNA-mediated knockdown of EZH2 decreased tumorigenicity of human HCC cells

xenografted into nude mice (Chen et al., 2007). In a testing cohort containing 121 HCCs and 121 nontumorous liver tissues, nuclear EZH2 immunoreaction was observed in 66% of tumor cells but only 2.4% of nonneoplastic hepatocytes, and by selecting an appropriate cut-off value, discrimination with 96% sensitivity and 98% specificity could be obtained (Cai et al., 2011). Subsequently, in a validation series consisting of core biopsies, EZH2 alone was able to discriminate between nonmalignant nodules and HCC with a sensitivity of 78% and a specificity of 93%. In fact, EZH2 as an individual marker outperformed both GPC3 and HSP70 in this study, and additional refinement of diagnostic accuracy could be achieved by combining EZH2 with the latter two markers (see below).

Glutamine synthetase (GS) is a metabolic enzyme that converts glutamate and ammonia into glutamine, a main fuel for tumor cells. In contrast with the normal liver where GS expression is restricted to pericentral and periportal hepatocytes, most HCCs exhibit upregulation of GS (Christa et al., 1994) and diffuse, intense cytoplasmic GS immunostaining (Di Tommaso et al., 2007). This is thought to be due to overactivation of the β-catenin pathway, since GS is among the target genes of β-catenin (Zucman-Rossi et al., 2007). Expression levels of GS were found to increase in parallel with HCC progression (Osada et al., 1999). GS as an individual marker yielded 70% sensitivity and 94% specificity in discriminating resected benign vs. malignant nodules in cirrhosis (Di Tommaso et al., 2007). Over 50% of tumor cells were strongly GS-positive in the majority of HCCs, including early tumors; however, to gain more sensitivity, GS immunostaining was already considered homogeneous and positive with little more than 10% of immunoreactive cells. Similar diagnostic efficacy was obtained with biopsy specimens (GS as an individual marker, sensitivity: 59%, specificity: 98%) (Di Tommaso et al., 2009).

Glypican-3 (GPC3), a glycosyl-phosphatidylinositol anchored cell surface heparan sulfate proteoglycan, is overexpressed in the majority of HCCs (Zhu et al., 2001), and is currently accepted as the best-performing individual IHC and serum marker of HCC (Capurro et al., 2003; International Consensus Group, 2009). GPC3 regulates multiple growth factor signaling pathways including those of Wnts, Hhs, IGF, FGF2, and BMPs, and is therefore thought to be directly involved in HCC pathogenesis (reviewed by Akutsu et al., 2010). Being an oncofetal antigen, it is absent from the healthy adult liver and becomes re-expressed upon hepatocytic transformation only. GPC3 immunostaining in malignant hepatocytes may present as granular or strong diffuse cytoplasmic pattern, and may also appear on the cell membrane. With the detection threshold set very low (lesions with a single GPC3-immunoreactive cell were treated as positive), a sensitivity of 77% and a specificity of 96% could be achieved when discriminating sHCC from benign hepatic nodules in resected specimens (Libbrecht et al., 2006). The same values were 83% and 100% for needle biopsies. Of course, like with any other method, some lesions were in the 'grey zone': occasionally, foci of hepatocytes with marked atypia and GPC3 positivity were discovered in HGDNs; and a proportion of HCCs, especially the well-differentiated and less aggressive ones, remained negative in spite of maximum possible sensitivity. Di Tommaso et al. (2007, 2009) applied somewhat stricter criteria for positivity (threshold was set at 5% immunoreactive cells), and could nevertheless nicely reproduce previous results, achieving 74% sensitivity and 96% specificity on resected specimens, and 71% / 94% on core biopsies. Wang et al. (2010) found 83% of HCC needle biopsies to be GPC3-positive. Impressive as these values are, GPC3 IHC also has its potential pitfalls and, not coincidentally, GPC3 is

currently recommended for use in combination with one or more additional markers (see below). As much as 40% of early HCCs arising in cirrhosis may be GPC3-negative (Wang et al., 2006). On the other hand, Abdul-Al et al. (2008) and Shafizadeh et al. (2008) pointed out that focal positive staining for GPC3 in active chronic hepatitis C is not an infrequent finding, and may correlate with the acquisition of a fetal-like phenotype during hepatocytic regeneration. Intriguingly, other authors claimed that cirrhotic nodules were invariably negative in core needle biopsies (Anatelli et al., 2008). In a high case number tissue microarray-based study, 9% of non-neoplastic and 16% of preneoplastic liver samples were GPC3-positive (Baumhoer et al., 2008). Thus, while the utility of GPC3 is undisputed, awareness of its limitations in terms of both sensitivity and specificity is advisable.

Heat shock protein 70 (HSP70). The 70-kDa member of the heat shock protein family was identified as the most abundantly upregulated gene during HCC progression by a gene expression array comparing early vs. progressed components of nodule-in-nodule type lesions (Chuma et al., 2003). Overexpression of HSP70, observed in other cancer types as well, enhances proliferation, and confers resistance to attacks of the immune system and apoptosis (Khalil et al., 2011). In IHC studies, HSP70 was strongly expressed by cholangiocytes that hence served as internal positive control, but not by normal hepatocytes, and only faintly and focally in cirrhotic nodules. The intensity of HSP70 immunostaining seemed to increase in parallel with the transition from DN through early HCC to progressed HCC (Chuma et al., 2003). Sensitivity and specificity of HSP70 alone in discriminating HGDN from early HCC was 78% and 95% in resected specimens (Di Tommaso et al., 2007). The criterion for positivity was nucleocytoplasmic staining in at least 5% of lesional hepatocytes. In needle biopsies, however, HSP70 recognized malignant nodules with a sensitivity as low as 48% (Di Tommaso et al., 2009). The fall in sensitivity was probably due to scattered focal positivity in highly differentiated lesions which made them especially prone to sampling error.

2.3 Marker panels for the discrimination of dysplastic nodules vs. small HCC

Agrin + CD34. The discriminative power of agrin IHC could be slightly improved by taking into account the CD34 immunostaining pattern of small nodular lesions. By handling only those nodules as malignant that exhibited complete immunostaining with both agrin and CD34, sHCC could be identified in 87% of the cases, and 100% specificity was attained (Tátrai et al., 2009).

Clathrin heavy chain (CHC) + formiminotransferase cyclodeaminase (FTCD). These two potential markers were identified by 2-dimensional fluorescence difference gel electrophoresis, and validated by IHC on a tissue array containing 83 HCC and 68 non-tumor liver tissue cores (Seimiya et al., 2008). CHC, as a member of the clathrin complex, is a ubiquitous protein involved in membrane trafficking and mitosis; however, CHC has also been shown to regulate p53 function, and is a gene fusion partner in several human tumor types (Ohmori et al., 2008; Blixt & Royle, 2011). FTCD, a bifunctional enzyme that couples histidine degradation to folate metabolism, is also present in every cell type but most abundantly in the liver (Mao et al., 2004). CHC was found to be upregulated and FTCD downregulated in early HCC relative to the surrounding parenchyma (Seimiya et al., 2008). Although the sensitivity of either CHC or FTCD alone was not sufficiently high for the detection of HCC (52% and 61%, respectively), a sensitivity of 81%, beside a specificity of 94%, could be

achieved by combining the two. The authors reported additional gains in efficacy when combining the novel markers with GPC3, and their findings were further evaluated in respect with CHC by Di Tommaso et al. (2011) who tentatively added CHC to the widely accepted 3-marker panel GPC3 + GS + HSP70 (see below).

GPC3 + EZH2 + HSP70. This panel of markers was recently shown to discriminate needle-biopsied benign and malignant nodules with a sensitivity of 81% and a specificity of 100% when cases with 2 positive markers out of 3 were regarded as malignant (Cai et al., 2011). This efficacy was significantly superior to that of any single marker alone, and better than the standard 3-marker panel with GS in the place of EZH2 (see below).

GPC3 + GS + HSP70: the standard 3-marker panel and its derivates. Combination of these three markers was first proposed by the Roncalli group (Di Tommaso et al., 2007), and has been quickly acknowledged as the best IHC panel for the discrimination of early HCC against benign nodules (International Consensus Group, 2009; Roncalli et al., 2011). With any 2 of the 3 markers being unequivocally positive, early HCC could be distinguished from HGDN with 72% sensitivity and 100% specificity in resected specimens, and 59% sensitivity and 100% specificity in needle core biopsies (Di Tommaso et al., 2007, 2009). In both cases, sensitivity and specificity were significantly improved as compared to the application of any of the three markers alone. Recently, inspired by the results of Seimiya et al. (2008), the group tested the added value of including CHC (see under 2.2) as a fourth marker into the diagnostic panel (Di Tommaso et al., 2011). Extension of the 3-marker panel to a 4-marker panel by the inclusion of CHC was shown to yield a further gain in diagnostic efficacy. E.g., by taking 2 positive markers out of the 4 as indicative of HCC, sensitivity of detection of sHCC in core biopsies was improved from 47% to 64%. To the same end, Longerich et al. (2011) complemented the 3-marker panel with ANXA2 (see under 2.2), and achieved 74% sensitivity coupled with 100% specificity in discriminating any benign lesion from HCC in resected specimens.

GPC3 + phenol sulfotranferase 1 (SULT1A1). SULT1A1 is a xenobiotic metabolic enzyme selected by 2-dimensional polyacrylamide gel electrophoresis and confirmed as HCC marker by Western blotting and IHC (Yeo et al., 2010). By IHC, SULT1A1 was found to be downregulated in roughly half of HCCs. By combining the results of GPC3 and SULT1A1 (the IHC-based diagnosis is HCC if GPC3 is positive or SULT1A1 is negative), sensitivity could be improved from 72% (GPC3 alone) to 79%. SULT1A1 was shown to differentiate between LGDN and HCC, but its utility was not specifically addressed in the discrimination of HGDN vs. sHCC.

3. Differentiation of HCC from benign liver tumors

3.1 Focal nodular hyperplasia and hepatocellular adenoma: Entities with distinct pathogenesis and clinical behavior

Focal nodular hyperplasia (FNH), as also suggested by its name, is not a true neoplastic lesion; rather, it is a polyclonal hyperplastic, tumor-like reaction to an intrahepatic vascular malformation or alteration (Schirmacher & Longerich, 2009). With an incidence of 3% in the total population, FNH is the second most common benign liver tumor after hemangioma. Classical morphologic features of FNH include a central stellate scar with abnormal arteries, and surrounding nodules separated by fibrous septa and florid ductular reaction. FNH, unlike HCA, is not prone to malignant transformation or hemorrhage; however, its

differential diagnosis by contrast-enhanced imaging techniques may be difficult under certain circumstances, making resection and pathologic examination necessary.

Hepatocellular adenoma (HCA) is 10 times less frequently encountered than FNH; it may nevertheless call for urgent attention due to the risk of hemorrhage and, especially in the β-catenin-mutated subtypes, transformation into HCC. Molecular classification of HCA has led to the identification of three groups: 1) classical HCA with inactivating mutation of hepatocyte nuclear factor 1α (HNF1α); 2) 'atypical' HCA with activating mutation of β-catenin; and 3) inflammatory HCA with inflammatory infiltrate and occasional activating mutation of β-catenin (Bioulac-Sage et al., 2007a). Less than 10% of all HCAs lack any characteristic profile and hence remain unclassified. Inflammatory HCA presents the highest risk of bleeding, while β-catenin-mutated HCA harbors a strong tendency toward malignant transformation, and approx. 40% of cases actually progress to HCC (Schirmacher & Longerich, 2009).

Current recommendations on IHC-assisted pathologic differential diagnosis of FNH and HCA have recently been covered by Bioulac-Sage et al. (2011); the present discussion is mostly based on this excellent review.

3.2 Individual markers and their combinations for the discrimination of HCC vs. benign liver tumors

Agrin. (See section 2.2 for introduction.) Agrin, just like CD34, is diffusely positive over the entire vascular network of HCCs, whereas the expression of agrin is more restricted than that of CD34 in HCAs lacking significant atypia. Thus, agrin IHC is more selective in discriminating typical HCA from HCC when compared to CD34. Strong and ubiquitous vascular agrin immunostaining was present in 26/27 HCCs but only 7/30 HCAs, with 3 of the 7 diffusely agrin-positive HCAs exhibiting marked to severe atypia (Tátrai et al., 2009). By quantitative evaluation of agrin immunostaining, HCA could be distinguished from HCC with a sensitivity of 80% and a specificity of 89%.

Annexin A2 (ANXA2). (See section 2.2 for introduction.) ANXA2 alone did not work as accurately in the discrimination of HCA and HCC as in the differentiation of benign vs. malignant nodules: the vascular network of 6/19 (32%) of HCAs showed diffuse ANXA2 immunostaining (Longerich et al., 2011). However, when combined with the 3-marker panel GPC3 + GS + HSP70, false positives could be eliminated, and satisfactory diagnostic efficacy was obtained (all benign lesions vs. HCC, sensitivity: 74%, specificity: 100%).

β-Catenin is the major target of Wnt signaling and a key transcriptional regulator with well-known functions in hepatic oncogenesis (reviewed by Dahmani et al., 2011). Activation of the β-catenin pathway may accompany both benign and malignant hepatocytic proliferation; mutations of β-catenin, however, are specifically found in the high-risk subtypes of HCA, as well as in up to 50% of HCCs. Overactivation of the β-catenin pathway is hallmarked by aberrant nuclear and/or cytoplasmic β-catenin immunostaining. Such positive nucleocytoplasmic β-catenin staining is absent from FNH but, although often weakly and focally, found in β-catenin-mutated HCA and HCC.

CD34. (See section 2.2 for introduction.) In both FNH and HCA, some sinusoids experience altered perfusion, and a shift toward arterial supply favors the neoexpression of CD34. CD34-positive, arterialized sinusoids are seen to radiate away from portal tract-like structures in FNH, and surround small tumor-supplying arteries in typical HCA

(Theuerkauf et al., 2001). On the other hand, in the majority of HCCs, and also in some cases of 'atypical' HCA (i.e., HCA showing marked cytologic and/or architectural atypia), the entire capillary network of the tumor is diffusely CD34-positive. Although not sufficiently specific on its own, the above features make CD34 a useful ancillary marker when applied in combination (see *Glypican-3* in this section).

C-reactive protein (CRP) and serum amyloid A (SAA) are acute phase proteins upregulated in inflammatory HCA, and as immunomarkers they have been shown to specifically identify this subtype of HCA (Bioulac-Sage et al., 2007b). Both are absent from FNH and non-inflammatory HCA, and only rarely positive in HCC.

Glutamine synthetase (GS). (See section 2.2 for introduction.) GS immunostaining, restricted to 1-2 cell thick hepatocyte plates around hepatic venules in the normal liver, is greatly broadened in FNH, resulting in a map-like pattern of large anastomosing immunopositive areas (Bioulac-Sage et al., 2009). Hepatocytes in the immediate vicinity of fibrous septa and arteries usually remain unstained. Although this is a typical finding, it may be less evident in rare cases with excessive steatosis or sinusoidal dilation. As a contrast, GS immunostaining is either missing or reminiscent of the normal liver in β-catenin non-mutated HCA, and diffusely present over the entire area of both β-catenin-mutated HCA and HCC. Thus, large, contiguous bands of strong GS immunopositivity with interspersed negative areas, together forming a map-like pattern, are indicative of FNH, whereas diffuse GS labeling, either homogeneous or heterogeneous, suggests β-catenin-mutated HCA or HCC.

Glypican-3 (GPC3). (See section 2.2 for introduction.) When evaluating tumors developed in the non-cirrhotic liver, sensitivity issues regarding GPC3 come to the foreground. HCCs that arise in a cirrhotic background, especially those reaching a progressed stage, are overwhelmingly (in up to 90% of cases) GPC3-positive, whereas 36% of HCCs discovered in normal livers were found to be GPC3-negative (Wang et al., 2006). A significant proportion (up to 50%) of well-differentiated HCCs are actually devoid of any GPC3-staining, which calls for extreme caution in the interpretation of GPC3 results, and warns against overestimation of its potency as a single marker in the HCA vs. HCC problem (Shafizadeh et al., 2008). Diffuse GS staining often observed in β-catenin-mutated HCA further obscures the fuzzy borderline between atypical HCA and well-differentiated HCC, and makes the 3-marker panel GPC3 + GS + HSP70 which is so helpful in cirrhosis virtually useless in this situation. Coston et al. (2008) proposed that GPC3 should be combined with CD34 to help identify HCCs with no or little GPC3 positivity but complete CD34 labeling of the vasculature; however, atypical HCAs, too, may show complete CD34-positive pattern.

Liver fatty acid binding protein (LFABP) is a target gene of the liver-specific transcription factor HNF1α (Akiyama et al., 2000); consequently, its expression is practically lost in classical HCAs that harbor biallelic inactivating mutations of HNF1α (Bioulac-Sage et al., 2007b). LFABP, on the other hand, is produced by normal hepatocytes, and its expression is retained in both FNH and HCC. Therefore, negative LFABP staining of a tumor against a background of LFABP-positive liver parenchyma is highly indicative of classical HCA.

4. Conclusion

IHC markers discussed so far are summarized in **Table 1** (DNs vs. sHCC) and **Table 2** (benign hepatocellular tumors vs. HCC).

Like in many other situations, biology knows no black and white, but the pathologist must come up with 'yes' or 'no'. There may be cases, both small nodules and large tumors, that do not embarrass a liver specialist who spends his whole life with examining hepatocellular lesions, but may perplex a less trained observer. For the latter, IHC markers, and marker panels in particular, which are unambiguous to interpret and offer clear guidance, may be invaluable. Moreover, cumbersome cases always turn up that puzzle even the most experienced specialist, who must then seek for external confirmation of his intuition.

Marker	Labeled structure in HCC	Typical pattern		Sens. / Spec.
		Non-malignant nodules	Small HCC	
α-SMA	wall of unpaired arteries	no or few unpaired arteries	many unpaired arteries	ND
agrin	blood vessel walls	ductular reaction positive; sinusoids negative	all blood vessels positive	resected: 87% / 97%
ANXA2	tumor hepatocytes and endothelial cells	proliferating hepatocytes positive	endothelium also positive	resected: 84% / 100%
CD31/34	endothelium	most sinusoids negative, unpaired arteries positive	all blood vessels positive	ND
CAP2	tumor hepatocytes	smooth muscle cells positive, hepatocytes negative	tumor cells positive	ND
CK7/19	none	inner / outer ductular reaction (DR) positive	missing DR in and around the nodule	resected: 83% / 98%; needle: 95% / 90%
EZH2	tumor hepatocytes	few hepatocytes positive	most tumor cells positive	needle: 78% / 93%
GS	tumor hepatocytes	positive staining restricted to periportal and pericentral areas	diffuse positive staining	resected: 70% / 94%; needle: 59% / 98%
GPC3	tumor hepatocytes	hepatocytes negative	tumor cells positive	resected: 74-77% / 96%; needle: 71-83% / 94-100%
HSP70	tumor hepatocytes	bile ducts/ductules positive, hepatocytes negative	tumor cells positive	resected: 78% / 95%; needle: 48% / 94%

ND, not determined

Table 1a. Individual markers for the discrimination of benign vs. malignant nodules in cirrhosis. See text for abbreviations and references.

Marker panel	Spec. / Sens.
agrin + CD34	resected: 87% / 100%
CHC + FTCD	tissue array: 81% / 94%
GPC3 + EZH2 + HSP70	needle: 81% / 100%
GPC3 + GS + HSP70	resected: 72% / 100%; needle: 59% / 100%
GPC3 + GS + HSP70 + CHC	needle: 64% / 100%
GPC3 + GS + HSP70 + ANXA2	resected: 74% / 100%
GPC3 + SULT1A1	resected: 79% / ND

ND, not determined

Table 1b. Marker panels for the discrimination of benign vs. malignant nodules in cirrhosis. See text for abbreviations and references.

Marker	Typical pattern		
	FNH	**HCA**	**HCC**
agrin	sinusoids negative	vascular network: no or incomplete positivity	vascular network: complete positivity
ANXA2	ND	vascular network: focal positivity	vascular network: complete positivity
β-catenin	no aberrant staining in hepatocytes	nucleocytoplasmic staning in β-catenin-mutated HCA	nucleocytoplasmic staning in β-catenin-mutated HCC
CD34	aberrant arteries in scar positive; some sinusoids positive	sinusoids in arterial inflow areas positive	entire vascular network positive
CRP / SAA	negative	positive in inflammatory HCA; negative in others	rarely positive
GS	positive in map-like pattern	negative, or positive in restricted areas only	diffusely positive
GPC3	negative	negative	positive in tumor hepatocytes
LFABP	positive	negative in HNF1α-mutated HCA; positive in others	positive

Table 2. Markers for the discrimination of benign vs. malignant liver tumors. See text for abbreviations and references.

Optimally, an IHC marker of HCC should show an unequivocal and consistent staining pattern, either positive or negative, throughout the entire malignant lesion, and thus produce a sharp contrast against the non-malignant background. Also, optimal sampling should cover a representative area of the lesion, include some surrounding tissue for reference and, of course, provide high-quality tissue. Although no individual marker meets these strict criteria, and sampling is not always ideal, combining IHC markers has been shown to increase diagnostic success rates even in needle core biopsies where both sample size and quality are limiting. The 3-panel marker consisting of GPC3, GS and HSP70 proved to be a powerful tool in the discrimination of HGDN and early HCC, and extension of the panel with additional markers such as ANXA2, CHC, or EZH2, has been shown to further improve diagnostic accuracy. Conventional markers such as CD31/CD34 or biliary cytokeratins, as well as emerging candidates like agrin or CAP2, may also find application when any doubt remains. Similarly, a characteristic pattern of IHC markers, some indicating malignant hepatocellular transformation and others reflecting arterialization of tumor sinusoids, may greatly facilitate pathologic differentiation of benign hepatocellular tumors vs. HCC.

However, dubious cases will continue to occur, and no degree of certainty can be too much; hence, the quest for new IHC markers is unlikely to come to an end soon. And, since HGDN and early HCC, just like atypical HCA and well-differentiated HCC, probably lie along a continuum of malignant behavior, the ultimate elimination of the word 'borderline' from our vocabulary may remain a hope.

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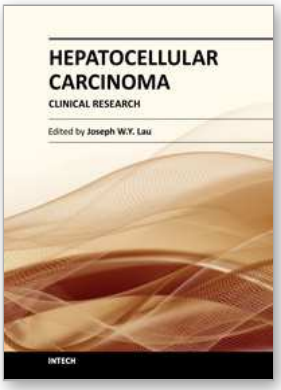

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This book covers the clinical aspects of hepatocellular carcinoma. This book is a compendium of papers written by experts from different parts of the world to present the most up-to-date knowledge on the clinical aspects of hepatocellular carcinoma. This book is divided into three sections: (I) Diagnosis / Differential Diagnosis; (II) Surgical Treatment; (III) Non-surgical Treatment. There are 19 chapters covering topics from novel diagnostic methods to hepatic lesions mimicking hepatocellular carcinoma, from laparoscopic liver resection to major hepatectomy without allogeneic blood transfusion, from molecular targeted therapy to transarterial radioembolization, and from local ablative therapy to regional therapy. This volume is an important contribution to the clinical management of patients with hepatocellular carcinoma. The intended readers of this book are clinicians who are interested in hepatocellular carcinoma, including hepatologists, liver surgeons, interventional and diagnostic radiologists, pathologists and epidemiologists. General surgeons, general physicians, trainees, hospital administrators, and instruments and drug manufacturers will also find this book useful as a reference.

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