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Abstract

Although the histological and cytological features of differentiated hepatocellular carcinoma (HCC) usually allow a reliable diagnosis, poorly differentiated HCCs and tumors found in small samples require immunohistochemical confirmation. Generally, cells of HCCs share with normal hepatocytes a rather wide array of lineage markers. These include Hep Par1, arginase 1, and glutamine synthetase, which are expressed in many, but not all, HCCs. A majority of HCCs are positive for the heparan sulfate proteoglycan, glypican-3. Many HCCs express and secrete typical export proteins, of which alpha-fetoprotein is the best-known example. Part of HCCs show reactivity for the canalicular domain markers, polyclonal CEA and CD10. The tumors share with hepatocytes the expression of cytokeratins 8 and 18, and sometimes 19, the latter defining a tumor subset with poor prognosis. In addition, HCCs express, however in a variable manner, markers of cellular differentiation, cell adhesion molecules, oncogenes, tumor suppressors, and a host of other markers related to various biochemical and metabolic functions.

Introduction

Immunohistochemistry has an important role in the diagnostic approach to HCC and for tumor monitoring (Varma and Cohen 2004; Wee 2006; Lo and Ng 2011; Masuda and Miyoshi 2011; Minguez and Lachenmayer 2011). The field of HCC markers is very active and the number of markers is steadily growing (Marrero and Lok 2004). The most important markers are compiled in the Table 1.

Hepatocyte Lineage Markers

As part of HCC consists of cells that are similar to normal or hyperplastic hepatocytes, immunohistochemical methods are useful for the diagnosis of these neoplasms (reviews: Varma and Cohen

Table 1 Markers for hepatocellular carcinoma

<i>Hepatocyte/HCC cell lineage markers</i>
Hep Par1
Arginase 1
Glutamine synthetase
<i>Heparan sulfate proteoglycan expressed by hepatocytes/HCC cells</i>
Glypican-3
<i>Hepatocyte/HCC-typical export proteins</i>
Alpha fetoprotein (AFP)
Alpha-1-antitrypsin (AAT)
Ferritin
Abnormal prothrombins (PIVKA-II)
<i>Canalicular domain markers</i>
CD10
Polyclonal CEA (pCEA)
<i>Intermediate filaments</i>
Cytokeratin 8
Cytokeratin 18
Cytokeratin 19
<i>Wnt/beta-catenin signaling pathway</i>
Beta-catenin
<i>Markers of proliferative activity</i>
PCNA
Ki-67
Cyclin D1
<i>Markers of cellular differentiation</i>
DEC1 (in part of HCCs)
MUC1 (in part of HCCs)
Squamous cell carcinoma antigen (SCCA; in part of HCCs)
<i>Cell adhesion molecules and cytoskeletal proteins</i>
CD44
N-cadherin (in part of HCCs)
EpCAM (epithelial cell adhesion molecule; in part of HCCs)
NCAM (neural cell adhesion molecule; in part of HCCs)
Clathrin heavy chain
<i>Oncogenes and tumor suppressor genes</i>
p53
p73
<i>Other markers (expressed in part of HCCs)</i>
Heat shock protein 70 (HSP70)
HCC-specific gamma-glutamyltransferase
Kazal type 1 proteinase inhibitor (SPINK1)
CD147 (EMMPRIN)
SALL4
Osteopontin
Osteonectin/SPARC
Importin-alpha 1
Aldo-keto reductase 1

2004; Wee 2006). There are several markers of the hepatocyte cell lineage that can be employed in HCC diagnosis, including antibodies directed against hepatocyte antigens and proteins exclusively expressed in hepatocytes (review: Roncalli et al. 2011).

HepPar1

Hep Par1 is a reliable hepatocyte lineage marker and is also regarded as a potent tool in the differential diagnosis of hepatocellular tumors (Leong et al. 1998; Zimmermann et al. 2001; Chu et al. 2002; Siddiqui et al. 2002; Lamps and Folpe 2003; Saad et al. 2004; Varma and Cohen 2004; Wang et al. 2006; Karabork et al. 2010; Al-Muhannadi et al. 2011; Shibuya et al. 2011). The antigen for Hep Par1 was reported to be the hepatocytic urea cycle enzyme, carbamoyl phosphate synthetase 1 located in mitochondria (Butler et al. 2008). Hep Par1 has a sensitivity for HCC of around 80–100 % and generally reflects hepatocyte-like differentiation in tumors. The reactivity manifests as a diffuse cytoplasmic granular staining pattern in normal and neoplastic hepatocytes (Figs. 1 and 2).

The frequency of Hep Par1 positivity depends on the tumor's grade. In an analysis of 96 cases of HCC, all 50 cases of nuclear grade 1 and nuclear

grade 2 HCC were positive for Hep Par1, while only 84 % with grade 3 and 50 % with grade 4 were positive, and a positive result was more common in HCC with a trabecular, pseudoacinar, or scirrhous growth pattern than in those with a compact pattern (Chu et al. 2002). Apart from differences related to grade, not all HCC show uniform Hep Par1 staining. Tumors containing steatotic, clear cell, or oncocytic components show reduced or lacking reactivity in these areas. Hep Par1 reactivity is also detectable in metastases of HCC and seems to be a prognostic factor (Mondada et al. 2006). As Hep Par1 is expressed in hepatoid adenocarcinoma and its metastases (Pitman et al. 2004), and hepatoid carcinomas histologically are more or less identical to HCC, Hep Par1 will not serve to distinguish these two entities. However, not all HCC stain uniformly, and not all Hep Par1-positive tumors are of hepatocyte lineage or arise in the liver (review; Wee 2006). In one analysis, 3/19 HCCs showed <5 % Hep Par1 immunostaining, and variable Hep Par1 positivity was detected in part of gastric carcinoma, cholangiocarcinoma, colorectal carcinoma, lung carcinomas, ovarian carcinomas, and adrenocortical carcinomas (Fan et al. 2003), but also in reactive lesions such as intestinal metaplasia (Chu et al. 2003).

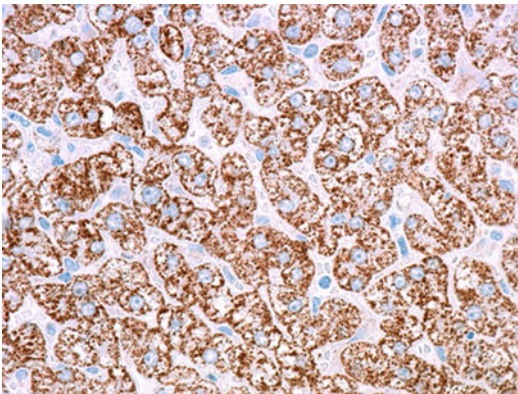


Fig. 1 Well-differentiated hepatocellular carcinoma with expression of Hep Par1 in a cytoplasmic granular pattern (Hep Par1 immunostain)

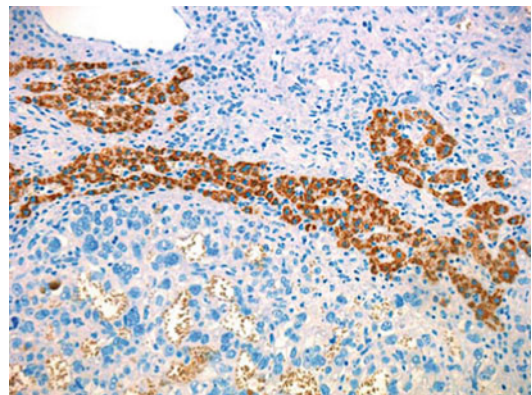


Fig. 2 Poorly differentiated hepatocellular carcinoma may downregulate expression of Hep Par1. In the present case, pleomorphic HCC cells (lower half of figure) are Hep Par1-negative, in contrast to compressed and partially atrophic hepatocytes (Hep Par1 immunostain)

Arginase-1

An important marker of the hepatocyte enzyme category comprises arginase-1 which is reactive in HCC (Yokoyama et al. 2004; Yan et al. 2010; Radwan and Ahmed 2012). Arginase-1 reactivity was demonstrated as a reliable marker of HCC in fine needle aspirates (McKnight et al. 2012) and was found to be superior in sensitivity to Hep Par1 and glypican-3 (Fujiwara et al. 2012). Arginase-1 reactivity was also useful in the diagnosis of scirrhous HCC (Krings et al. 2013). However, expression of arginase-1 is not restricted to hepatocytes and hepatocytic neoplasms, as this enzyme is expressed in myelocytes/metamyelocytes and localized in gelatinase granules of human neutrophils (Jacobsen et al. 2007).

Glutamine Synthetase

Expression of glutamine synthetase (GS) is an early marker for HCC based on immunohistochemical and proteomic analyses (Matsuno and Goto 1992; Dal Bello et al. 2010; Long et al. 2010; Shin et al. 2011) and is expressed in most of these neoplasms. GS expression is regulated by ubiquitin-dependent proteolysis (Osada et al. 1999). GS immunostaining is characterized by diffuse reactivity of the cytoplasm. GS showed an increased expression and phosphorylation in well-differentiated HCC, GS-positive cells sometimes growing in nodule-in-nodule manner (Kuramitsu et al. 2006). GS staining was correlated with large tumors, low histologic grade, formation of pseudoacini, bile deposition, and reduced specific and overall mortality (Del Bello et al. 2010). On the other hand, there is evidence that GS expression may enhance the metastatic potential in HCC (Osada et al. 1999, 2000). GS immunostaining is also present in focal nodular hyperplasia/FNH, regardless of size or a steatotic component. However, the staining pattern is different from that of HCC in that it is not diffuse, but

characteristically anastomosed in a map-like cytoplasmic pattern (Bioulac-Sage et al. 2009).

Glypican-3

Glypican-3 is a valuable marker for hepatocyte-derived malignancies, including HCC and hepatoblastoma (Zhu et al. 2001; Coston et al. 2008; Wang et al. 2012b; Filmus and Capurro 2013; Krings et al. 2013; Witjes et al. 2013) but also for several other malignancies. Immunostaining for GPC3 can distinguish HCC from benign liver cell neoplasms, preneoplastic lesions, cholangiocarcinoma, metastases, and hyperplastic cells of cirrhosis. That enhanced GPC expression differentiates the majority of HCCs from non-HCC lesions has been shown by us in 2001 (Zhu et al. 2001) and has since been confirmed several times (Nakatsura et al. 2003; Filmus and Capurro 2004; Man et al. 2005; Liu et al. 2010; Suzuki et al. 2010; Yan et al. 2011; Yorita et al. 2011; review: Kandil and Cooper 2009; Fig. 3).

In contrast to HCCs, which show an upregulation of GPC3 in the majority of cases, GPC3 is downregulated in cholangiocarcinomas (Man et al. 2005) and nonmalignant hepatocellular lesions (Enan et al. 2013). Expression of GPC3 is a valuable diagnostic element in early HCC (Chen et al. 2014) and in HCC that are negative for AFP (Li et al. 2013). Immunohistochemically,

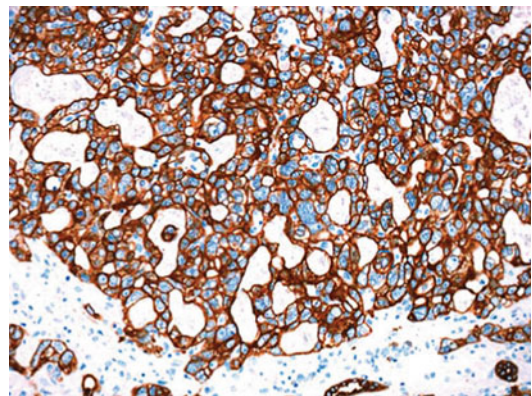


Fig. 3 Glypican-3 expression by HCC cells (glypican-3 immunostain)

GPC3 is visualized as a more or less diffuse cytoplasmic staining of cells in moderately and well-differentiated HCCs. In poorly differentiated HCC, membranous GPC3 immunostaining is seen, and membranous staining is also prevalent in metastatic lesions of HCC when compared with the primary tumors (Suzuki et al. 2010). HCC patients with circumferential cell surface GPC3 immunostaining revealed worse prognosis of their tumor disease (Yorita et al. 2011). This immunophenotype of HCCs is also visualized in needle biopsies (Kandil et al. 2007). The yield of GPC3 positivity in HCCs may exceed 85 % (Wang et al. 2006). In a microarray study of 54 HCCs and adjacent liver tissue, GPC3 staining was observed in 90 % of 21 HCC cases with cirrhosis and in 64 % of 28 HCC cases associated with non-cirrhotic liver. In cases with adenomas, only malignant foci were positive. Among 94 macronodules, GPC3 immunostaining was noted in 48 % of high-grade dysplastic nodules or early HCCs and in only 3 % of benign or low-grade dysplastic nodules (Wang et al. 2006).

Similar to classical HCC, fibrolamellar hepatocellular carcinoma (FL-HCC) can express GPC3 (Shafizadeh et al. 2008; Abdul-Al et al. 2010; Ward et al. 2010; Ward and Waxman 2011). In a comparative immunohistochemical study of 26 cases of FL-HCC and 62 classical HCCs, 39 % of HCC and 59 % of FL-HCC cases were positive for GPC3 (Ward et al. 2010). In contrast to this study, another investigation found that GPC3 was more often and more strongly expressed in HCCs (72 %) than in FL-HCCs (17 %) (Abdul-Al et al. 2010). Immunoreactivity for GPC3, together with that of Hep Par1, was detected in lymphoepithelioma-like hepatocellular carcinoma (Nemolato et al. 2008). GPC3 is also involved in the biology of hepatic cells other than hepatocytes and neoplasm derived thereof. M2-polarized tumor-associated macrophages which promote the progression and metastasis of HCC are recruited by CCL5, CCL3, and CSF1 under involvement of GPC3 (Takai et al. 2009).

Glypican-3 (GPC3) is a member of the membrane-anchored heparan sulfate proteoglycan family. Glypicans are linked to the

cell surface membrane by a glycosylphosphatidylinositol (GPI) anchor. So far, six members of this family (GPC1 – GPC6) have been identified in mammals, and two in *Drosophila*. All glypicans share the same basic structure, characterized by a core domain, an N-terminal secretory signal peptide, and a hydrophobic domain required for the addition of the GPI anchor (review: Filmus and Selleck 2001). Glypican-type heparan sulfate proteoglycans are coordinators of several cell functions (reviews: David and Bernfield 1998; Tumova et al. 2000; De Cat and David 2001; Fransson 2003), represent important mediators of developmental processes, and play distinctive roles in carcinogenic pathways. GPC3 has an important role in several developmental processes. Loss of GPC3 function causes growth factor-dependent defect in cardiogenesis (Ng et al. 2009), and loss-of-function mutations of the GPC3 gene (localized to chromosome Xq26) are the cause of Simpson-Golabi-Behmel syndrome type 1 (SGBS1; OMIM 312870, synonyms: Bulldog syndrome, Golabi-Rosen syndrome, Simpson dysmorphia syndrome; dysplasia gigantism syndrome, X-linked). Silencing of the glypican-3 gene regulates invasion and migration of human HCC cells (Qi et al. 2014). GPC3 is normally expressed in fetal tissues, including fetal liver and placenta, but not in the normal adult human liver. In the postnatal liver, GPC3 mRNA is repressed by the transcription factor, zinc fingers, and homeoboxes 2 (Zhx2) (Morford et al. 2007). In the liver, GPC3 is involved in hepatocyte proliferation and regeneration. GPC3 mainly operates in pathways regulating cell proliferation and the function of the cytoskeleton. The expression of GPC3 itself is regulated by the action of specific microRNAs (miRs; Maurel et al. 2013). miR-96, belonging to the miR-182/183 cluster, downregulates GPC3 expression by targeting its mRNA 3'-untranslated region and interacting with the predicted site.

Glypican-3 is also the source of a potential peptide vaccine (Sawada et al. 2012, 2013) and for recombinant humanized antibodies to treat HCC (Zhu et al. 2013). Glypican-3 is particularly useful in the diagnosis of well-differentiated

HCC (Sakamoto et al. 2008; Shafizadeh and Kakar 2011). In HCC patients, glypican-3 can circulate in the blood, and it is comparable to AFP as a serum marker for the diagnosis of HCC (Liu et al. 2013; Xu et al. 2013; Yao et al. 2013b) or for monitoring tumor recurrence (Fu et al. 2013). GPC3 has been considered as a potential target for the antibody therapy of liver cancer (reviews: Ho 2011; Feng and Ho 2014). By use of RNA interference it was shown that suppression of GPC3 in cultured HCC cells inhibited cell proliferation with cell cycle arrest at the G1 phase, associated with upregulation of TGF-beta2 (Sun et al. 2011). GPC3 can be targeted to HCC cells using multifunctional nanoparticles (Park et al. 2011) or liposomes containing GPC-3-targeting peptide ligands (Lee et al. 2011).

Heat Shock Protein 70

Expression of heat shock protein 70/HSP70 has proven to be a reliable marker for HCC (Di Tommaso et al. 2009). In one series, HSP70 was found in 71.9 % of HCC (Shin et al. 2011). HSP70 can synchronously be co-expressed with AFP in HCC cells (Wang et al. 2007).

Alpha-Fetoprotein (AFP) and Other Proteins Secreted by Hepatocytes

Alpha fetoprotein (AFP), an embryonal/fetal type of albumin, is produced and secreted by the majority of HCC (Fig. 4). AFP can be visualized in HCC cells both by immunofluorescence and immunohistochemistry methods (Purtilo and Yunis 1971; Nishioka et al. 1972; Thung et al. 1979; Espinoza et al. 1984; Imoto et al. 1985; Roncalli et al. 1985; Okushin et al. 1987; Brumm et al. 1989; Hurlimann and Gardiol 1991).

Also AFP messenger RNA is detectable in human HCC expressing the AFP gene (Di Bisceglie et al. 1986). In cases with AFP positivity, AFP is not present in distinct cell populations but rather shows a randomly

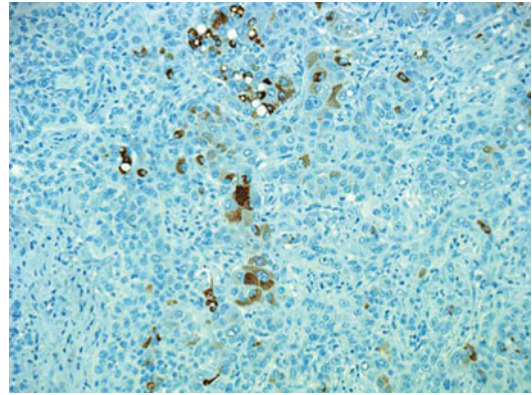


Fig. 4 A subset of hepatocellular carcinoma cells are reactive for alpha-fetoprotein (AFP) in a highly variable pattern (AFP immunostain)

heterogeneous distribution (Kinoyama et al. 1986). By use of immune-electron microscopy, intracellular AFP was mainly found in perinuclear space, cisternae of the rough endoplasmic reticulum, Golgi complex, and secretory vesicles (Okada et al. 1987). It was reported that the number of positive cells and the intensity of cytoplasmic AFP staining was roughly proportional to serum AFP levels in most cases (Kojiro et al. 1981), but depending on the preservation status of tissue and antibodies used, this phenomenon is not always observed. Expression patterns for AFP may be related to tumor grade, although reported percentages of positivity per a given grade vary considerably. In small HCC with a diameter of <3 cm, AFP immunoreactivity was less frequent than that of vitamin K absence or antagonist II/PIVKA-II. There is evidence that AFP-positive HCC are biologically more malignant than those neoplasms that are AFP-negative or PIVKA-II-positive (Fujioka et al. 2001).

Part of HCC cells are reactive for alpha-1-antitrypsin (AAT; Palmer and Wolfe 1976; Thung et al. 1979; Cohen et al. 1982; Nakopoulou et al. 1982; Fernandez-Izquierdo and Lombart-Bosch 1987). In a series of 63 HCC cases, AAT reactivity of cancer cells was found in 82.5 % (Busachi et al. 1986). Some HCC showed atypical PAS-positive diastase-resistant globules resembling those in Z-gene AAT deficiency, and these globules were in part reactive for AAT (Palmer

and Wolfe 1976; Palmer et al. 1977; Reintoft and Hägerstrand 1979). In one investigation, atypical AAT-positive globules were found in 5.4 % of HCC, whereas a diffuse fine granular pattern of AAT distribution was detected in 31 % of HCC cases (Nakopoulou et al. 1982). A second study described AAT positivity in tumor cells in a finely granular pattern in 73 % of cases (Thung et al. 1979). In contrast to globules in normal hepatocytes in inherited AAT deficiency, where the inclusions are round and regular and show a ringlike AAT immunoreactivity, AAT inclusions in HCC were both intracellularly and extracellularly as round, but often distorted and less uniform globules (Palmer et al. 1980). However, other studies failed to detect AAT globules in HCC cells in patients with AAT deficiency who had inclusions in normal hepatocytes (Blenkinsopp and Haffenden 1977).

HCC cells are capable to synthesize and secrete ferritin. Serum ferritin concentrations are elevated in up to 100 % of patients with HCC. In part of these cases, ferritin can immunohistochemically be demonstrated in tumor tissue (Cohen et al. 1984). However, high serum ferritin in patients with HCC can also be caused by associated hepatic iron overload. Immunohistochemically, reactivity for ferritin was found in up to 70 % of cases, but predominantly in well-differentiated neoplasms (Imoto et al. 1985). HCC show an upregulated expression of transferrin receptors (Sakurai et al. 2014). PIVKA-II, an abnormal prothrombin, is secreted by part of HCC and serves as a serum marker for this neoplasm. About half of HCC showed cytoplasmic reactivity for PIVKA-II (Koda et al. 1993).

HCC-Specific Gamma-Glutamyltransferase

HCC cells can express a tumor-specific form of gamma-glutamyltransferase (GGT; hepatoma-specific GGT; HS-GGT; Yao et al. 2004). Analysis of HS-GGT bands in serum can serve as a diagnostic means for HCC (Yao et al. 1998). Overexpression of HS-GGT in HCC may be

related to an altered methylation status of the HS-GGT gene (hypomethylation of CCGG sites; Yao et al. 2000).

Canalicular Domain Markers and Organelle Markers

CD10 (other names : neprilysin, CALLA/common acute lymphoblastic leukemia antigen, atriopetidase, endopeptidase 24.11, enkephalinase, fibroblast metalloelastase, kidney-brush-border neutral peptidase, membrane metalloproteinase A, neutral endopeptidase; MEROPS peptidase code M13.001) is thermolysin-like zinc metalloendopeptidase of the neprilysin/NEP family that can also act as a secretase and plays an important role in tuning off peptide signaling events at the surface of several cell types, targets including enkephalins, tachykinins, chemotactic peptides, natriuretic peptide, and Alzheimer beta-amyloid peptide (Turner et al. 2001; Xiao et al. 2001). The canalicular domain, which is usually present in well-differentiated HCC, is immunoreactive for CD10 (CD1can), similar to polyclonal CEA antibody (Borscheri et al. 2001; Wee 2006). CD10 was detectable in 52 % of HCC cases (Chu et al. 2002), and is more often expressed by moderately to well-differentiated HCC cells than poorly differentiated HCC cells (Dragovic et al. 1997; Lau et al. 2002; Ahuja et al. 2008). Reactivity for CD10 is suitable to distinguish HCC from metastatic carcinomas resembling liver cancer (Ahuja et al. 2008). Expression of CD10can (but not its cytoplasmic counterpart, CD10cyt) in HCC was found to be a favorable prognostic factor (Mondada et al. 2006). Similar to CD10, polyclonal CEA antibody (pCEA) stains the canalicular domain of moderately to well-differentiated HCC (Varma and Cohen 2004), and reactivity was found in up to more than 80 % of cases (Wee and Nilsson 1997; Morrison et al. 2002; Al-Muhannadi et al. 2011). pCEA expression is related to tumor grade, in that canalicular staining becomes infrequent and irregularly distributed with increasing anaplasia. However, pCEA immunostaining did not separate

malignant, dysplastic, or benign hepatocytes (Wee and Nilsson 1997).

Intermediate Filament Expression Patterns

HCC cells can be distinguished from normal hepatocytes by differing patterns of organellogenesis, including mitochondria (Fig. 5).

Cytokeratins

Cytokeratins 8 and 18

HCC cells are strongly and consistently stained by CAM 5.2, which reacts with keratins of molecular weights 50, and 43, corresponding to cytokeratins 8 and 18 (Fig. 6; Hurlimann and Gardiol 1991; Wee 2006). Fifty-five percent of pure, classical HCC expressed cytokeratins of the hepatocyte lineage (D'Errico et al. 1996). In one study, intensity and extent of CAM 5.2 immunostaining did not correlate with the histologic grade of HCC (Johnson et al. 1988). In contrast to the strong expression of lineage-typical cytokeratins, most HCC do not show reactivity for vimentin, but

vimentin may be positive in poorly differentiated HCC with ambiguous cell lineages. In HCC cells, the vimentin gene is aberrantly methylated (Kitamura et al. 2011), causing silenced expression via an epigenetic mechanism.

Cytokeratin 19

Part of HCC are reactive for cholangiocyte markers, including CK7 and CK19, a phenomenon probably related to transdifferentiation (Van Eyken et al. 1988; Shibuya et al. 2011). Expression of CK19 can also be found among morphologically pure HCC (D'Errico et al. 1996). Expression of CK19 in HCC as a stemness feature associated with high-risk biology (HCC of progenitor cell type). Expression of cytokeratin 19 (CK19; Keratin 19) is a feature typical for many adenocarcinomas, in particular also cholangiocarcinomas, while it was formerly held that CK19 is not a feature characterizing hepatocellular carcinomas (HCC). A subset of HCCs is characterized by the expression of stemness-related markers, including CK19 (Kamohara et al. 2008) and ezrin (Okamura et al. 2008). Such tumors have been termed, "dual-phenotype HCC/DPHCC" (Lu et al. 2011). However, expression of CK19 in HCCs not only represents

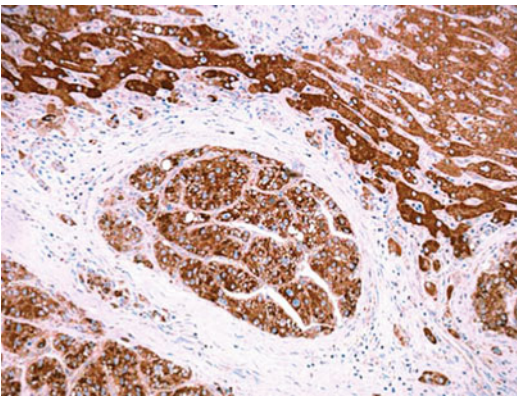


Fig. 5 Similar to normal hepatocytes, HCC cells contain variable amounts of mitochondria and are therefore reactive for a mitochondrial antigen (tumor focus in the center of the figure). The adjacent, strongly positive hepatocytes exhibit perifocal atrophy (mitochondrial antigen immunostain)

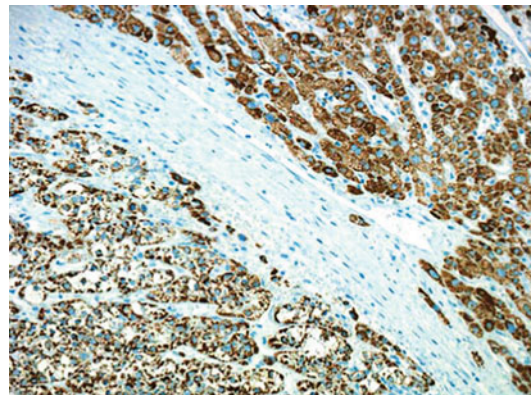


Fig. 6 Hepatocellular carcinoma cells (*left bottom corner*) show strong positivity for cytokeratins 8 and 18, but the granular reaction product is less compact than that of normal hepatocytes seen to the right and top (CAM 5.2 immunostain)

the acquisition of a biliary phenotype but is also related to stemness features. It has been proposed that HCC expressing a cholangiocyte phenotype is a novel subtype of HCC with a highly aggressive behavior. CK19 expression predicts accelerated progression of HCC and poorer survival (Uenishi et al. 2003; Yang et al. 2008; Yamada et al. 2011; van Malenstein et al. 2012; Wang et al. 2012a; Greenhill 2013; Lee et al. 2013; review: Izumi 2012). Among 155 HCCs, tumors with CK19 expression accounted for 10.1 %, and patients with this type of neoplasm a significantly lower overall survival and recurrence-free survival (Lu et al. 2011). 35.7 % of 210 surgically resected HCCs were CK19-positive, and this feature predicted early tumor recurrence and poor prognosis (Yuan et al. 2011). CK19-expressing HCCs with stemness-related marker expression demonstrated more frequent large vessel invasion, increased tumor size, microvessel invasion, poor tumor encapsulation, and poor survival (Kim et al. 2011). The adverse effect of CK 19 on prognosis, with early tumor recurrence, has also been found in small HCC after curative resection (Zhou et al. 2010), and particularly small HCCs (diameter <3 cm) additionally expressing mucin as an indicator for biliary differentiation form a high-risk group (Aishima et al. 2007). CK19-positivity in HCCs is an independent risk factor for developing lymph node metastasis (Ding et al. 2004; Zhuang et al. 2008). Part of HCCs expressing CK19 and showing aggressive behavior coexpress other stemness-related markers, such as CD133, nestin, CD44, and ATP-binding cassette subfamily G member 2/ABCG2 (Yang et al. 2010). In one investigation, CK19 was most frequently expressed in combination with at least one other stemness-related marker, such as CD133, EpCAM, and c-kit. HCC expressing CK19 in part also expressed Yes-associated protein 1, a potential oncogene known to promote stem cell proliferation (Kim et al. 2013). Expression of CK19 was also significantly associated with expression of proteins characterizing epithelial to mesenchymal transition (EMT), including vimentin, S100A4, uPAR, and ezrin (Kim et al. 2011). There is a significant inverse correlation between the expression of the organic anion

transporter peptides (OATP) 1B1 and 1B3 and of CK7 and CK19, in that all HCCs expressing OATP 1B1/1B3 were CK7/CK19 negative (Vasuri et al. 2011). One stemness-related marker, epithelial cell adhesion molecule (EpCAM), is highly expressed in premalignant hepatic tissues and in a subset of HCCs (De Boer et al. 1999). EpCAM-positive HCCs display a distinct molecular signature with features of hepatic progenitor cells (Yamashita et al. 2008). The aggressive phenotype of EpCAM-positive HCCs may be related to the observation that expression of EpCAM shifts the state of cadherin-mediated adhesions from strong to weak (Winter et al. 2003). The reason why expression of CK19 is associated with higher aggressivity of HCCs is not yet known. From other tumor models there is evidence that CK19 and other intermediate filaments play an important role in tumor cell migration, invasion, and metastasis (Hendrix et al. 1996). Keratin 19-positive HCC highly expressed invasion-related/metastasis-related markers and showed expression of members of the miRNA 200 family. Furthermore, primary human keratin 19-positive HCC showed increased invasiveness in vitro (Govaere et al. 2014). HCC expressing “stemness”-related proteins were characterized by increased telomere length, augmented expression hTERT, and shelterin complex proteins, associated with increased chromosome instability, alterations favoring an aggressive tumor phenotype (Kim et al. 2013).

Proliferative Activity

Proliferative activity of HCC cells is usually assessed by means of determination of the mitotic index or immunohistochemical assessment using PCNA or Ki-67 (MIB1) staining (Figs. 7 and 8; Grigioni et al. 1989; Ojanguren et al. 1993; Ng et al. 1995). The proliferative activity of HCC assessed through PCNA and MIB1 labeling is significantly related to tumor differentiation (Ng et al. 1995). Whereas PCNA immunostaining of normal and regenerative livers showed no or only a minimal proliferative activity, even well-differentiated HCC exhibited a labeling index

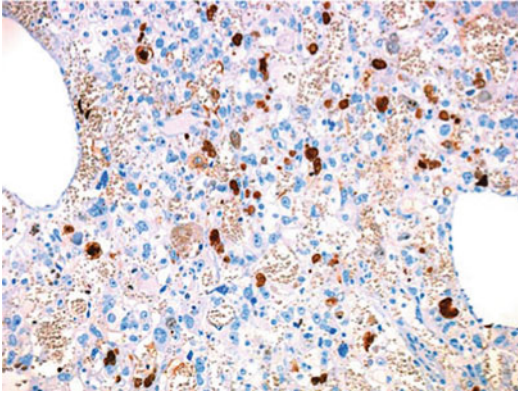


Fig. 7 Hepatocellular carcinoma, grade 3. There is increased proliferative activity (MIB1 immunostain)

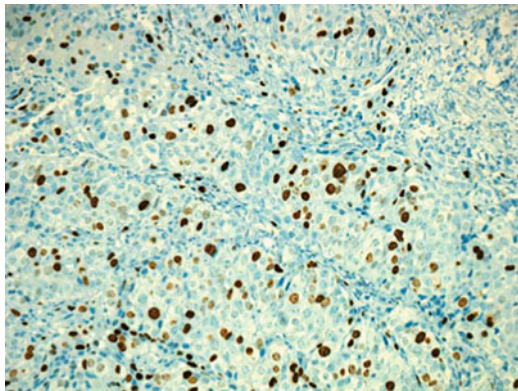


Fig. 8 This hepatocellular carcinoma with a diffuse growth pattern and grade 4 differentiation exhibits a markedly increased proliferative activity (MIB1 immunostain)

exceeding 15 % (Ojanguren et al. 1993). Ojanguren and coworkers (1993) classified PCNA labeling into several categories, i.e., absent; minimal, <5 % positive nuclei; grade 1, 1.5–25 % positive nuclei; grade 2, 26–50 % positive nuclei; grade 3, 51–75 % positive nuclei; and grade 4, 76–100 % positive nuclei. These categories are now known as Ojanguren grades. In cirrhotic liver harboring HCC, the PCNA labeling index was higher in perineoplastic cirrhotic liver than in perineoplastic non-cirrhotic liver (Mun et al. 2006). Based on Ki-67 immunostaining, HCC had a proliferative index ranging from 15 % to 50 %, dependent on Edmondson-Steiner grade

(Grigioni et al. 1989). HCC express factors involved in the promotion of cell proliferation, such as cyclin D1 (expression associated with tumor progression), other cyclins (Choi et al. 2001), and S100A6 (Hua et al. 2011).

Markers of Cellular Differentiation

Apart from the synthesis and secretion of certain distinct proteins, such as AFP, other proteins produced by HCC cells reflect variable grades of differentiation and lineage features. The protein, differentiated embryo chondrocyte 1/DEC1, is expressed in the cytoplasm of hepatocytes and HCC, but well-differentiated HCC cells also display nuclear reactivity of DEC1, while low DEC1 expression indicates poor histologic differentiation (Shin et al. 2011). A subset of HCC expresses mucin core protein 1 (MUC1). In one study, MUC1 immunoreactivity was demonstrated in 85/186 HCCs, and MUC1 positivity in HCC was significantly associated with serum AFP concentrations, tumor differentiation, bile duct invasion, lymph node metastasis, cytokeratin 19 expression, and higher rate of first recurrence, suggesting that MUC1-expressing HCC form a high-risk group of neoplasms (Ichikawa et al. 2006). HCC express the squamous cell carcinoma antigen (SCCA) or variants/isoforms thereof, a protein that is also secreted into serum (Pontisso et al. 2004; Guido et al. 2008; Schmilovitz-Weiss et al. 2011). Immunohistochemically, the antigen is expressed in the cytoplasm of HCC cells, with an uneven distribution of positive tumor cells within HCC nodules, either scattered or in irregular clusters (Guido et al. 2008). A further expression pattern reflecting cell differentiation is the production of intercellular junctions and the associated proteins. Tight junctions are involved in numerous important processes in hepatocytes and cholangiocytes (reviews: Lee and Luk 2010; Lee 2012). In the course of carcinogenesis, including hepatocarcinogenesis, tight junction components undergo marked alterations which allow cancer cells to individualize and to dissociate, prerequisites for invasion (Swift et al. 1983). While occludin and ZO-1, two typical tight junction components, are

strongly expressed in canalicular domains of normal hepatocytes, they are not expressed in HCC cells. As colorectal metastases show strong occluding and ZO-1 expression, analysis of these two proteins may be diagnostically helpful (Orban et al. 2008). Tight junction proteins can be used in differential diagnostic approaches. Claudins have a complex role in liver cell function and play a role in HCV biology. Claudins are members of the tetraspanin family of proteins. During HCV infection, claudin-1 is highly expressed in liver and is required for HCV entry (Ahmad et al. 2011). Claudin-1 and claudin-4 are highly expressed in colorectal cancer metastasis, but claudin-1 has low expression in HCC and claudin-4 is virtually absent (Holczbauer et al. 2013). Lack of claudin-4 expression in HCC and high expression in cholangiocarcinoma has been described in another analysis (Lodi et al. 2006). Conversely, claudin-7 was expressed in HCC cells (Brokalaki et al. 2012). High expression of claudin-10 in HCC determined by molecular methods was associated with tumor recurrence (Cheung et al. 2005). Claudin-5 is expressed in sinusoidal endothelial cells, portal vein, and arteries, but not central veins, while it is downregulated in tumor vessels of HCC (Sakaguchi et al. 2008). Claudins play a role in acquisition of an invasive phenotype. For example, claudin-1 acts through a c-Abl-protein kinase C delta signaling pathway to promote cell invasion (Yoon et al. 2010).

Markers Related to Adhesion Molecules, Cytoskeletal Structures, and Tumor Cell Organelles

HCC express diverse cell adhesion molecules in differential patterns. Normal hepatocytes express E-cadherin but are only weakly stained for alpha-catenin, whereas bile duct cells show high-level expression of alpha-catenin. Conversely, E-cadherin and alpha-catenin expression is often reduced in HCC (Kozyraki et al. 1996; Zhao and Zimmermann 1998). Loss of E-cadherin expression on the surface of HCC cells is mainly found in poorly differentiated neoplasms (Shimoyama and Hirohashi 1991). Part of HCC express neural

cadherin/N-cadherin, whereby overexpression of this adhesion molecular is a negative prognosticator (Seo et al. 2008). Epithelial cell adhesion molecule (EpCAM) is expressed by part of HCCs and is regarded as a stem cell marker in these neoplasms. EpCAM was preferentially expressed in HCC with a nodular growth pattern and high tumor grade (Bae et al. 2012). HCCs with progenitor cell features express the adhesion molecule, NCAM (Tsuchiya et al. 2011). A cell surface glycoprotein called MOC31 is consistently positive in cholangiocarcinomas and metastatic adenocarcinomas, but is negative in HCC (Proca et al. 2000).

An adhesion molecule that plays a significant role in HCC is CD44. The CD44 (cluster differentiation 44) isoform group is a conserved family of several transmembrane glycoproteins serving as cell surface adhesion molecules. CD44 mainly acts as a receptor for hyaluronan, but can also bind other glycosamino-glycans, glycoproteins, and proteins with lower affinity, including chondroitin sulfate, fibronectin, serglycin, and osteopontin. CD44 as an adhesion molecule is widely expressed in lymphoid cells, other leukocytes, and epithelial cells, and is an important component of lymphocyte homing. The cytoplasmic domain of CD44s interacts with several components of the cytoskeleton via ankyrin and proteins of the ezrin-moesin-radixin family. Upon binding of hyaluronan, CD44 activates the Rho GTPase signaling pathway. Interaction of CD44 isoform with cytoskeletal proteins is required for cell locomotion and plays a critical role for cell migration, specifically in cancer cell systems. Cells expressing CD44v(3,8–10) are capable to form membrane spikes or invadopodia required for locomotion, and CD44v(3,8–10) is closely associated with the actomyosin contractile system and with the active form of metalloproteinase-9. The activity of two metalloproteinases, MMP-2 and MMP-9, was stimulated by the interaction of the variant CD44, CD44st, with hyaluronan, associated with an invasive phenotype (Fang et al. 2011). Expression of CD44s is a well-known prognostic factor in various cancers, based on its functions as a tumor growth factor and adhesion

molecule affecting tumor cell invasion and spread. CD44 is not expression by normal hepatocytes (Mathew et al. 1996), but is expressed in HCCs in a membranous staining pattern (Washington et al. 1997;). CD44 expression in HCCs depends of differentiation and is an important prognicator. In a tissue array study of 260 HCC samples employing a primary mouse CD44s monoclonal antibody, expression of CD44s correlated with poor differentiation of HCC (high grade in Edmondson-Steiner grading) and shorter disease-free survival time (Ryu et al. 2011). A correlation with higher tumor grade was found in other studies (Beckebaum et al. 2008). In contrast, use of an antibody that does not distinguish between standard CD44 and splice variants did not show a correlation between CD44 expression and HCC grade (Washington et al. 1997), and reactivity for CD44 was not correlated with the proliferation index of HCC cells (Mathew et al. 1996). Immunoreactivity of CD44 in HCC is significantly correlated with vascular invasion (Mathew et al. 1996) and extrahepatic metastasis (Ogawa et al. 2004). CD44s expression in HCC is associated with higher cancer stage and the presence of lymph nodes metastases (Beckebaum et al. 2008). Apart from CD44 effects mediated by the modulation of tumor cell adhesion, the altered biologic behavior of HCC cells expressing CD44 and its variants may also be caused by a different cellular composition of CD44-expressing HCCs. Cancer stem cells/progenitor cells are highly enriched in CD133+/CD44+ populations of HCC (Zhu et al. 2010; Chan et al. 2014), suggesting the emergence of distinct cell lineages in these tumors.

Clathrin heavy chain positivity is a marker for HCC that already works in the diagnosis of small HCC (Di Tommaso et al. 2011). Golgi protein 73 (GP73) is expressed in HCC (Yao et al. 2013a) and appears in serum, whereby there is evidence that serum GP73 has a either a comparable or lesser accuracy to AFP for the diagnosis of HCC (Ozkan et al. 2011; Zhou et al. 2012). In contrast to cholangiocarcinomas, HCC cells are not reactive for epithelial

membrane antigen/EMA, positive cases representing mixed hepato-cholangiocarcinomas (Sacho et al. 1991).

Oncogenes and Tumor Suppressor Genes

A majority of HCC revealed nuclear reactivity for p53 protein related to p53/TP53 gene mutations (Fig. 9; Cohen and DeRose 1994; Zhao et al. 1994; Kang et al. 1998).

The TP53 gene encodes a tumor suppressor protein, p53, composed of several domains, including transcriptional activation, DNA-binding, and oligomerization domains. P53 protein is a cellular stress response protein that regulates the expression of several genes affecting cell proliferation, apoptosis, DNA repair, and cell senescence. In a recent whole-genome sequencing analysis, TP53 was the most frequently mutated tumor suppressor in HCC (35.2 %; Kan et al. 2013). Mainly in East Asia and southern Africa, p53 protein expression is linked to a mutational hot spot at codon 249 of the p53 gene (Murakami et al. 1991; Debuire et al. 1993). However, p53 protein overexpression is frequent in European HCC largely independent of the codon 249 hotspot mutation (Volkman et al. 1994). The TP53

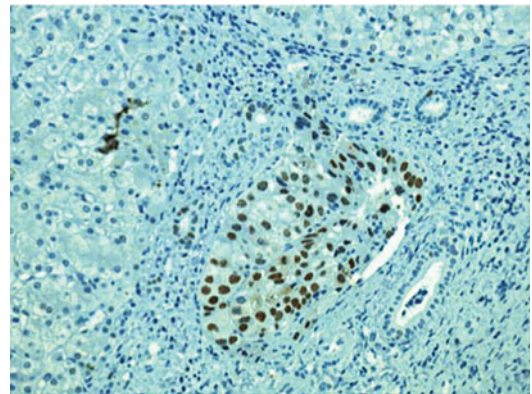


Fig. 9 This hepatocellular carcinoma, which has invaded a venous branch in a portal tract, shows strong nuclear reactivity for p53 protein. The lesion is surrounded by several small interlobular bile ducts (p53 immunostain)

gene mutational hot spots R249S and V157F were significantly associated with worse prognosis (Villanueva and Hoshida 2011). The patterns of p53 mutations have been shown to vary among tumors within the same liver (Tullo and Sbisà 2002), suggesting the emergence of several clones with a different genomic composition. Immunohistochemically, p53 protein reactivity presents as a strong nuclear positivity that may be patchy or diffuse. Nuclear staining restriction depends on the antibody used and was mainly found with the antibody CM-1, whereas other antibodies may also result in variable degrees of cytoplasmic staining (Zhao et al. 1994). There was no relationship between p53 immunostaining and type or grade of HCC (Zhao et al. 1994). However, one study showed a correlation between p53 reactivity and significantly higher Ki67 scores (D'Errico et al. 1994).

HCCs can express a tumor suppressor protein related to p53, p73 (Herath et al. 2000). The p73 gene is located at chromosome 1p36.3. Similar to p53, p73 is involved in the regulation of apoptosis. HCC which express p73 protein belong to an aggressive, high-risk group (Tannapfel et al. 1999).

Components of the Wnt/Beta-Catenin Signaling Pathway

Whole-genome sequencing demonstrated that beta-catenin, a key component of the Wnt signaling pathway, is the most frequently mutated oncogene (15.9 %; Kan et al. 2013). There is a tight correlation between beta-catenin gene mutations and the expression of Wnt target genes in HCC. In one investigation, 36 % of HCC displayed beta-catenin immunostaining, and this immunophenotype was associated with certain signification features, including a homogeneous microtrabeculo-acinar pattern, low-grade cellular atypia, and cholestasis, the latter visualized in the form of numerous bile plugs in acinar or canaliculus-like lumina (Audard et al. 2007). Abnormal beta-catenin reactivity in the cytoplasm and/or nuclei of HCC cells was also

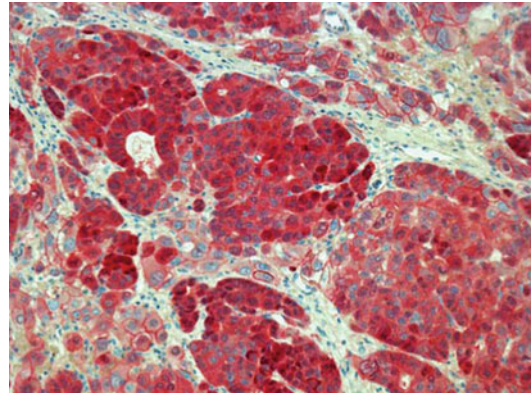


Fig. 10 Hepatocellular carcinoma with mixed cytoplasmic, membranous, and nuclear reactivity for beta-catenin (beta-catenin immunostain)

found at higher rates, e.g., 72.9 % (Fig. 10; Li et al. 2014).

Similar to hepatoblastoma, overexpressed beta-catenin is correlated with cytoplasmic overexpression of cyclin D in HCC, and is associated with increased proliferative activity of tumor cells (Ueta et al. 2002). HCC that harbor exon-3 mutations of the beta-catenin/CTNNB1 gene showed a distinct phenotype with frequent macro- and microvascular invasion, a larger tumor size, a less common association with cirrhosis, and an aggressive behavior, as beta-catenin plays a significant role in the metastatic cascade (Cieply et al. 2009; Lai et al. 2011). In comparison with normal liver, HCC show much less reactivity for Wnt-5a (Li et al. 2014).

Extracellular Matrix of HCC

HCC possess, depending on the amount of stroma formation, a complex extracellular matrix which shares many proteins and glycoproteins with the normal hepatic matrix. Typical components of this matrix include interstitial collagens and mucopolysaccharides, basement membrane proteins (laminin, type IV collagen, nidogen, fibronectin), tenascin, protein receptors such as laminin receptors, and proteases and growth factors that are stored in

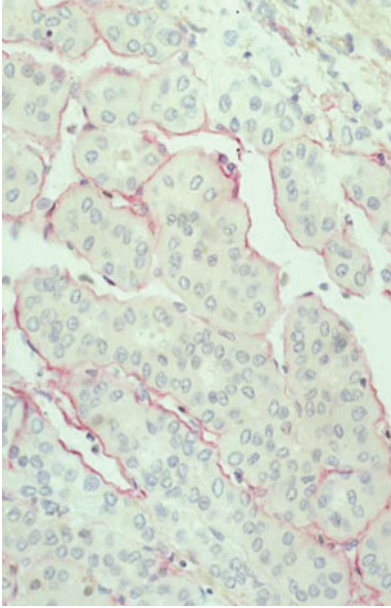


Fig. 11 This trabecular hepatocellular carcinoma shows a thin rim of peritubercular extracellular matrix, situated between tumor cell plates and vascular channels. This matrix reveals reactivity for laminin (*red* reaction product; laminin immunostain)

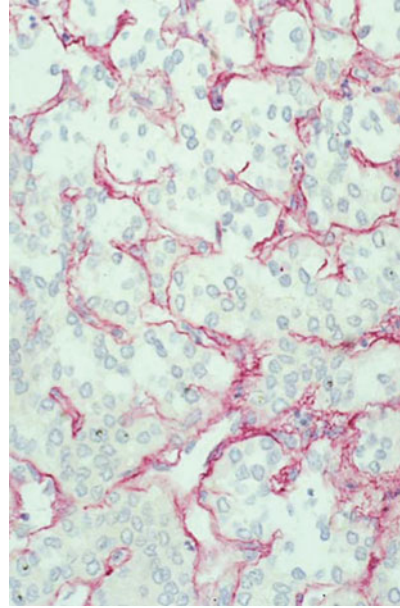


Fig. 12 As a marker for tumor extracellular matrix there is reactivity for tenascin in the peritubercular space of this hepatocellular carcinoma (*red* reaction product; tenascin immunostain)

the matrix. Part of these proteins are present both within cancer cells and in the extracellular space, e.g., fibronectin (Okushin et al. 1987; Figs. 11 and 12).

The composition of the extracellular matrix affects the biologic behavior of HCC cells (Grigioni et al. 1991). Type IV collagen is predominantly present along sinusoid-like vascular channels in well-differentiated HCC, whereas laminin and type IV collagen in poorly differentiated HCC is mainly restricted to large intratumoral vessels (Grigioni et al. 1987). Most HCC show expression of tenascin, this expression being stronger than that of accompanying cirrhosis (Zhao et al. 1996) and the basement membrane protein nidogen (Cheng et al. 2012). Proteins of basement membrane substance, including type IV collagen and laminin, are differentially expressed in various types of HCC. As the extracellular matrix is less well developed in poorly differentiated HCC, such neoplasms showed a reduced expression of

type IV collagen (Zhao et al. 1996). Agrin, a basement component, is a multifunctional heparan sulfate proteoglycan, is present in minor amounts in hepatic blood vessel walls and around bile ducts, but is upregulated in cirrhosis and HCC (Tatrai et al. 2009; Somoracz et al. 2010), the source probably being myofibroblasts (Tatrai et al. 2006). The stroma of HCC can contain heparan sulfate proteoglycans, e.g., syndecan-3 and perlecan, proteoglycans that may be involved in angiogenesis (Roskams et al. 1998). HCC can express various types of matrix metalloproteinases and their inhibitors. Tissue inhibitor of metalloproteinases (TIMP) is detectable in myofibroblasts, smooth muscle cells and endothelial cells of blood vessels, and bile ducts cells of normal livers, and is strongly expressed in the capsule of HCC (Fukuda et al. 1991). HCC also express factors involved in the production of an extracellular matrix, including several types of fibrokinases and their regulators.

CD34 Expression in Vascular Endothelial Cells: An Important Diagnostic Feature

In normal liver tissue, CD34 immunostaining is restricted to portal tracts and few sinusoids in the periportal zone 1 of the lobule; whereas in HCC a diffuse and strong endothelial cell reactivity is present (Figs. 13, 14, 15, 16, and 17; Tanigawa et al. 1997).

CD34 immunostaining is in fact a highly sensitive method for the diagnosis of HCC, as it clearly outlines the abnormal growth pattern of

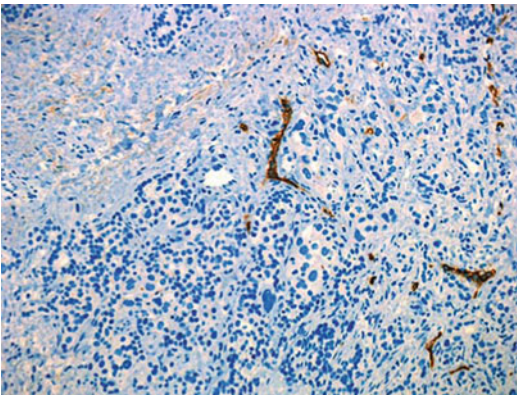


Fig. 13 CD34-reactive small feeding vessels at the periphery of hepatocellular carcinoma (CD34 immunostain)

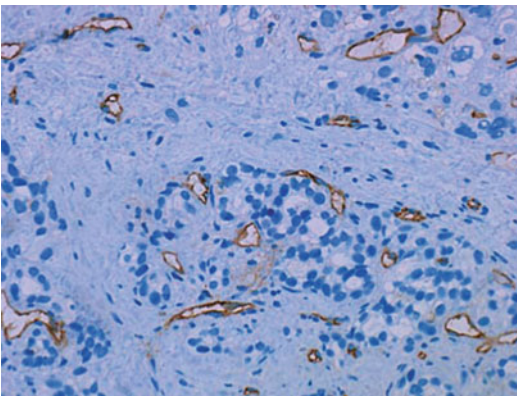


Fig. 14 Angiogenesis in hepatocellular carcinoma results in an intricate relationship between small tumor vessels (*brown*) and clusters of tumor cells (CD34 immunostain)

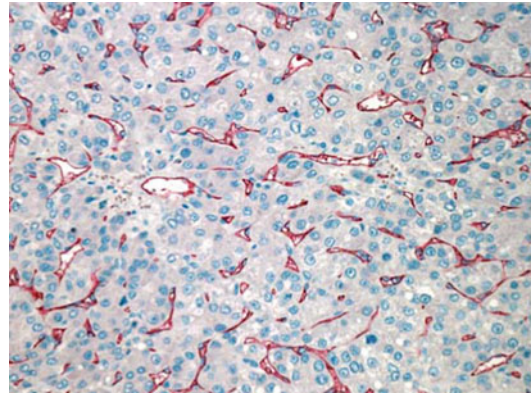


Fig. 15 The vascular channels of this trabecular hepatocellular carcinoma are lined by diffusely CD34-positive endothelial cells (CD34 immunostain)

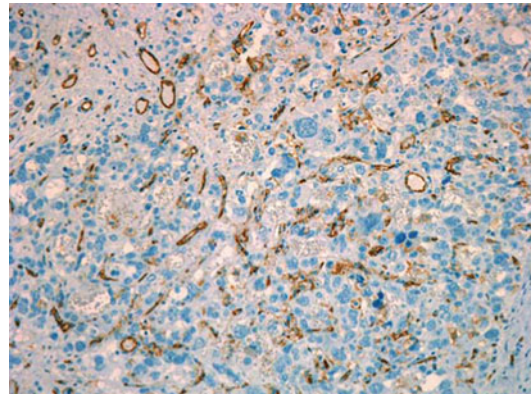


Fig. 16 At the invasion front of this hepatocellular carcinoma (upper third of figure), vigorous angiogenesis has taken place (CD34 immunostain)

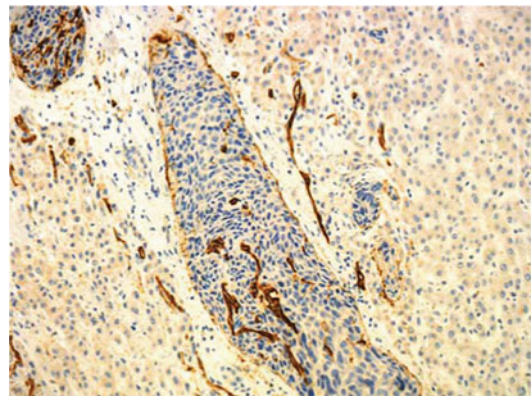


Fig. 17 An intravascular plug of hepatocellular carcinoma (“tumor thrombus”) is secondarily vascularized by CD34-positive vascular sprouts (CD34 immunostain)

HCC, mainly well or moderately differentiated forms (Gottschalk et al. 1998; Wee and Nilsson 2003; Varma and Cohen 2004; Coston et al. 2008; Tatrai et al. 2009; Yao et al. 2013a). However, diffuse CD34 immunostaining is not specific for HCC, as it also occurs in part of liver cell adenomas and focal nodular hyperplasia (de Boer et al. 2000). These authors proposed that the reticulin stain may be superior to CD34 immunostaining in the diagnosis of HCC. There is evidence that CD34-positive endothelial cells may play a significant role in hepatocarcinogenesis. These cells may, at least in part, originate from circulating progenitor cells capable to transform into a distinct population of endothelial cells (Ohmori et al. 2001).

Markers Related to HCC Angiogenesis

As described in more detail in a separate chapter, angiogenesis is a prominent feature of HCC and exerts a strong influence on tumor biology (Semela and Dufour 2004; Fernandez et al. 2009). Angiogenesis in HCC is derived from endothelial cells of feeding arteries and sinusoid-like vascular channels, but liver-specific pericytes also play a role, mainly as mediators of sinusoidal remodeling (Lee et al. 2007). Several factors involved in angiogenesis are expressed, or upregulated, in HCC. Vascular endothelial growth factor/VEGF is expressed in HCC cells, but also in hepatocytes and vascular endothelial cells, but VEGF expression in endothelial cells is numerous times more common in HCC than in the surrounding liver, suggesting that VEGF plays an important role in tumor angiogenesis (An et al. 2000). CD105 (endoglin) is strongly expressed in tumor vessels and is related to prognosis (Duff et al. 2003; Yang et al. 2006). Endoglin is a co-receptor for several TGF-beta family cytokines, is expressed in dividing endothelial cells alongside ALK1 and ACVRL1, and is required for BMP9 (bone morphogenetic protein 9)/pSMAD1 signaling in endothelial cells. Endothelial cells of HCC, and mainly those of microvessels, are reactive for the chemokine receptor, CXCR7 (Monnier et al. 2012).

Other Markers

Part of HCC cells revealed reactivity for thrombospondin in the cytoplasm, but were also present in accompanying fibroblasts and endothelial cells (Hayashi et al. 1997). Reactivity for serine peptidase inhibitor, Kazal type 1 (SPINK1), was found in more than 90 % of HCC, but was not detectable in normal or cirrhotic liver tissues and in dysplastic nodules (Marshall et al. 2013). Early HCC were found to be reactive for CD147 (EMMPRIN) (Mamori et al. 2007). Few cases of HCC were immunoreactive for inhibin (McCluggage et al. 1997; Vrettou et al. 2005), inhibin as such not being a marker of the hepatocyte lineage or of most HCC, and positive results usually being caused by endogenous biotin (Iezzoni et al. 1999). Part of HCC are reactive for the transcription factor SALL4, which is a well-known marker for yolk sac tumor. In contrast to yolk sac tumor, which displays a diffuse finely granular nuclear staining pattern, HCC show a distinct punctuate/clumped nuclear pattern of SALL4 staining (Gonzalez-Roibon et al. 2013). Part of HCC express osteopontin, a secreted phosphoprotein implicated in cancer progression and metastasis (Pan et al. 2003; Hua et al. 2011), and osteonectin/SPARC, which is mainly expressed in stromal fibroblasts of HCC showing a high grade (Le Bail et al. 1999). Importin-alpha1 is produced by HCC cells and is a factor associated with progression of disease (Yoshitake et al. 2011). Aldo-ketoreductase family 1 member B10 (AKR1B10) reactivity seems to be promising diagnostic marker for HCC (Schmitz et al. 2011). Subsets of HCC are reactive for multidrug resistance-conferring transporters. Multidrug resistance-associated protein 1 (MRP1) expression was high in HCC with poor differentiation, large tumor size, and microvascular invasion and may reflect tumors with progenitor cell features and an aggressive biology (Vander Borghet et al. 2008). Another immunohistochemical marker associated with poor tumor differentiation is p21 (Schoniger-Hekele et al. 2005). The PTEN phosphatase was expressed in 64–5 % of HCC, but immunoreactivity was lower and weaker than that in

surrounding non-neoplastic liver tissues (Wu et al. 2007). Prothymosin alpha, normally positive in the nuclei of bile duct cells but not in quiescent hepatocytes, is intensely expressed in regenerating hepatocytes and HCC cells, confirming that this protein is related to the regulation of liver cell proliferation (Fraga et al. 1993). Expression of the insulin-like growth factor-I receptor may participate in hepatocarcinogenesis (Yan et al. 2013). HCC, but also regenerating hepatocytes, overexpress hepatic prothymosin alpha, which may be a marker for this neoplasm. Reactivity is exclusively present in nuclei (Fraga et al. 1993; Wu et al. 1997).

Immunohistochemical Panels Serving for Diagnosis of HCC

The specificity of lineage marker expression can be augmented by combining the most efficient markers, namely arginase-1, Hep Par1, heat shock protein 70, glutamine synthetase, and glypican-3 (Sakamoto et al. 2008; Shin et al. 2011; Timek et al. 2012; Tremosini et al. 2012; Lagana et al. 2013), and eventually other markers. Histologic diagnosis of well-differentiated HCC can be difficult, especially in biopsies, as tumor cells may only slightly deviate in their morphology from normal hepatocytes or benign hepatocellular tumors. Apart from a careful analysis of cell morphology, reticulin patterns, and vascular architecture, panels of immunohistochemical stains are helpful in critical situations. A panel including glypican-3, heat shock protein 70, and glutamine synthetase have proven to be of use in the diagnosis of HCC, including small and well-differentiated HCC (Di Tommaso et al. 2009), early HCC (Tremosini et al. 2012), and to distinguish low-grade HCC from hepatocellular adenoma (Lagana et al. 2013). For the distinction of small HCC from dysplastic nodules, a combination panel of aminoacylase-1, glypican-3, and sequestosome-1 has been proposed (Jin et al. 2013). For separating HCC from intrahepatic cholangiocarcinoma, a combination of glypican-3 and CK19 was proposed as a first-line marker, with an accuracy rate of 73.5 %, and adding

claudin 4 and MOC31 increased this rate to 88.5 % (Ryu et al. 2012), MOC31 mainly serving to distinguish HCC from cholangiocarcinoma and metastatic adenocarcinoma (Porcell et al. 2000).

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