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Diagnostic Algorithm of Hepatocellular Carcinoma: Classics and Innovations in Radiology and Pathology

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<http://dx.doi.org/10.5772/intechopen.76136>

Abstract

In the global cancer statistics, hepatocellular carcinoma (HCC) ranges sixth by incidence and second by oncological mortality. The risk factors comprise hepatitis B and C virus infection, non-alcoholic steatohepatitis, as well as long-lasting peroral exposure to alcohol or aflatoxins. Liver cirrhosis is the most important single predisposing factor. Ultrasonography once per 6 months is recommended for surveillance in cirrhotic patients. Computed tomography (CT) and magnetic resonance imaging (MRI) represent the gold standard of non-invasive diagnostics while core biopsy and/or immunohistochemistry (IHC) are indicated for controversial and non-cirrhotic HCC cases. Molecular classification is under development. At present, classics of HCC diagnostics is based on evaluation of risk factors, surveillance in cirrhotic patients, preference for CT or MRI-confirmed non-invasive diagnosis and biopsy proof in equivocal cases. Diffusion-weighted imaging and hepatobiliary phase contrasting represent significant recent developments in MRI. Contrast-enhanced ultrasonography is recommended by some but not all guidelines. Positron emission tomography is advocated before liver transplantation to detect extrahepatic metastases but has limited role in the initial diagnostic evaluation of liver nodule. Innovations are expected in the field of molecular diagnostics, including IHC panels and novel antigens, e.g. clathrin and bile salt export pump protein, and development of molecular classification.

Keywords: hepatocellular carcinoma, HCC, diagnosis, imaging, CT, MRI, contrast-enhanced ultrasound, CEUS, positron emission tomography, PET, liver biopsy, immunohistochemistry

1. Introduction

Hepatocellular carcinoma (HCC) is a primary malignant liver tumour exhibiting hepatocellular differentiation [1]. It is well known for the strong association with preceding chronic liver disease and liver cirrhosis [2]. However, nowadays an increasing proportion of HCCs develops in non-fibrotic liver or on the background of mild fibrosis [3, 4]. The changing patterns of presentation influence the diagnostic approach both because of alterations in risk groups that could be targeted by surveillance and limits of non-invasive diagnostics in non-cirrhotic cases. In addition, the differential diagnostics of liver nodule differs in regard to the presence or absence of background liver cirrhosis. In cirrhotic liver, 59–94% (depending on size) of new mass lesions are malignant [5]. Thus, in patients with liver cirrhosis or preceding chronic liver disease, new nodule favours the diagnosis of HCC, as metastases and benign liver tumours are uncommon in cirrhotic liver [6, 7]. Hence, any mass lesion in cirrhotic liver must be considered HCC until proven otherwise [7].

In the global cancer statistics, HCC represents a frequent and aggressive tumour although different geographic regions face various burden of it. Worldwide, liver cancer is estimated to range sixth by incidence and second by oncological mortality causing 5.6% of global cancer incidence and 9.1% of mortality [8]. HCC is the most frequent primary liver cancer (>90%) being significantly more widespread than cholangiocarcinomas, hepatoblastomas and other primary liver malignancies [1]. Number of death cases per year (recently assessed by Ferlay et al. for the year 2012 as 745,000) is virtually identical to the incidence throughout the world (782,000; the same source) underlining the unfavourable course. The high ratio of mortality to incidence (0.95) reflects the dismal prognosis. As the geographical patterns of incidence and mortality closely follow each other [8], liver cancer is still an unsolved problem in the whole world.

According to the data provided by Surveillance, Epidemiology and End Results (SEER) Program of the National Cancer Institute, the 5-year survival for liver cancer is 16.6%, ranging from 30.5% in localised stage to 10.7% in regional stage and 3.1% in distant stage [2]. Different but similarly discouraging estimates have been reported, including 1-, 5- and 10-year survival of 31.3, 5.1 and 0.8%. The median survival is 6 months. However, significantly better outcome can be reached in early cases. Thus, median survival reaches 107 months in patients receiving liver transplantation for early HCC [9].

The incidence of liver cancer is high in Eastern and Southeastern Asia, followed by Northern and Western Africa [8]. China, Mongolia and Japan experience high occurrence [10]. In Europe, the highest age-standardised incidence rate of liver cancer is observed in Southern Europe [8] including Italy and France [10]. Although more developed regions generally show lower incidence of liver cancer (except Japan, France and Italy), its incidence is growing in many countries [8, 10]. Thus, although the total cancer incidence in the United States of America (USA) decreased in males and remained stable in females over time period 2003–2012, liver cancer incidence rates increased in both genders: 3.7% yearly in males and 3.0% in females. According to the National Program of Cancer Registries and SEER database, liver cancer incidence rate (2008–2012) in USA has increased by 2.3% per year [2]. The incidence rate of

histologically proved HCC in USA has increased from 1.4/100,000 persons per year between 1976 and 1980 to 2.4/100,000 persons per year between 1991 and 1995 [11] followed by further growth of HCC incidence rate reaching 6.2/100,000 persons per year in 2011 as shown by SEER-based analysis [9].

Similarly, although death rates attributable to other frequent cancers, including lung, breast, colorectal and prostate cancers, are declining in USA, mortality from liver cancer has increased by 2.8% per year (2003–2012) in males and by 2.2% per year in females. The growing mortality from liver cancer in USA contrasts with the general decline in cancer mortality reaching 1.5% per year. Among all cancers, HCC is the fastest growing cause of death in the USA [2] and poses a significant economic burden on healthcare [10].

The spectrum of risk factors in HCC (see **Table 1**) explains the geographic heterogeneity. Awareness of these factors is important to understand the incidence and the associated needs for diagnostics and treatment. Worldwide, men have a higher incidence than women; gender ratio ranges around 3:1 both in global epidemiological studies of liver cancer [8] and more targeted analysis of HCC [9]. Incidence starts to increase at the sixth decade of life [2].

Liver cirrhosis of any aetiology represents the single largest risk factor of HCC and is found in 70–90% of cases. Worldwide, hepatitis B virus (HBV) infection accounts for more than 50% of HCC cases. In comparison to non-infected individuals, the relative risk of HCC is increased 100-fold in HBV-infected persons, and the risk further increases if HBV-infected patient develops cirrhosis, has longer duration of infection and higher virus burden in blood. The yearly risk of HCC in HBV-infected patients is 2% [18]. In East Asia and sub-Saharan Africa, HBV is the most common risk factor for HCC [12].

Hepatitis C virus (HCV) infection is implicated in 25–31% of patients [13, 19]. Although the presence of HCV infection holds 17-fold risk of HCC in comparison with non-infected persons [13], risk is significantly higher in cirrhotic patients. Thus, surveillance is limited to those having

Risk factor	Risk assessment	References
Hepatitis B virus infection	100-fold higher	[13]
Hepatitis C virus infection	17-fold higher	[13]
Alcohol consumption	2.2 times higher in people who consume at least 50 g of alcohol per day	[15]
Non-alcoholic fatty liver disease	More than 4 times higher	[16]
Aflatoxin exposure	Of the 550,000–600,000 new HCC cases worldwide each year, about 25,200–155,000 may be attributable to aflatoxin exposure	[14]
Primary biliary cirrhosis	Incidence of HCC is 3–5% per year	[12]
Primary sclerosing cholangitis (PSC)	The risk of HCC for PSC patients with cirrhosis is up to 2% per year	[17]
Hemochromatosis	Approximately 20-fold higher	[12]

Table 1. Risk factors of hepatocellular carcinoma [12–17].

HCV-associated cirrhosis or advanced fibrosis [12]. Annually, HCC develops in 2–8% of HCV-infected patients [13]. In North America, Latin America, Europe and Japan, HCV infection, together with alcohol abuse, represent the main risk factors [3, 13]. In Europe and Japan where HCV infection spread earlier than in the United States, HCC incidence has almost reached a plateau, while in the United States it is still increasing. HCV infection may have a synergistic effect with other risk factors, such as non-alcoholic fatty liver disease [3].

Globally, 15% of HCC cases can be attributed to alcohol-induced liver damage and non-alcoholic steatohepatitis [19], although the estimates range between 4 and 22% [20]. Non-alcoholic fatty liver disease (NAFLD) is the major hepatic manifestation of metabolic disturbances including obesity, type 2 diabetes mellitus, dyslipidaemia and metabolic syndrome [4]. As prevalence of these conditions is increasing, NAFLD has become the most common liver disorder in industrialised countries [21]. In NAFLD, HCC incidence reaches 44 (range, 29–66) per 100,000 person-years [22] contrasting with the general incidence of 6 per 100,000 in USA population [20]. The proportion of HCC related to NAFLD and non-alcoholic steatohepatitis (NASH) is increasing worldwide, especially in Western countries [20]. Although previously it was considered that HCC risk was limited to patients with liver cirrhosis, nowadays a significant fraction of NASH-associated HCC is found in non-cirrhotic liver or liver showing mild fibrosis [4].

Aflatoxins are a group of mycotoxins produced by *Aspergillus* fungi (*A. flavus*; *A. parasiticus*), which can contaminate food products such as grains, rice, cassava, soybeans, corn and peanuts, stored in hot climate and high moisture. Aflatoxins are major risk factors of HCC in sub-Saharan Africa and Eastern Asia [23]. Chronic exposure to aflatoxin results in DNA damage, including mutation of the tumour suppressor gene *TP53* in hepatocytes [13]. In people subjected to aflatoxin ingestion and chronic HBV infection, HCC risk is 30- to 60-fold higher, *versus* HBV-uninfected people exposed to aflatoxin alone. Synergistic action is observed also between aflatoxin and HCV [14, 23, 24].

Planning the surveillance for individual patient, the presence of known risk factors must be considered and the relative risk must be taken into account. Organising surveillance measures in the society, population-attributable fraction (PAF) is also important. PAF depends both on relative risk and population prevalence of the corresponding risk factor. Thus, in USA, the risk increase of HCC is highest in HCV infection (odds ratio (OR), 39.9), followed by HBV infection (OR, 11.2), alcohol-induced liver disease (OR, 4.1) and diabetes mellitus and/or obesity (OR, 2.3). However, considering the prevalence of these conditions, diabetes and/or obesity are associated with the highest population attributable fraction (36.6%), followed by alcohol (23.5%), HCV (22.4%) and HBV (6.3%) as reported by Welzel et al. [25]. PAFs differ by the population. Worldwide, 54% of HCC occur in HBV-infected patients, 31% can be attributed to HCV and 15% to alcohol and NASH [19].

Considering the serious prognosis, early diagnosis is crucial, however, not always easy. Thus, correctly interpreted radiological findings, combined with biopsy when necessary, and appropriate immunohistochemical examination of biopsied tissues have diagnostic value. The molecular portrait of the tumour as well as easily available markers of the systemic inflammatory response, such as neutrophil-to-lymphocyte ratio or platelet-to-lymphocyte ratio, are recently reported to have prognostic and predictive value in HCC.

The aim of the present chapter is to highlight the current approach and innovations for diagnostic evaluation of a liver nodule, suspected to be hepatocellular carcinoma. Non-invasive radiologic assessment represents the gold standard in certain patients. In contrast, difficult cases need biopsy evaluation, supplemented by immunohistochemistry, and may remain controversial even then.

2. Radiology

Radiological imaging and functional evaluation are significant in screening and diagnostics of HCC [26]. The gold standard techniques comprise ultrasonography (US), computed tomography (CT) and magnetic resonance imaging (MRI). A major advance in the diagnostics of HCC was reached in 2001, when non-invasive criteria were developed and accepted by the European Association for the Study of the Liver (EASL) to diagnose HCC in cirrhotic liver [27]. In addition to the presence of liver mass, radiologic studies of HCC evaluate the typical vascularity. HCC receives enhanced arterial blood supply reflected histologically by unpaired arteries. The blood supply via portal vein decreases in comparison with surrounding parenchyma. However, in early stages of development, HCC can be hypovascular if the portal flow has already decreased but the arterial supply has not yet fully developed.

According to the guidelines, ultrasonography is advocated for screening and surveillance of patients having high risk to develop HCC due to HBV or HCV infection, cirrhosis or other known risk factors [28]. The specificity is mostly higher than 90%, ranging 45–94% [5, 27]. The reported sensitivity ranges widely from 33 to 96%, at least partially because of differences in the equipment and qualification of radiologists [18]. The sensitivity decreases in advanced chronic liver disease because of the coarse cirrhotic nodularity seen both grossly and by US [5]. In a large group of 200 patients undergoing US and liver transplantation, the sensitivity for HCC diagnostics was 29.6% in regard to patients and only 20.5% counting the tumours themselves. Even a large tumour exceeding the diameter of 5 cm was missed [29].

The typical US presentation of HCC is a hypoechoic nodule although iso- or hyperechogenicity is possible as is nodule-in-nodule appearance [7]. Small HCCs (less than 2 cm in the greatest diameter) are mostly hypoechoic with or without posterior enhancement. Hyperechoic appearance is seen in 17% of small HCCs and can be associated with fat accumulation. Larger HCCs are heterogeneous reflecting necrosis (hypoechoic), calcifications and fibrosis. If hypoechoic halo (seen in 50% of HCC) and posterior enhancement is evident, these findings increase the specificity of diagnosis [5, 7, 18]. HCC in dysplastic nodule might seem hyperechoic within a larger hypoechoic area. If a nodule is identified on US, either CT or MRI is indicated for masses larger than 20 mm, while both methods are advocated for pathologic foci measuring 10–20 mm. If either CT or MRI confirms HCC, the diagnosis is reliable. Biopsy is indicated only for lesions that remain controversial after both imaging modalities. Nodules measuring less than 10 mm are followed up by US every 4 months [18].

By Doppler US, HCC is characterised by so-called basket pattern reflecting rich arterial vascularisation. Benign cirrhotic nodules feature either low vascularity or arterial vessels with

low frequency (high in HCC: >1 kHz) and normal resistive index (elevated in HCC: >0.71). However, the typical Doppler pattern is seen only in 50% of small HCC [7].

A significant innovation in ultrasonography is the application of contrast enhancement by stabilised gaseous microbubbles. Consequently, three phases can be assessed analogously to CT and MRI: arterial phase (beginning 20 seconds after injection and lasting for 30–45 seconds); portal venous phase (starting at 30–45 seconds and lasting for 2–3 minutes) and late phase (4–6 minutes). Some contrast agents display additional post-vascular phase characterised by contrast uptake in Kupffer cells (10–60 minutes). To avoid overlap with late phase, the post-vascular phase must be assessed not earlier than 10 minutes after contrast injection. The typical pattern of HCC upon contrast-enhanced ultrasound (CEUS) examination is arterial hyperenhancement followed by washout in the late phase. The evaluation of washout is important in order to exclude arterial hyperenhancement in hemangioma or dysplastic cirrhotic nodule. However, well-differentiated HCC can remain isoechoic in portal venous or late phase; such pattern is suspicious for HCC, but CT or MRI is mandatory [7]. The benefits of CEUS include the easy procedure and high safety as the technique is not associated with ionising radiation or renal toxicity. Pitfalls include false positives in cholangiocarcinoma [30]. The lack of specificity is associated with the intravascular location of microbubbles in contrast to CT or MRI contrast agents that reach the extravascular extracellular space. At present, CEUS has been excluded from diagnostic guidelines provided by the European Association for the Study of the Liver (EASL) and the American Association for the Study of the Liver Diseases (AASLD) but is advocated by Asian Pacific and Japanese guidelines [27].

US can be applied to recognise benign or secondary liver tumours. Sensitivity of US to detect liver metastases varies between 40 and 80%, again depending on experience of radiologist and available US equipment. Metastases can be hypovascular, e.g., gastric or colorectal carcinoma, or hypervascular as malignant melanoma or sarcoma [18].

If US or CEUS discloses a suspicious nodule, in-depth evaluation by CT or MRI is indicated [31] based on the risk of malignancy. Nodules smaller than 1 cm are mostly benign. Risk of HCC is 66% in nodules measuring 10–20 mm, 80% in nodules 20–30 mm in size and 92–95% in nodules larger than 3 cm [7]. Both methods (CT and MRI) are advocated for lesions measuring 10–20 mm while one is sufficient for larger nodules (>20 mm). The diagnosis of HCC is confirmed by the typical pattern of arterial hypervascularity and late washout [27].

For CT, dynamic multidetector row, multiphase contrast-enhanced computed tomography approach is recommended [27, 32] as the diagnosis is based on dynamic evaluation of blood flow. However, contrasting is not possible in all patients in order to avoid anaphylactic reactions, acute renal failure or hyperthyroidism [33]. To disclose HCC, CT must be evaluated in three phases (late arterial, portal venous and equilibrium) in addition to the first unenhanced image. HCC classically is hypervascular, characterised by high contrast in the arterial phase, followed by washout in portal and/or equilibrium phases [27]. Portal venous phase can be useful in some cases of HCC when the tumour is otherwise not visible in CT. Portal venous phase is generally less informative for HCC because the tumour shows rarefaction similar as liver parenchyma. This phase is most useful for detecting hypovascular metastases, e.g., from colorectal carcinoma [34].

Examination of hypovascular or hypervascular liver metastases with multidetector CT is similar to CEUS. Hypovascular metastasis presents as rounded and uniformly hypoattenuating mass in portal venous phase and peripheral rim in arterial phase. Hypervascular metastasis is characterised by homogeneous late arterial enhancement. Inhomogeneous enhancement can develop in necrosis or haemorrhage [35].

MRI has excellent results for detection and characteristics of HCC. By meta-analysis, MRI was characterised by sensitivity of 88% and specificity of 94%, exceeding the characteristics of multidetector CT [36]. Contrast usually is applied in liver MRI, most frequently by gadolinium compounds. The gadolinium-based contrast agents can be classified as extracellular *versus* hepatobiliary. Extracellular agents are small molecules that can reach interstitium moving out from vascular space. In turn, hepatobiliary contrast agents move even further becoming absorbed by hepatocytes [37, 38].

Classic MRI protocol for HCC includes a 3D T1-weighted fat saturated sequence with intravenous contrast. The first phase is called late arterial phase. It is seen 25–30 seconds after injection of contrast. This phase is followed by portal venous phase, at 65–70 seconds. In this phase, there is a dense contrast enhancement in portal vein, and hepatic veins also become highlighted. Finally, delayed phase develops 3 minutes after injection [38]. Before contrasting, classical HCC is hypointense in T1-weighted and hyperintense in T2-weighted images. Contrasting reveals similar enhancement pattern as in CT with arterial enhancement and subsequent washout [18]. In addition, MRI can be applied to disclose tumour thrombus in portal venous system [39].

Most metastases show mild-to moderate high signal intensity on T2-WI. In some cases, e.g., in cystic or necrotic metastases, T2 signal increases.

The sensitivity of MRI can be further improved by diffusion-weighted imaging, based on the assessment of Brownian motion of water molecules and water diffusion within a voxel (a tridimensional pixel). Cell membranes limit the diffusion, therefore greater cellularity, seen also in malignant tumours, results in diffusion restriction [40]. However, the fibrosis also decreases the mobility of water molecules. By different modalities, diffusion-weighted imaging can increase the sensitivity for HCC detection, the liver-to-lesion contrast and the specificity in the differential diagnosis with benign cirrhotic nodules [27].

Another advance in liver pathology is represented by hepatobiliary phase MRI using contrast agents that are absorbed by hepatocytes and excreted in biliary system, e.g., gadoxetate disodium and gadobenate dimeglumine. These agents undergo dual elimination via biliary excretion (50%) and renal glomerular filtration, while the traditional agents, as gadopentetic acid, are almost completely excreted via kidneys [41]. The hepatobiliary phase of MRI corresponds to the peak parenchymal enhancement due to contrast uptake in hepatocytes. Depending on the agent, the hepatobiliary phase develops either 10–20 (gadoxetate) or 60 (gadobenate dimeglumine) minutes after injection [42]. Most of HCCs are hypointense in hepatobiliary phase [18].

MRI can be applied to distinguish between HCC and benign lesion in non-cirrhotic liver. In such patients, HCCs are hypointense in T1, hypo- or hyperintense in T2, lack central

enhancement in the tumour, exhibit satellite lesions and do not uptake liver-specific contrast agents [43].

Positron emission tomography (PET) is a non-invasive radiologic visualisation that demonstrates metabolic activity in normal or pathological tissue. It is usually performed in combination with CT to ensure both anatomical imaging and metabolic evaluation. 18-fluorodeoxyglucose (FDG) is one of the radiopharmaceuticals used in PET/CT. It discloses areas of high glucose uptake as many tumours including HCC are characterised by aerobic glycolysis: the Warburg effect [44].

The significance of FDG PET/CT in HCC evaluation is not unequivocal. The distinction between small, well-differentiated HCC *versus* regenerative or dysplastic nodules can be difficult. The positive aspect of PET/CT is the ability to detect extrahepatic metastases of HCC. Considering that PET/CT provides whole-body examination, it is recommended before liver transplantation [45, 46]. Hypothetically, prognostic role of PET/CT in HCC has been discussed as well as the ability to predict response to treatment [46]. Other radiopharmaceuticals are also under discussion, including lipid radiotracer on choline base, like 11C-choline or 18F-fluorocholine [47]. 68Ga-labelled prostate-specific membrane antigen, that is used to diagnose prostate cancer, is present in other tumours, including HCC [48].

3. Pathology

Needle biopsies followed by morphologic and immunohistochemical examination can be invaluable for the characterisation of liver masses. However, nowadays clear-cut radiologic diagnostic criteria have been established for the non-invasive diagnostics of HCC; therefore, the advantages and indications of the biopsy should be considered against the risks and contraindications. Liver biopsy is recommended only in selected patients, thoughtfully evaluating the diagnostic yield [6].

Currently, three general groups of indications (see **Table 2**) for liver biopsy are known: to establish the diagnosis, to assess the prognosis and/or to assist in the management of patient

Diagnosis:

- Identification and staging of parenchymal and cholestatic liver diseases (alcohol-induced liver disease; non-alcoholic steatohepatitis; primary biliary cirrhosis; primary sclerosing cholangitis; Wilson's disease, haemochromatosis)
- Evaluation of persistent abnormal liver biochemical tests
- Evaluation of the type and extent of drug-induced liver injury
- Diagnosis of multisystem infiltrative disorders
- Identification and determination of the nature of focal/diffuse intrahepatic abnormalities on imaging studies

Prognosis and management:

- Pre-treatment evaluation and staging of chronic hepatitis, e.g. chronic viral hepatitis B and C
 - Evaluation of pre-transplant living-related donor
 - Evaluation of post-transplant patient with abnormal liver tests (rejection *versus* infection)
 - Evaluation of treatment efficacy for liver diseases
-

Table 2. Indications for liver biopsy [49].

with known liver disease [49]. Percutaneous liver core biopsy is most frequently performed to evaluate the presence and activity of inflammation and extent of fibrosis/stage of frequent liver diseases, mostly chronic viral hepatitis, alcohol-induced liver disease and NAFLD. Regarding focal liver lesions, biopsy can yield the diagnosis. Molecular analyses of tissue may help determine the most appropriate individual treatment strategy for the patient with HCC [50] but are still under development for HCC. At present, biopsy from a nodule in cirrhotic liver is indicated if the findings of radiological imaging are controversial [6].

Although biopsy is often essential, sometimes it may be difficult to undertake because of associated risks (see **Table 3**). Percutaneous, ultrasound-guided liver biopsy (the Menghini method) has become the worldwide standard [51]. However, it is appropriate only in cooperative patients. Thus, if the patient refuses from the procedure, it is absolutely contraindicated. Although precise blood clotting parameters are unsettled, coagulopathies should be mentioned as a serious contraindication [49]. In this case, mini-laparoscopy or transjugular liver biopsy might be considered [51]. Among relative contraindications, ascites should be pointed out, as it may prevent adequate sampling of tissue, as well as increase the risk of bleeding [49]. Biopsies of malignant liver lesions also carry a low risk of tumour seeding.

Significant complications due to liver biopsy arise in about 1% of cases, with less than 0.1% mortality [51]. The main complications are post-interventional haemorrhage and bile leakage; others, like injuries to gall bladder, lung, kidney, as well as bacteraemia are rare [49, 51].

The initial assessment of liver tissue starts with the overall evaluation of parenchymal architecture. Haematoxylin and eosin represents the generally accepted standard stain in liver pathology [6]. Helpful additional visualisation methods in liver pathology include Masson's trichrome to assess fibrosis, Gordon and Sweets reticulin to evaluate lobular architecture and hepatocyte plate thickness, Perl's iron to detect hemosiderin deposits and periodic acid-Schiff (PAS) stain to identify glycogen, mucus or chitin of certain liver parasites.

Microscopically, cells of classical HCC resemble normal hepatocytes. The similarity to normal liver is most notable in well to moderately differentiated tumours. In such cases, the loss of the normal liver cell plates and plate thickness change from 1 to 2 cell nuclei to 3 or more nuclei

Absolute contraindications

- Uncooperative patient
- History of unexplained bleeding
- Tendency to bleed (prothrombin time more than 3–4 seconds over control; platelet count $<50,000 \text{ mm}^3$; prolonged bleeding time (≥ 10 minutes))
- Unavailability of blood transfusion support
- Recurrent use of aspirin or other non-steroidal anti-inflammatory drugs within last 7–10 days

Relative contraindications

- Ascites
 - Morbid obesity
 - Infection in the right pleural cavity or below the right hemidiaphragm
 - Suspected haemangioma or other vascular tumour
 - Suspected hydatid disease (Echinococcal cysts)
-

Table 3. Contraindications of liver biopsy [49].

across a single neoplastic cord is a feature of malignancy. In healthy liver, narrow cords of hepatocytes are running in parallel, but even well-differentiated HCC shows a disorganised pattern secondary to the increased thickness of the hepatocyte cords (usually more than 3 cells thick), that can be highlighted by reticulin staining. The invasive growth of HCC disrupts and destroys the liver plate architecture, leading to decreased amount of reticulin and disorganised pattern of it. However, the loss of reticulin is not complete. HCC is characterised by the absence of normal portal tracts and/or naked or unaccompanied arteries in accordance with the radiologic hypervascularity and high contrast in the arterial phase of contrast-enhanced CT [6]. Invasion in connective tissues is diagnostic. However, except scirrhous and fibrolamellar HCC, stroma is usually scant in HCC. Loss of perinodular ductular proliferation is a manifestation of invasive growth [6]. Vascular invasion is diagnostic if evident.

Cytologically, HCC shows both signs of hepatocellular differentiation that serves as the clue to hepatocellular origin of the tumour and atypia indicating malignant behaviour. Regarding tumour differentiation, bile production is a reliable indicator of hepatocellular origin. Bile can be found in the cytoplasm of neoplastic cells or in lumina of acinar complexes. Similarly to benign counterparts, steatosis, Mallory bodies and hyaline globules can develop in cytoplasm of tumour cells. HCC cells can have intranuclear inclusions and/or optically clear cytoplasm. Giant cells are occasionally present. Iron accumulation in cells of hepatocellular carcinoma is not seen, even in the setting of hereditary hemochromatosis. In hepatocytes, nuclear pleomorphism can be a feature of regenerative changes; therefore, mitotic activity is more suspicious of malignancy, and the presence of atypical mitoses definitively confirms the presence of a malignant tumour. However, in well-differentiated HCC, abnormal mitoses are rare and are not mandatory for diagnosis [6].

The histologic patterns of HCC include trabecular (the most common pattern), acinar (pseudoglandular), solid and scirrhous patterns. Trabecular HCC resembles normal liver architecture. In acinar or pseudoglandular HCC, the neoplastic cells are arranged in gland-like tubules containing bile or fibrin. Solid HCC is characterised by compact, sheet-like arrangement of neoplastic cells. Scirrhous HCC exhibit marked desmoplasia; it will be described in detail later. HCC is characterised by significant inter- and intratumoural heterogeneity, manifesting as variability of grade and growth patterns [3]. Grade progression can be present even in a single patient and, in fact, reflects the biology of HCC. Hepatocellular carcinoma frequently develops in foci with equivocal biological potential, e.g., dysplastic cirrhotic nodule. Such early HCC typically is well differentiated. Over the disease course, well-differentiated HCC progresses to advanced dedifferentiated tumour. The heterogeneity can lead to diagnostic problems and failures in biopsy due to sampling errors. For instance, if a small suspicious nodule was evident by radiological imaging and a biopsy was obtained, the differential diagnosis between dysplastic nodule and HCC will frequently imply the necessity to distinguish between premalignant process and well-differentiated tumour, usually lacking marked cell atypia or clear-cut invasion. In addition, both processes can be adjacent in the tissues.

HCC has several histologic variants, including fibrolamellar, sarcomatoid, scirrhous, steatohepatic and clear cell HCC, presenting with peculiar morphological features. Some cases display lymphoepithelioma-like morphology. In addition, correlations between histological and molecular subtypes have been reported [52].

Fibrolamellar HCC is a rare subtype accounting for less than 1% of HCC. Typically, fibrolamellar carcinoma is diagnosed in young adults lacking liver cirrhosis or other known predisposing factors [3]. The mean age of diagnosis is 26 years [53]. Association with germline pathogenic variants of *TP53* gene has been reported suggesting that some cases of fibrolamellar HCC might represent Li-Fraumeni syndrome. Interestingly, in the case described by Andrade et al., a germline mutation of *TP53* was identified not only in proband affected by fibrolamellar HCC but also in her asymptomatic mother [54].

The presence of fibrous septae and central scar with possible calcification leads to architectural similarity with focal nodular hyperplasia [3, 6]. Histologically, the neoplastic cells are arranged in trabeculae and sheets, separated by collagen fibres that undergo hyalinisation and show the typical lamellar pattern [3]. Fibrolamellar HCC is defined by triad of histologic features: (1) large, polygonal neoplastic hepatocytes with wide eosinophilic granular cytoplasm. Ground glass pale bodies and PAS-positive cytoplasmic globules can be present [3, 53] but are neither sufficient nor necessary for diagnosis. (2) Prominent single eosinophilic macronucleoli should be present, and frequently are seen on the background of vesicular chromatin structure [3, 6]. (3) Lamellar fibrosis, usually present in at least half of the tumour tissue [53].

The immunophenotype of fibrolamellar HCC is also unusual, showing expression of hepatocellular markers in combination with biliary, progenitor and stem cell features as well as macrophage markers (CD68). The granular or dot-like expression of CD68 in association with appropriate morphology is helpful in diagnosing fibrolamellar HCC [6].

Prognosis of fibrolamellar HCC is poor. The 5-year survival is similar to conventional HCC arising in non-cirrhotic liver [53]; however, it is better than for classical HCC arising in cirrhotic liver [3].

Sarcomatoid HCC can occur either primarily or within classical HCC [3]. This subtype, comprising 1.8–3.9% of HCC, is partially or fully composed of malignant spindle-shaped cells, occasionally showing heterologous (rhabdoid, osteoid or chondroid) differentiation [53]. If there is no adjacent area of classical HCC, it is difficult to distinguish sarcomatous HCC from true sarcomas, including primary or metastatic tumours, e.g., metastatic gastrointestinal stromal tumour, leiomyosarcoma or fibrosarcoma. Haematoxylin-eosin stain alone can be insufficient, necessitating immunohistochemistry [3]. Considering the high grade and remarkable anaplasia of sarcomatoid HCC, hepatocellular markers should be supplemented with pancytokeratin and specific markers for sarcoma, including CD117, DOG, actin, desmin, S-100, CD34 and CD31. Hepatocellular antigens are frequently negative, and even pancytokeratin is expressed only in 23–63% cases of sarcomatoid HCC [53]; therefore, complex assessment of morphology is mandatory along with clinical history and IHC for sarcoma.

Scirrhous HCC is a rare type, accounting for 0.2% to 4.6% of HCC. It can develop beneath liver capsule leading to pedunculated gross view [3, 53]. Microscopically, scirrhous HCC is characterised by diffuse fibrosis surrounding thin trabeculae of neoplastic cells. Such fibrosis can occur either after various regimens of oncologic treatment (chemotherapy, transarterial chemoembolization, irradiation) or in untreated patients [3]. However, HCC exhibiting post-treatment fibrosis should not be classified as scirrhous [53]. The marked desmoplasia and morphology of the tumour cells, displaying clusters, strands and tubules, can lead to misdiagnosis as cholangiocarcinoma or

metastasis both in biopsy and in preoperative imaging. While conventional HCC is characterised by CT enhancement in the arterial phase and washout in the venous phase, scirrhous HCC can present with peripheral ring-like enhancement in the arterial phase and delayed central enhancement in the venous phase [53]. In addition, expression of cytokeratin 19 is frequent [52]. Haemorrhage or necrosis is usually absent. Marked CD8-positive lymphocytic infiltrate can be present [3, 53]. Regarding molecular profile, scirrhous HCC is associated with mutations in *TSC1/TSC2* genes, lack of *CTNNB1* mutations, presence of epithelial to mesenchymal transformation and stem cell profile [52].

Lymphoepithelioma-like carcinoma is characterised by the presence of rich lymphocytic infiltrate surrounding pleomorphic, small, polygonal neoplastic cells that might show syncytial growth [1].

Steatohepatic HCC is remarkable for similarity to steatohepatitis that can even lead to missed diagnosis in well-differentiated cases [53]. This subtype HCC is characterised by the presence of fat vacuoles in more than 5% of the tumour. The neoplastic cells also show Mallory bodies and ballooning degeneration. The stroma features pericellular and trabecular fibrosis as well as inflammatory infiltrate, consisting of neutrophils, plasma cells and lymphocytes [3]. Infiltrative borders are characteristic. Within the tumour, fibrosis can be prominent [53]. The patients can have underlying steatohepatitis due to metabolic syndrome/NASH [3] or alcohol-induced liver disease [53]. However, this phenotype of carcinoma is also seen in patients without steatohepatic changes in the non-neoplastic liver tissue [3]. Molecularly, IL6/JAK/STAT molecular pathway is frequently activated along with immunohistochemical C-reactive protein expression. In contrast, mutations in *CTNNB1* gene or activation of Wnt/Beta-catenin pathway are not evident. Regarding immunophenotype, low expression of glutamine synthetase has been observed [52].

Clear cell HCC features optically clear cytoplasm due to the presence of glycogen and fat vesicles in the neoplastic cells. The architecture is mostly trabecular [3].

The differential diagnosis of HCC includes benign pathological processes, for instance, dysplastic nodule in a cirrhotic liver while hepatic adenoma, focal nodular hyperplasia and bile duct adenoma should be considered in non-cirrhotic liver. Parasitic infestations, e.g., echinococcosis and infrequent benign tumours, e.g., angiomyolipoma occasionally need to be ruled out. The malignant tumours that enter the spectrum of differential diagnoses of hepatocellular carcinoma include metastases of extrahepatic tumours as well as cholangiocarcinoma, hepatoblastoma and non-epithelial liver tumours.

3.1. Immunohistochemistry and differential diagnosis

Benign and malignant liver tumours may share morphologic similarities; thus, immunohistochemical assessment is crucial to set the correct diagnosis. The two challenging tasks are (1) to distinguish low-grade/early HCC from benign lesions like liver adenoma, focal nodular hyperplasia or dysplastic nodule and (2) to differentiate high-grade HCC from metastatic tumours in the liver.

The differential diagnosis of HCC varies also depending on the underlying liver pathology. In cirrhotic liver, primary tumours such as HCC and cholangiocarcinoma are much more common than secondary tumours [3]. In contrast, in non-cirrhotic liver, HCC accounts only for about 2% of tumours and metastatic lesions predominate over primary liver neoplasms. Metastasis can mimic HCC, especially in case of clear cell renal cancer, clear cell adenocarcinoma of the female genital organs, hepatoid gastric carcinoma, adrenal carcinoma and melanoma. Metastatic gastrointestinal neuroendocrine tumours can be challenging to differentiate from HCC, especially if trabecular architecture is present [3]. In the evaluation of HCC diagnosis, arginase-1, hepatocyte paraffin-1 antigen, glypican-3, carcinoembryonic antigen by polyclonal primary antibody, CD10, glutamine synthetase and CD34 are frequently assessed. Alfa-fetoprotein is partially replaced by new markers showing higher expression frequency and less background. However, it is still helpful in some cases. Clathrin and bile salt export pump protein represent promising novel targets.

Arginase-1 (Arg1) is occasionally considered the most sensitive and specific marker of hepatocellular differentiation [55], characterised by sensitivity and specificity of approximately 90% [55]. Arginase-1 represents manganese metalloenzyme involved in the urea cycle [56]. It catalyses the hydrolysis of arginine to ornithine and urea. Arg1 is expressed in normal human liver [6] and hepatocellular tumours, including HCC. Arg1 shows better sensitivity and specificity diagnosing HCC, compared to HepPar1 and glypican 3 [55], although other researchers prefer HepPar1 (see further) to identify hepatocellular differentiation [3]. Regarding the types of HCC that might cause diagnostic difficulties—high-grade HCC and scirrhous HCC—Arg1 is characterised by sensitivity of 85 and 85%, exceeding the sensitivity of HepPar1 (64 and 26%, respectively). Arg1 displays diffuse nuclear and cytoplasmic expression pattern in HCC [6, 55]. Most other tumours are negative for Arg1, but focal or weak expression can occur in colorectal, pancreatic, breast and prostatic carcinomas, cholangiocarcinoma or hepatoid tumours [55].

Hepatocyte paraffin-1 (HepPar1) antigen is another marker of hepatocellular differentiation. Some authors prefer HepPar1 as the best marker to confirm the hepatocellular origin of a tumour [3]. HepPar1 is a carbamoyl phosphate synthetase 1: another enzyme involved in urea synthesis. In contrast to Arg1, it is expressed not only in the liver but also in non-neoplastic small intestinal mucosa and Barrett's oesophagus [56]. HepPar1 has diffuse granular cytoplasmic staining pattern. The sensitivity and specificity in HCC reaches 80%. HepPar1 is expressed in almost all well-differentiated HCCs. However, only less than 50% of high-grade cases express HepPar1 [3]. Most of metastatic and/or non-hepatocellular tumours, including adenocarcinomas, neuroendocrine tumours, renal cell carcinoma, adrenocortical carcinoma, melanoma and angiomyolipoma, are negative for HepPar1. However, focal reactivity is occasionally observed. Strong expression can be present in cholangiocarcinomas and metastatic oesophageal, gastric and pulmonary adenocarcinomas [55]. Positive reaction has also been reported in non-ampullary small intestinal adenocarcinomas (60%) and ampullary adenocarcinomas with intestinal (73%) differentiation while expression in ampullary adenocarcinomas exhibiting pancreatobiliary (14%) morphology or colonic (9%) adenocarcinomas is rare [56].

Glypican-3 (GPC3) is a member of the glypican family of heparan sulphate proteoglycans. It is bound to the external surface of plasma membrane through a glycosyl-phosphatidyl-inositol

anchor. Glypicans regulate signalling via Wnt, Hedgehog, fibroblast growth factor and bone morphogenetic protein pathways. Thus, glypicans are involved in the control of cell proliferation. In HCC, GPC3 promotes cancer growth by stimulating Wnt signalling. The GPC3 molecule can be released to extracellular environment after it has been cleaved off by lipase [57]. Hence, the functional activity of GPC3 explains its role as possible serum marker or treatment target for HCC. GPC3 is normally found in foetal liver and placenta but is absent from healthy adult liver and benign hepatocellular lesions including focal nodular hyperplasia and liver adenoma [55]. Thus, expression of GPC3 in liver biopsy is highly suggestive of HCC. The staining pattern is (1) granular or diffuse cytoplasmic, possibly with membranous enhancement; (2) membranous or (3) Golgi complex-related [6, 55]. Heterogeneity can lead to focal lack of expression; therefore, negative result in biopsy does not exclude HCC. The sensitivity of GPC3 ranges from 56 to 62% in low grade (G1) HCC to 80–83% in intermediate grade (G2) HCC, 85–89% in high grade (G3) HCC and 79% in scirrhous HCC [55]. GPC3 is expressed in many extrahepatic tumours that can spread to the liver, including metastatic adenocarcinoma, squamous cell carcinoma, non-seminomatous germ cell tumours (choriocarcinoma, yolk sac tumour) and malignant melanoma (5%). Cholangiocarcinoma can be positive (5%) as well [6, 55]. The strong advantages of GPC3 include the absence of it from non-malignant liver as well as high sensitivity in high-grade HCC. Lack of specificity is the greatest pitfall [55].

Carcinoembryonic antigen (CEA) family represents a class of different glycoproteins belonging to immunoglobulin superfamily. Within CEA family, adhesion molecules and pregnancy-specific glycoproteins are distinguished. The functions of CEA family include cell adhesion, as well as cell interaction in pregnancy, immune reactions and angiogenesis [58]. By immunohistochemistry, CEA is found in foetal and adult epithelial cells [6]. In liver pathology, CEA assessment by polyclonal antibody (pCEA) is strongly advised. In HCC, distinct specific canalicular or so called chicken-wire fence pattern can be observed. Metastatic adenocarcinomas show diffuse membranous, luminal and/or cytoplasmic positivity [55]. In higher grade HCC, the specific canalicular pattern is progressively lost and replaced by unspecific membranous expression [6].

CD10 is a zinc-dependent metalloproteinase, located in cell surface membranes. It exhibits neutral endopeptidase activity: cleavage of peptides at the amino side of hydrophobic residues. CD10 inactivates several hormones, as glucagon, oxytocin and bradykinin. In HCC, CD10 shows canalicular expression similarly to pCEA. However, the sensitivity of CD10 for HCC is lower, around 50% [55].

Alpha-fetoprotein (AFP), the protein encoded by *AFP* gene on 4q25, is the major plasma protein in developing foetus. It is produced by liver and yolk sac and might represent the foetal analogue of albumin. AFP can bind metal ions, fats and bilirubin. In adults, AFP is found in HCC and germ cell tumours but normal liver tissue does not express AFP [3]. Although the sensitivity of AFP for HCC is only 30–50% and high background can frequently limit the interpretation [55], truly positive cases in our experience were easy to recognise. In contrast to HepPar1 and pCEA, AFP positivity increases with dedifferentiation of HCC [3].

Glutamine synthetase (GS) is an enzyme that catalyses the condensation reaction between glutamate and ammonia resulting in glutamine. GS is regulated by beta-catenin molecular pathway. In normal liver tissue, immunohistochemical expression of glutamine synthetase is

found only in a thin central perivenular (zone 3) area. In contrast, extensive diffuse cytoplasmic expression is present in 70% of HCC [6].

CD34 has multiple diagnostic roles. Within its wide expression spectrum, endothelial cells are also positive. Sinusoidal expression of CD34 is increased in both benign and malignant hepatocellular lesions, contrasting with limited expression in periportal sinusoids within normal liver [55] or in parenchymal capillaries close to fibrous septa within cirrhotic tissues [6]. In HCC, the endothelial expression of CD34 increases, until capillarisation of the sinusoids becomes complete. The capillarisation develops due to higher oxygen tension in HCC. Although incomplete CD34 expression does not exclude HCC, diffuse positive reaction is strongly suggestive of HCC. However, limited sampling in biopsy can lead to pitfalls as foci of complete CD34 expression are seen in adenomas and in periphery of cirrhotic nodules. If such foci are predominantly sampled within the biopsy, false overestimation of CD34 reactivity is possible [6].

Clathrin is one of the novel markers appearing in the differential diagnostics between malignant and non-malignant hepatocellular nodules. Clathrin is a protein that forms airscrew-like triskelion consisting of three light chains and three heavy chains. When these molecules assemble between themselves, clathrin-coated vesicles arise and participate in endocytosis and exocytosis. Thus, clathrin participates in cell communication and signalling, in the transport of nutrients, receptors and other macromolecules. During mitosis, clathrin stabilises mitotic spindle. The heavy chain of clathrin is significantly upregulated in HCC. In the initial reports, striking contrast in the immunohistochemical staining was found between tumour and surrounding tissues suggesting high affinity and low background. The expression pattern was cytoplasmic and membranous. Expression of the heavy chain of clathrin was tested for the distinction between HCC and benign nodules. The sensitivity and specificity of the heavy chain of clathrin was 41.2 and 77.2%, and the sensitivity increased to 61.1% in combination with glypican-3 [59].

Bile salt export pump protein is a transport molecule that is present in bile canaliculi. By immunohistochemistry, bile salt export pump protein was expressed in 89.6% HCC, mostly (76.7%) in canalicular pattern. In comparison with cholangiocarcinomas and metastatic tumours, expression of bile salt export pump protein had 90% sensitivity and 100% specificity for HCC. The performance of bile salt export pump protein was comparable to arginase-1 showing both sensitivity and specificity of 94% and slightly better than HepPar1 characterised by sensitivity 90% and specificity 97% [60].

3.2. Well-differentiated hepatocellular carcinoma *versus* adenoma

Hepatocellular adenoma (HCA) is defined as benign monoclonal proliferation of well-differentiated hepatocytes. The most common risk factor for HCA is exposure to high oestrogen levels in oral contraceptives, thus the disease has strong female predominance (9:1). Adenomas are typically small, solitary lesions in non-cirrhotic liver. Occasionally, multiple tumours are observed [61]. In HCA, the neoplastic hepatocytes are arranged in cords and sheets, typically two layers thick [3, 62]. The portal triads and interlobular bile ducts are absent from adenoma tissue [63]. Pseudoglandular architecture can be observed, especially in adenomas associated

with anabolic use. HCA cells appear larger due to intracellular glycogen or fat accumulation. Nuclear atypia is absent [3].

Several molecular subtypes of hepatocellular adenomas are known [62, 64], including hepatocyte nuclear factor 1 α (HNF1 α) inactivated type (H-HCA); β -catenin activated type (B-HCA); inflammatory HCA (I-HCA) and the unclassifiable type (U-HCA). Not surprisingly, beta-catenin activated subtype is associated with malignant transformation [62]. Beta-catenin mutations are reported in 20% of HCCs, especially in patients with underlying hepatitis C virus infection. HCC arising from B-HCA is usually well to moderately differentiated and lacks vascular invasion or satellite nodules [3]. Mutations lead to remarkable overexpression of *GLUL* gene (coding for glutamine synthase), thus beta-catenin activation can be assessed by intense homogeneous cytoplasmic expression of glutamine synthase and by aberrant nuclear localisation of beta-catenin [62, 63]. H-HCA shows decreased expression of liver fatty acid-binding protein, and presence of fat in neoplastic cells can be seen histologically. I-HCA is characterised by immunohistochemical positivity for serum amyloid A and C-reactive protein. Marked inflammatory infiltrate, ductular reactions and sinusoid dilation can be present in the tissue as well. U-HCA lacks gene mutations or specific immunohistochemical findings, but is diagnosed as HCA by histology [61]. Liver adenomas express hepatocellular markers and have lower proliferation activity than HCC [63]. To discriminate between adenoma and HCC, the following parameters are of importance: (1) clinical history in order to disclose risk factors that might indicate either HCA or HCC; (2) structure of surrounding liver as presence of cirrhosis favours HCC; (3) expression of HCA subtype-specific proteins; (4) presence or absence of cell atypia and invasion; (5) hepatocyte plate thickness and (6) expression of malignancy-associated HCC markers, e.g., GPC3.

3.3. Well-differentiated hepatocellular carcinoma *versus* focal nodular hyperplasia

Focal nodular hyperplasia (FNH) is a hyperplastic hepatocellular proliferation resulting from blood flow abnormalities. It is a pathological focus characterised by nodular architecture, hypervascular central scar associated with thick fibrous septa between hepatocyte nodules, inflammatory infiltrate, presence of ductular reaction and sinusoid dilation [55, 61–63].

To distinguish FNH from HCC, GPC3, heat shock protein 70 (HSP70) and reticulin network can be assessed. Loss of reticulin framework, immunohistochemical expression of GPC3 and/or diffuse nuclear expression of HSP70 favours HCC. Such immunohistochemical evaluation has 100% specificity for HCC although the sensitivity is only 43–46%. Typical “map-like” pattern of GS expression is evident in FNH. It is characterised by wide central positive areas in the middle of nodules. The positive foci interconnect between themselves, while periseptal areas remain negative. This reactivity pattern contrasts with normal liver showing limited perivenular reactivity in the middle of lobules [55].

3.4. Well-differentiated hepatocellular carcinoma *versus* high-grade dysplastic cirrhotic nodule

Dysplastic cirrhotic nodules (DNs) are characteristic precursors of HCC in the setting of chronic liver disease and/or liver cirrhosis. Most but not all dysplastic nodules are small, not

exceeding the diameter of 1 cm [6]. Morphologically DNs are classified into high-grade DN and low-grade DN. Low-grade DN, carrying low risk of transformation to HCC, is generally characterised by monotonous cell population when compared with the surrounding cirrhotic liver, mildly increased cell density and minimal cell atypia. The nuclear/cytoplasmic ratio is mildly increased, nuclear atypia is slight, mitoses are absent and cell plates are 1–2 cells thick. The reticulin network is retained. The borders of low-grade dysplastic nodule are rounded, but the adjacent liver parenchyma is not compressed [3]. In contrast, high-grade dysplastic nodules can have many of classical HCC features. The nuclear/cytoplasmic ratio is increased. Nuclei show hyperchromasia and irregular borders and can be peripherally located. Occasional mitoses can be present. Cell plates are thicker than 2 cells. Cytoplasm switches to basophilic staining. Pseudoglandular structures start to appear. Occasional unpaired arteries have been observed. Lack of invasion is the most reliable criterion in the differential diagnosis with early HCC [3]. This trait is both important and biologically substantiated as the invasion is the hallmark of malignant tumours. However, it can be notoriously difficult to apply practically. In early HCC, invasion can be absent from biopsy due to sampling error. Regarding high-grade dysplastic nodule, entrapment of perinodular hepatocytes into fibrous tissues mimics invasion. To classify the entrapped hepatocytes correctly, immunohistochemical investigation of ductular proliferation can be helpful, as further described, because these non-neoplastic intraseptal hepatocytes and ductular proliferation stem from common progenitors [3].

Expression of GPC3 points towards malignant hepatocellular tumour, as it was previously noted. However, GPC3 expression has been reported in 3–76% of dysplastic nodules. Glutamine synthetase is expressed in 69.8% of HCC contrasting with 13.6% in high-grade DN. Heat shock protein 70 is found in 73.5% of HCC and only exceptional dysplastic nodules [3]. To distinguish high-grade DN from early HCC, immunohistochemical panel comprising heat shock protein 70, glypican-3 and glutamine synthetase has been recommended. Expression of one marker is compatible with DN, while HCC expresses at least two markers. The sensitivity of this panel is estimated as 60–78% [55].

In addition, cytokeratin (CK) 7 and/or CK19 and CD34 can be useful in the assessment of architecture and reactive changes. HCC is characterised by more diffuse expression of CD34 and loss of ductular reaction at the nodule interface. Dysplastic nodule shows only focal CD34 expression in the periphery of the nodule and more marked proliferation of CK7-positive ductules surrounding DN [55]. In the ductular reaction, CK7 and CK19 usually are coexpressed. Thus, gradual loss of CK7 and CK19 positive ductular reaction in perinodular stroma correlates with progression of cirrhotic to dysplastic nodule and further to HCC. Ductular reaction is present around $\geq 50\%$ of perimeter of a DN, while it is almost lost in HCC [3].

Different systems for complex evaluation of the biological potential of hepatocellular nodule have been proposed. Integrated evaluation of haematoxylin-eosin findings together with reticulin stain and immunohistochemistry for CD34 has been suggested. A hepatocellular nodule should be classified as HCC if at least three features from the following are present: necrosis; cellular atypia; thickness of trabeculae more than 4 cells; mitotic activity or diffuse expression of CD34 in the sinusoidal endothelium [6]. Alternatively, stromal invasion, loss of reticulin network and positivity for at least two out of three markers (HSP70, GS, GPC3)

are considered the strongest parameters discriminating HCC from high-grade dysplastic nodule [3].

3.5. Hepatocellular carcinoma *versus* metastasis

If high-grade malignant tumour is found in the liver, the differential diagnosis includes metastatic malignancy *versus* HCC and cholangiocarcinoma. Any malignant tumour can ultimately spread to the liver via bloodstream, lymphogeneous dissemination or transperitoneal spread. In some biopsy series, metastatic lung, colorectal, pancreatic and breast carcinomas have been the most common secondary liver tumours [3]. However, frequency of different metastatic malignant tumours in liver biopsies depends on many factors, including the biological potential of the tumour and its incidence in the population as well as institutional approach to liver biopsy in different oncological patients. This, in turn, may depend on the patient's general status, presence of contraindications for biopsy or significant oncological treatment and the availability of effective treatment.

In order to distinguish HCC from metastatic tumours, it is advisable to combine at least two hepatocellular markers and at least two antigens that are more frequently seen in adenocarcinomas. Among hepatocellular markers, Arg1 should be combined with either HepPar1 or GPC3. Most of adenocarcinomas express cytokeratin (CK) 19, MOC-31 and CK7 [55]. The spectrum of immunohistochemical panel should be planned in accordance with tissue availability within the biopsy. The suggested minimal panel includes ARG1 and CK19 [55], while maximal investigation might include several HCC markers accounting for different grades of HCC, several adenocarcinoma markers and antigens that are characteristic for certain tissues (neuroendocrine or melanocytic differentiation) or epithelia of specific organs, e.g., breast, large bowel, lung, thyroid, kidney and others. Panels of immunohistochemical markers can disclose the location of primary tumour giving rise to metastasis. Thus, CK20 and CXD2 are typical for metastatic colorectal carcinoma; CDX2 and CK7 for gastric carcinoma; TTF-1 and napsin A for lung adenocarcinoma and oestrogen receptor, mammaglobin, GATA3 or GCDFP-15 for breast cancer [65]. The expression frequencies of different tissue- and organ-specific antigens in metastases and corresponding primary tumours are further outlined in **Table 4**.

Antigen	Tumour	Frequency, %	References
CDX2	Colorectal carcinoma	100	[66]
CDX2	Metastatic colorectal carcinoma	96.7–100	[67, 68]
SATB2	Primary colorectal carcinoma	96.0	[68]
SATB2	Metastatic colorectal carcinoma	92.2	[68]
CK20	Metastatic colorectal carcinoma	97.1	[68]
TTF-1	Lung adenocarcinoma	83.3	[69]
Napsin A	Lung adenocarcinoma	86.7	[69]
HMB-45	Metastatic melanoma	76–81	[70, 71]
MART-1	Melanoma	48.4–83	[72, 73]

Antigen	Tumour	Frequency, %	References
MART-1	Metastatic melanoma	63–82	[70, 72, 73]
Tyrosinase	Melanoma	71	[72]
Tyrosinase	Metastatic melanoma	63	[72]
PAX-8	Ovarian cancer	80	[69]
PAX-8	Endometrial cancer	100	[69]
PAX-8	Renal cancer	83–93.3	[69, 74]
PAX-8	Metastatic renal cancer	93.9	[74]
Napsin A	Renal cancer	50	[69]
Gross cystic disease fluid protein-15	Breast carcinoma	23.9–60	[75, 76]
Gross cystic disease fluid protein-15	Primary triple negative breast carcinoma	10–14	[75, 77]
Gross cystic disease fluid protein-15	Primary non-triple negative breast carcinoma	69	[77]
Gross cystic disease fluid protein-15	Metastatic triple negative breast carcinoma	21	[75]
Mammaglobin	Breast carcinoma	46.6–80	[75, 76]
Mammaglobin	Primary triple negative breast carcinoma	17–25	[75, 77]
Mammaglobin	Primary non-triple negative breast carcinoma	61	[77]
Mammaglobin	Metastatic triple negative breast carcinoma	41	[75]
GATA3	Invasive breast cancer	82.5–94	[76, 78]
GATA3	Primary triple negative breast carcinoma	20.2–87	[77–80]
GATA3	Metastatic triple negative breast carcinoma	44	[79]
GATA3	Luminal A breast carcinoma	99.5	[80]
GATA3	Luminal B breast carcinoma	97.7	[80]
GATA3	HER2-positive breast carcinoma	59.6–68.5	[76, 80]

CDX2, caudal type homeobox 2; SATB2, special adenosine-thymidine-rich-binding protein 2; CK, cytokeratin; TTF-1, thyroid transcription factor 1; HMB-45, melanosome protein human melanoma black 45; MART-1, melanoma antigen recognized by T cells 1; PAX-8, paired box gene 8; GATA3, guanosine-adenosine -thymidine -adenosine nucleotide sequences binding protein 3.

Table 4. Frequency of immunohistochemical expression of selected tissue- or organ-specific markers [66–80].

When differentiating between HCC and metastasis, the peculiar immunophenotype of fibrolamellar HCC must be recognised promptly. Fibrolamellar HCC expresses hepatocellular proteins, such as HepPar1, GPC3 or pCEA; biliary (CK7), progenitor and stem cell (CK19, CD44) antigens and macrophage markers (CD68). The granular or dot-like expression of CD68 in a tumour featuring appropriate morphology is helpful in diagnosing fibrolamellar HCC [6].

4. Molecular analysis

The molecular classification of hepatocellular carcinoma is still developing. Thus, different approaches have been proposed. Although the present tools of molecular analysis assure the

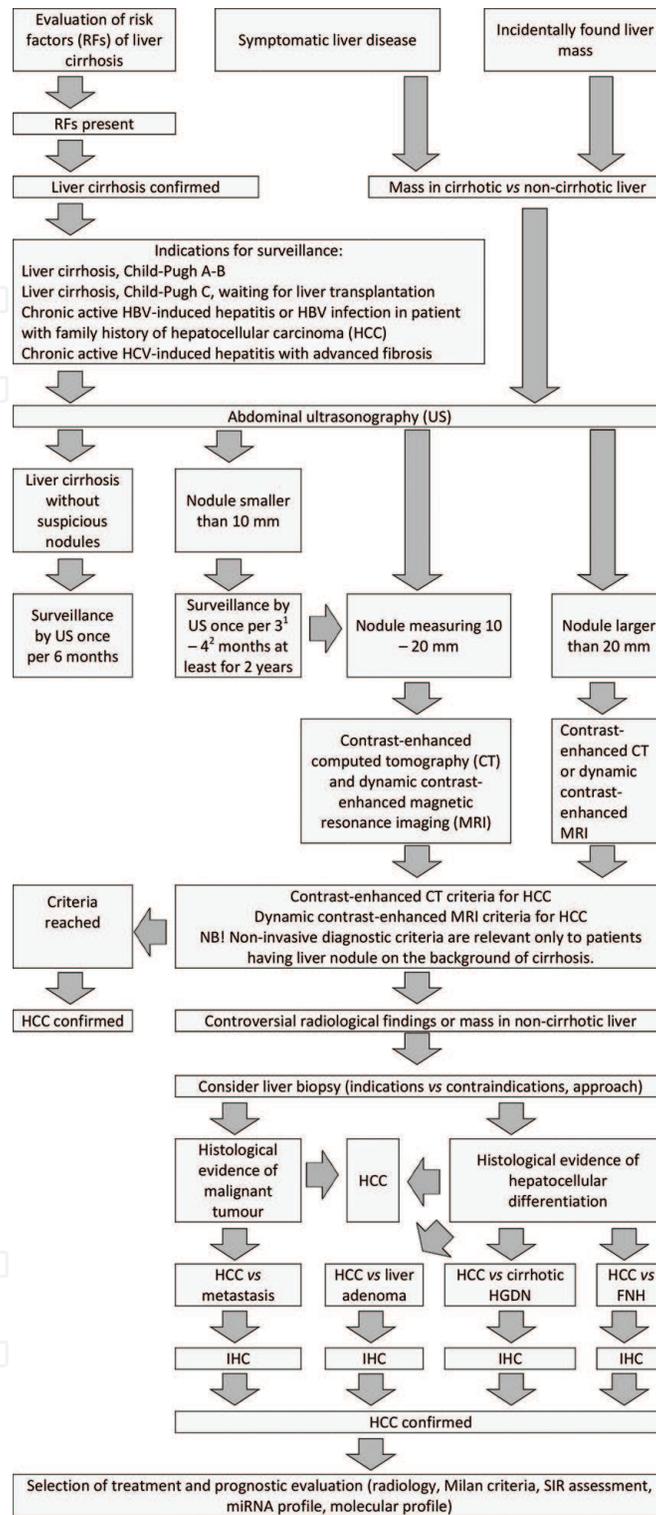


Figure 1. Diagnostic algorithm of hepatocellular carcinoma. 1—Recommended by the American Association for the Study of the Liver diseases (AASLD). 2—Recommended by the European Association for the Study of the Liver (EASL). Abbreviations: RFs, risk factors; *vs*, versus; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; US, ultrasonography; CT, computed tomography; MRI, magnetic resonance imaging; HGDN, high-grade dysplastic nodule; FNH, focal nodular hyperplasia; IHC, immunohistochemistry; SIR, systemic inflammatory response; mi, micro; RNA; ribonucleic acid.

technical background for in-depth studies, HCC might be more difficult target for the systematisation of molecular findings than other tumours. The problems are associated with heterogeneity of etiological factors and their geographic distribution in different populations with diverse genetic background [81].

A trans-ancestry study has been carried out involving 608 cases of HCC. The cohort was created to reflect both etiological and geographic/genetic diversity of HCC. The main identified molecular targets were TP53–Rb pathway, Wnt pathway, modulators of chromatin and transcription, mTOR–PIK3CA pathway and mutations in genes regulating telomere maintenance [82].

French research team has recently proposed molecular classification into six subtypes, designated as G1–G6. The first three subtypes are characterised by *TP53* mutations and are high-grade tumours. G1–G2 share *AXIN1* and *ATM* mutations, while G1 also possesses *RPS6KA3* mutations. G3 is characterised by mutations in *TSC1/TSC2* and *FGF19*. G3 is also associated with haemochromatosis, macrovascular invasion, macrotrabecular and compact histological pattern as well as presence of multinucleated and pleomorphic cells. Sarcomatoid changes are more frequent in G1–G2, but clear cells—in G1. G4–G6 lack *TP53* mutations and are low-grade tumours. G5–G6 exhibit mutations in *CTNNB1* gene, while G4 lacks both mutations in *TP53* and *CTNNB1*. G4 tumours are more frequently characterised by small size, steatohepatic morphology and inflammatory infiltrates as well as absence of satellite nodules and vascular invasion. G5–G6 carcinomas display microtrabecular pattern, cholestasis and lack inflammatory infiltrates. By immunohistochemistry, these HCCs are characterised by nuclear expression of beta catenin and strong positivity for glutamine synthetase [52].

5. Diagnostic algorithm of hepatocellular carcinoma

Nowadays, the classic diagnostic algorithm of HCC (see **Figure 1**) includes the evaluation of risk factors in a given patient to assess the need for surveillance. Cirrhotic patients are referred to ultrasound examination once per 6 months. Suspicious nodules are further evaluated by CT and MRI. Characteristic findings by CT and MRI including arterial hypervascularisation represent the basis of non-invasive diagnostics. In controversial and non-cirrhotic cases, biopsy is indicated that might need supplementation by immunohistochemistry according to the morphological features. Innovations are expected in the field of miRNA-based liquid biopsy to support radiological diagnosis, addition of SIR assessment and miRNA profile to select the optimal treatment, e.g. possibly broadening Milan criteria (see also chapter “Innovative Blood Tests for Hepatocellular Carcinoma: Liquid Biopsy and Evaluation of Systemic Inflammatory Reaction”), and novel immunohistochemical markers for cases that still remain ambiguous.

6. Conclusions

HCC is a frequent and aggressive malignant tumour, estimated to range sixth by incidence and second by mortality in the global cancer statistics. The high ratio of mortality to incidence (0.95)

and the close geographic correlation between incidence and mortality reflects the dismal prognosis. However, longer survival can be reached in early diagnosed and properly treated cases.

Awareness of the risk factors of HCC is helpful both in diagnostics and in order to set up the surveillance. Liver cirrhosis is the main risk factor; surveillance is indicated in these patients. A tumour found in cirrhotic liver is more likely to be HCC than metastasis or liver adenoma. However, the differential diagnosis includes a dysplastic cirrhotic nodule.

The other risk factors act mainly through inducing cirrhosis although a fraction of HCC can precede the development of cirrhosis in a patient affected by chronic liver disease or develop in non-fibrotic liver. Thus, the complete list of the risk factors of HCC includes chronic active hepatitis B or C, liver damage by alcohol and/or aflatoxins, as well as NASH. The risk factors can act synergistically. Evaluating the HCC risk in any patient, the relative risk must be considered in accordance to the risk factors that are identified in that individual. However, to estimate the expected cancer burden in the population, population attributable fractions are of importance; these parameters depend both on relative risk and population frequency of each particular factor.

Non-invasive radiological approach is the gold standard in the diagnostics of HCC in contrast with most of other malignant tumours necessitating confirmation by a biopsy. Biopsy is indicated only in radiologically controversial cases or to prove HCC in non-cirrhotic liver.

Ultrasonography is used for surveillance and the initial step of diagnostics. For surveillance of cirrhotic patient, US is carried out once in 6 months. If a suspicious focus is disclosed, the further approach is based on the size. Either CT or MRI is indicated for mass lesions larger than 20 mm, while both methods are recommended for a nodule measuring between 10 and 20 mm. Nodules that are smaller than 1 cm are followed up by US once in 4 months. Hypervascularity is a characteristic trait of HCC in CT and MRI. PET and CEUS may have additional role in HCC diagnostics.

If biopsy is carried out, HCC can be diagnosed if both signs of hepatocellular differentiation and cellular atypia or invasion are present. Low-grade tumours must be differentiated from dysplastic nodule, focal nodular hyperplasia and adenoma while high-grade HCC must be distinguished from metastasis. Mass lesion in cirrhotic liver is most probably a dysplastic nodule or HCC while adenomas and metastases usually develop in non-cirrhotic liver. In a Western patient, clearly malignant tumour in a non-cirrhotic liver has higher probability to represent a metastatic carcinoma.

Regarding immunohistochemistry, arginase-1 and HepPar antigen are reasonable hepatocellular markers that are used to distinguish HCC from metastases. Novel immunohistochemical markers of HCC include bile salt export pump protein and heavy chain of clathrin. Glypican should be used with caution due to the reported expression in a wide range of extrahepatic tumours. In order to discriminate between low-grade HCC and FNH, reticulin network, glypican-3 and heat shock protein 70 can be assessed. The differential diagnosis between high grade dysplastic nodule and low grade HCC can be very complicated as both processes share several morphological features and can coexist, biologically representing subsequent stages of HCC development. The features favouring malignancy over dysplastic nodule, include (1)

expression of at least two markers in a panel consisting from glypican-3, heat shock protein 70 and glutamine synthetase; (2) diffuse expression of CD34 due to higher oxygen tension in HCC and (3) loss of perifocal CK7- and CK19-positive ductular reaction as a sign of invasive growth.

Regarding molecular classification of HCC, reasonable success has been reached by French research group and trans-ancestry study team. However, no unified classification has been established yet. Molecular profile can have both diagnostic and prognostic value.

Acknowledgements

BS was financially supported by post-doctoral research project 1.1.1.2./VIAA/1/16/242.

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