REVIEW

Surveillance and diagnosis of hepatocellular carcinoma in patients with cirrhosis

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Abstract

Early identification of hepatocellular carcinoma (HCC) is more frequent because of surveillance programs for HCC worldwide. The optimal strategy of surveillance in cirrhosis is a current topical issue. In terms of diagnosis, recent advances in non-invasive imaging technology, including various techniques of harmonic ultrasound, new ultrasound contrast agents, multi-slice helical computed tomography and rapid high quality magnetic resonance, have all improved the accuracy of diagnosis. Consequently the role of liver biopsy in diagnosis of HCC has declined. The imaging diagnosis relies on the hallmark of arterial hypervascularity with portal venous washout. However, with recent advances in genomics and proteomics a great number of potential serum and tissue markers have been identified and are being developed as new candidate markers for both diagnosis and prognosis of hepatocellular carcinoma, and may increase the need for liver biopsy.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary hepatic malignancy worldwide and it represents the leading cause of death in patients with cirrhosis in Europe[1]. Malignant hepatic cell transformation is more frequent in cirrhotic livers, accounting for 80%-90% of overall autopsied series[2]. Between 59% and 94% of new diagnosed nodules in cirrhosis are histologically characterized as malignant[3,4] and about 50% of the hemangioma-like lesions in cirrhosis are shown to be HCC[5]. It is reasonable and common clinical practice to consider any lesions in a cirrhotic liver, as malignant until proven otherwise.

In developed countries there are surveillance programs for "at-risk" people, including those with cirrhosis, to identify the malignant lesions when they are small. For this purpose differentiating between early HCC and a dysplastic nodule is an important issue in a routine clinical setting.

Recent advances in non invasive imaging technology for the diagnosis of hepatocellular carcinoma include various techniques of tissue harmonic ultrasound (US) imaging, new US contrast agents, multi-slice helical computed tomography (CT) and rapid high quality

magnetic resonance (MRI) with new, tissue-specific contrast agents.

Ultrasonography is the first line of investigation in the detection of focal liver lesions, particularly as it used for surveillance of HCC in patients with cirrhosis, as it has relatively low cost, is non invasive and has wide spread availability[6].

SURVEILLANCE AND SCREENING

Surveillance of HCC in patients with cirrhosis in most centres is performed using 6-monthly US and in some centres this is combined with α-fetoprotein (AFP).

Data to support the effectiveness of ultrasound surveillance are sparse because of ethical problems of not performing ultrasound as it is part of current clinical practise[7-10].

The value of using AFP for surveillance has not been validated but once a nodule has been detected is useful[11]. The AFP test, with a cut-off value of 20 ng/mL, has a sensitivity from 41% to 65%[11-16]: lowering the cut-off value and changing it for different etiologies of liver disease, such as in HBV carriers results in greatest sensitivity[17]. Currently, HCC screening with AFP alone is not recommended, except when US either is not available or of poor quality[18]. Moreover AFP measurement together with US screening, is not cost-effective[19], as it only increases sensitivity by about 10% compared to US screening alone[20].

However, high levels of AFP can identify an "at risk" category of patients with cirrhosis that require surveillance[21] (Table 1).

In the sole randomized controlled trial performed in China, which also included individuals without cirrhosis, the survival rate at five years after enrolment was 46.4% in the surveillance group (AFP plus US scan every 6 mo) against 0% in the control group[22].

Indirect proof of the utility of a surveillance strategy is the resulting change in presentation of HCC, with an increased rate of detection of tumors < 2 cm in diameter. In fact while tumors less then 2 cm in diameter represented less than 5% of the cases in the early nineties in Europe, now they represent up to at least 30% in Japan[23]. However, the increased diagnosis of HCC does not necessarily mean an improvement in survival[24], although a well documented cohort study does suggest this[10]. More data are need to substantiate the value of this strategy. Based on the estimated HCC doubling time and cost-effectiveness estimates, the recommended screening interval is 6 mo, although a 1 year interval seems to be as effective[25].

An additional consideration is the fact that ultrasound imaging requires good equipment and skilled operators. In a retrospective study in patients with cirrhosis five-year survival was better in the group screened in a specialized centre (52%) *versus* the group screened in non-specialized centres (40%)[26]. Surveillance programs

Table 1 At risk population for HCC surveillance: AASLD guide lines[21]

Hepatitis B carriers
  Asian males 40 years or more
  Asian females 50 years or more
  All cirrhotic hepatitis B carriers
  Family history of HCC
  Africans over age 20
  For non-cirrhotic hepatitis B carriers not listed above the risk of HCC varies depending on the severity of the underlying liver disease, and current and past hepatic inflammatory activity. Patients with high HBV DNA concentrations and those with ongoing hepatic inflammatory activity remain at risk for HCC
Non-hepatitis B cirrhosis
  Hepatitis C
  Alcoholic cirrhosis
  Genetic hemochromatosis
  Primary biliary cirrhosis
  Group with lack of evidence. Although the following groups have an increased risk of HCC no recommendations for or against surveillance can be made because a lack of data precludes an assessment of whether surveillance would be beneficial: α1-antitrypsin deficiency, non-alcoholic steatohepatitis, autoimmune hepatitis

for HCC would benefit from the same organizational setup as breast programs, with recall facilities and dedicated centres.

Ultrasound surveillance as it is currently practised has an acceptable sensitivity of 65%-80% and has a upper level of specificity of more than 90%[7,21,27]. Tumor size significantly affects the sensitivity of US in detecting HCC. Sensitivity ranges from 42% for lesions smaller than 1 cm[28,29] to 95% for tumors of larger size[30]. In pre-transplant screening, the US sensitivity is poor because of the coarse echotexture of the liver, the frequent presence of ascites and the high rate of malignant lesions present in end-stage liver disease[31]. In a retrospective study on 200 patients who underwent liver transplantation, within 3 mo of previous screening, the US scanning was correlated with explanted livers, and had a sensitivity of only 13.6% to 50% for lesions of 1-5 cm in diameter[32]. Therefore in liver transplant candidates, CT or MRI scanning should be performed[33].

Using grey-scale US more than 76% of hepatocellular cancers smaller than 2 cm appear as hypoechoic, with or without posterior enhancement[34]. About 17% of small HCC show an hyperechoic appearance[34,35], features related to the fatty changes occurring during the evolution of the borderline lesion. Fewer small HCC lesions appear isoechoic[34,35]. Lesions larger than 2 cm in diameter show a more heterogeneous pattern than smaller lesions because of the changes during the growth of the lesion (i.e. development of necrotic hypoechoic areas, calcifications and pseudocapsule). In these cases the presence of the "halo sign" and posterior enhancement increase the specificity of diagnosis[21,27,31].

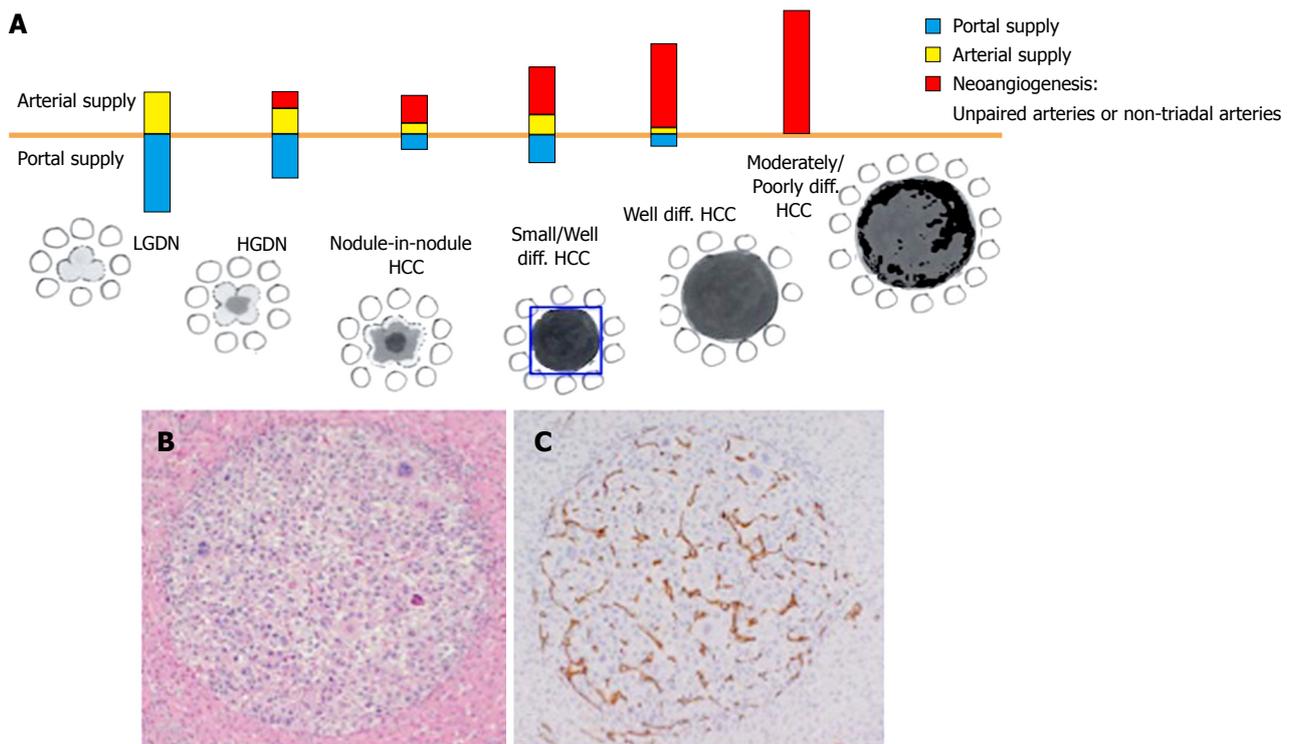The use of doppler US or power doppler US may help establish the nature of the lesion by detecting

Figure 1 **The diagram shows the changes of intranodular blood supply that characterises HCC (A)**; **The sampled small/well differentiated HCC shows in HE (B) compact carcinomatous tissue well circumscribed from dysplastic tissue; CD34 immunostain of the same nodule (C) demonstrates arteries not confined to portal tracts in HCC.** diff.: Differentiated.

arterial vascularization. However, in small HCC and in lesions located deep within the liver parenchyma, the sensitivity of doppler is low and a typical arterial pulsatile flow arterial pattern is detected in only 50% of nodules [31,36]. Colour or power Doppler US of large HCC often demonstrate a basket pattern, with a fine blood network surrounding the nodule and flowing into it[37].

When new nodules are found in a cirrhotic liver, these must be characterized with a contrast-enhanced imaging technique. During surveillance using double harmonic-equipped US machine, the same operator can also diagnose an HCC with contrast-enhanced US (CEUS). This make CEUS more cost-effective[38]. However, MRI or CT are needed whenever a second technique of imaging is needed, particularly if the lesions is 1-2 cm in diameter, and to image the whole liver to ensure there are no other lesions. A chest CT can be done at the same time, to exclude the presence of metastases.

DIAGNOSIS-IMAGING BASED ON BLOOD SUPPLY AND TISSUE CHARACTERIZZATION

In 2001 the European Association for the Study of the Liver (EASL) published a consensus statement defining histological and radiological criteria for the identification of HCC, and categorizing patients with cirrhosis and HCC on the basis of the size of presumed nodule[18]. In 2005, practice guidelines deriving from EASL, American Association of Study for the Liver Diseases (AASLD) and Japanese Society of Hepatology (JSH) revised the 2001 statements[21], giving prime diagnostic importance to arterial hypervascularization together with portal venous washout, and adding CEUS as a non invasive diagnostic technique[21,39].

In the multistep process of hepatocarcinogenesis there is a progressive change in the vascular supply that consists of an increase in arterial blood supply and loss of portal blood. Contrast enhancing agents can characterize the vascular pattern of focal liver lesions secondary to hepatocellular transformation with a good correlation between grade of HCC malignancy and intranodular hemodynamics[40,41] (Figure 1).

The typical feature of HCC, demonstrated by using intravenous contrast media that show extracellular-vascular distribution, consists of arterial enhancement with early portal and venous phase wash-out. Imaging techniques recommended in EASL/AASLD/JSH guidelines[21] are: triphasic CT with iodinated contrast media (but not lipiodol because of its low sensitivity), dynamic MRI enhanced by gadolinium or manganese based media (that have also a slightly hepatocellular uptake with biliary excretion) and CEUS with microbubble contrast agents[21]. Conventional angiography and CT hepatic arteriography-portography can be used, but are rarely necessary for diagnosis.

The average sensitivity, specificity and especially the predictive positive values of these techniques are currently comparable (Table 2).

Table 2 Overall sensitivity, specificity and predictive value of imaging technique for the diagnosis of hepatocellular carcinoma

Study	Number of patients (*n*)	HCC patients/ HCC instances	Gold standard reference	Imaging technique	Sensitivity (%)	Specificity (%)	PPV (%)	Ref.
Ward *et al* (2000)	145	25/76	Explant/ biopsy	MR (SPIO)	66	NA	NA	[72]
				MR (Double)	80	NA	93.5	
Rode *et al* (2001)	43	18/13	Explant	SDCT	53.8	92.9	94.3	[58]
				MR (Gd)	76.9	57.1	42.3	
Krinsky *et al* (2001)	71	10/19	Explant	MR (Gd)	53	NA	96.9	[69]
Krinsky *et al* (2002)	24	24/> 118	Explant	MR (Gd)	33	NA	NA	[70]
De Ledingen *et al* (2002)	34	21/54	Explant	MDCT	51.9	84.6 pts[2]	89.5	[55]
				MR (Gd)	61.1	100[2]	100	
Libbrecht *et al* (2002)	49	17/77	Explant	MDCT	70 pts[1]	82[2]	NA	[57]
				MR (Gd)	50[1]	79[2]	NA	
Zacherl *et al* (2002)	23	23/50	Explant	MDCT	75	NA	64	[62]
Barthia *et al* (2003)	31	14/32	Explant	MR (Double)	78	NA	NA	[31]
Burrell *et al* (2003)	50	29/76	Explant	MDCT	61	NA	87	[3]
				MR (MRA)	76	NA	90	
Teefey *et al* (2003)[3]	22	9/18	Explant	MDCT	57-67 pts[1]	69-75[2]	NA	[60]
				MR (Gd)	50-56[1]	63-81[2]	NA	
Battakiarjya *et al* (2004)	30	30/46	Explant	MDCT	67.4	78.9	NA	[53]
				IOCT	68	88.6	NA	
Kim *et al* (2004)	27	27/50	Biopsy/clinical/ radiological	MR (Gd)	91.3	NA	92.6	[68]
				MR (SPIO)	77.3	NA	NA	
Valls *et al* (2004)	85	51/85	Explant	MDCT	78.8 pts	NA	88	[61]
Kim *et al* (2006)	46	31/53	Biopsy/clinical/ radiological	MDCT	77.4-79.2	NA	95-97	[56]
				MR (Gd)	92.5-94.3	NA	92-96	
Hecht *et al* (2006)	38	18/19	Explant	MR (Gd)	68.4	65.7	NA	[67]
Ronzoni *et al* (2007)	88	48/139	Explant	MDCT	64	NA	66.9	[59]
					73.3	NA	79	
Lauenstein *et al* (2007)	115	27/36	Explant	MR (Gd)	77.8	NA	NA	[71]
Forner *et al* (2008)	89	60/60	Biopsy	MR (Gd)	61.7	96.6	NA	[42]
				CEUS	51.7	93.1	NA	
Dai *et al* (2008)	498	NA/56	Biopsy/ resection	MDCT	80.4	97.9	NA	[52]
				CEUS	91.1	87.2	NA	
Choi *et al* (2008)		47/41	Explant	MDCT	65	NA	NA	[54]
				MR (Gd)	83	NA	NA	

[1]Patient-based sensitivity; [2]Patient-based specificity; [3]Two observers; [4]Three observers. CEUS: Contrast-enhanced ultrasonography; Gd: Gadolinium; HCC Hepatocellular carcinoma; IOCT: Ionized oil computed tomography; MDCT: Multi-detector computed tomography; MR: Magnetic resonance; MRA: Breath-old 3D gadolinium-enhanced angiography; MR (Double): Double-contrast MR with gadolinium and superparamagnetic iron oxide agents; NA: Not applicable; PPV: Positive predictive value; pts: patients; SDCT: Single-detector computed tomography; SPIO: Superparamagnetic iron oxide.

Part of the current recommendations[21], is that the diagnosis of hepatocellular carcinoma when the focal liver lesion is larger than 2 cm in diameter can be confidently made using one dynamic imaging technique which demonstrate the typical pattern of HCC (Table 3). Moreover if AFP serum level is > 200 ng/mL and the radiological appearance of the lesion is suggestive of HCC, the likelihood that the lesion is HCC is high, even without classical vascular enhancement and washout[21].

When there are nodules between 1 and 2 cm in diameter two concordant dynamic imaging techniques are needed to confirm HCC[21] (Table 3).

Only one prospective study has been published validating the international guidelines for nodules smaller than 20 mm in diameter[42]. In this study, MRI and CEUS were concordant for HCC in only 33.3% of cases, showing a poor predictive negative value of 42% (slightly higher if only considering lesions of more than 1 cm in diameter). In addition, commenting on the above-quoted study, Caturelli *et al*[43] suggest that the

Table 3 Newly found focal liver lesion in patients with cirrhosis. Screening and diagnosis: AASLD guide lines[21]

Focal lesion < 1 cm diameter: screen every 3-4 mo
Focal lesion 1-2 cm diameter: HCC diagnosed when 2 dynamic imaging techniques are concordant for HCC feature
Focal lesion > 2 cm diameter: HCC diagnosed with feature of HCC on 1 dynamic imaging technique

first diagnostic approach to a newly found liver lesion smaller than 20 mm in diameter should be a fine-needle aspiration, as this technique reaches a higher diagnostic accuracy than that reported for the two concordant imaging techniques[42,43]. Otherwise these lesions should not be treated as HCC, without histological evidence, as the false positive rate is about 20%[44]. Thus a biopsy of the lesion is required if confirmation of diagnosis of HCC is needed. Alternatively repeat imaging in 2-3 mo may resolve the issue.

Lesions < 1 cm in diameter may be especially difficult

to characterize, even with the best imaging techniques. These small nodules are less likely to be HCC, even if they show hypervascularity with imaging techniques. These nodules need to be followed-up with US every 3-4 mo in order to determine if there is growth suggestive of malignant transformation (Table 3). If the nodule enlarges during follow-up, the criteria related to the particular size reached, pertain[21]. For this size of lesion, histology may not able to confirm the diagnosis.

Regenerative nodules and borderline lesions, such as dysplastic nodules and early HCC, show an inconsistent pattern of vascular enhancement. Benign regenerative nodules can also be hypervascular and if their diameter is between 1 and 2 cm, they should biopsied to resolve the diagnosis.

In addition, in smaller lesions the amount of Kupffer cells and fatty changes can be very variable, in comparison to overt HCC[45]. MRI with the use of super paramagnetic iron oxide (SPIO) enhancement, can be useful to characterize these lesions.

Characterisation of these smaller nodules poses a diagnostic challenge as they are more difficult to characterize even with pathological examination[23], although stromal invasion by "carcinomatous" cells, is associated with malignancy.

DIAGNOSIS - CEUS, CT AND MRI AS SINGLE TECHNIQUES: ADVANTAGES AND LIMITS

CEUS
In ultrasonography the main advance has been contrast-enhanced ultrasonography, which has improved the accuracy of ultrasound in detecting focal lesions, combining morphological aspects with functional perfusion ones[46]. CEUS is also very useful for assessment of HCC after treatment and has a good correlation with CT findings[47]. After trans-arterial embolization using lipiodol-based compounds that leave a radio-opaque shadow, only CEUS or dynamic MRI can detect residual vascularity.

Several reports have shown that CEUS is a good tool to show arterial hypervascularity of HCC. Two studies showed that CEUS has a higher detection rate compared to CT for lesions ≤ 2 cm (53.6% *vs* 42.9% and 61% *vs* 49%)[48,49]. However, a more recent study, comparing CEUS with MRI, found that CEUS was slightly inferior to dynamic MR imaging in showing the presence of arterial hypervascularity (78% *vs* 85%)[42]. The sole detection of arterial hypervascularity without contrast wash-out in a small solitary lesion of ≤ 2 cm in the setting of cirrhosis has a specificity of 86.2% and a positive predictive value of 92.2% for the diagnosis of HCC and thus cannot be considered as a conclusive finding[42]. Therefore, to increase the specificity of diagnosis it is necessary to evaluate contrast wash-out during the portal venous and the late phase as is currently recommended. Wang *et al*[50] found that the combination of arterial phase enhance-

ment and the contrast wash-out during the portal venous and the late phase determined by CEUS are more specific for HCC if nodules < 2 cm, compared with the use of either arterial phase enhancement or the absence of delayed phase enhancement considered separately (91.7% *vs* 66.7%).

Considering CT as the gold standard, the sensitivity of CEUS in detecting HCC decreases with smaller tumoral lesions. Two studies have found that lesion base sensitivity was 89.3% for nodules < 2 cm and 67% for nodules < 1 cm[51]. In a recent study, 72 patients with cirrhosis with 103 small hepatic nodules (1-2 cm) detected on US, underwent CEUS and CT. Nodules which had contrast enhancement during the arterial phase and contrast wash-out during the late phase on CEUS or CT were diagnosed as HCC. According on these diagnostic criteria the sensitivity of CEUS was 91.1% and specificity 87.2%[52].

CT
Helical CT and more recently multi-detector helical CT (MDCT), which has improved spatial and temporal resolution, has increased the accuracy of CT in diagnosis of HCC[47]. Several studies, most using correlation with explanted liver after transplantation, show that the overall sensitivity, specificity and positive predictive value of CT in diagnosis of HCC ranges from 51.9%-80.4%, 78.9%-97.9% and 88.6%-92.9% respectively[52-62].

Among studies that have assessed the sensitivity of CT in diagnosis of HCC and stratified for tumor size, the sensitivity for HCC < 2 cm in diameter was 61%, for HCC of 1-2 cm it ranged from 53.3% to 76%[3,53,58,59,61], and for HCC <1 cm in diameter it ranged from 10% to 57%[3,53-56,58,59].

CT requires intravenous iodinated contrast material and exposes patients to ionizing radiation. Although induction of renal failure by contrast is low, patients with liver cirrhosis frequently have renal dysfunction limiting its use[63], and the efficacy of N-acetylcysteine for preventing contrast induced-nephrotoxicity is not substantiated[64].

MRI
The application of MRI for liver imaging continues to expand with the recent progress of rapid, high-quality scanning techniques and the development of new tissue-specific contrast agents. Although initially used to complement CT in selective applications, MRI now plays an important primary role (after US) for the detection and characterization of liver tumors[65]. Extracellular intravenous contrast agents and novel tissue specific contrast agents are used to assess patterns of enhancement. Mostly, gadolinium-chelates are used for MRI, but hepatocyte-targeted and reticolo-endothelial system-targeted compounds are also used. Many studies have been published on the sensitivity and specificity of MRI imaging for diagnosis of HCC[3,42,54,55,57,58,60,66-72]. Among studies with liver explant correlation, the sensitivity, specificity and positive predictive value ranged from 33%

to 83%, from 57.1% to 100% and from 42.3% to 100% respectively[3,54,55,57,58,60,66,67,69-72]. Studies which evaluated the sensitivity of MRI in detecting HCC, using only liver biopsy or clinical and radiological findings as gold standard, have shown an overall sensitivity, specificity and positive predictive value of 77.3% to 94.3%, 96.6% and of 92.6% to 97% respectively[42,56,68].

For MRI, the lesion-based sensitivity stratified for tumor size was 55.6% for < 2 cm in diameter; for HCC 1-2 cm in diameter it ranged from 52% to 89%[3,58,69,70], and for HCC <1cm in diameter it ranged from 4% to 88.2%[3,54-56,58,66,69,70,72]. Super paramagnetic iron oxide particles used alone[56] or combined with gadolinium-based contrast agents[66,2] are highly sensitive for the diagnosis of small HCC. Ward *et al*[72] found a sensitivity of 91% for HCC $\geq$ 1 cm and of 46% for HCC $\leq$ 1 cm with double-contrast MRI. Another study [66] showed a sensitivity of 92% for HCC between 1 and 2 cm and 38% for HCC $\leq$ 1 cm. The sensitivity of MRA was shown to be superior to CT, but it also decreased with size of tumor (84% for nodules between 1 and 2 cm; 32% for nodules < 1 cm) [3]. In contrast when gadolinium-enhanced multiphase dynamic MRI was compared with MDCT scanning, for detecting small HCC, the sensitivity of detection for HCC <1 cm was higher with MDCT than with MRI (90%-95% *vs* 70%-85%)[73].

Whether MRI or CT should be the first imaging technique to characterise a nodule after ultrasound depends on the availability and characteristics of either technology in any one centre. Comparison of these is complicated by comparison of different generation machines[74] and different types of tumor. In general modern MRI appears more sensitive for the diagnosis of smaller nodules < 1.5 cm in a cirrhotic liver and in distinguishing regenerative/dysplastic nodules *versus* malignant ones. The importance of the experience of the reporting radiologist should not be underestimated here. Moreover every diagnostic imaging tool has some specific advantage and combination of more than one imaging technique, for difficult focal liver lesions, often increases diagnostic yield.

DIAGNOSIS-LIVER LESION BIOPSY AND TISSUE MARKERS

Many variables affect the accuracy of pathological diagnosis, with sampling being the most important. Many studies evaluating and/or validating immunostaining techniques, have used specimens obtained from resected or explanted livers. However, in clinical practice it is percutaneous biopsy specimens that are available. Biopsy specimens often are small, and represent a fraction of the tumor, so that a lack of immunostaining may simply be the result of inadequate sampling. Using standardized panels with more than one immunostaining method, may result in more confident histological diagnosis of HCC, with a better reproducibility and accuracy, but this needs to be evaluated formally using biopsy specimens.

The role of liver biopsy, in diagnosis of HCC in patients with cirrhosis, has declined during the past few years because better radiological techniques have enormously improved the accuracy of diagnosis. However, when the imaging characteristics are not typical together with AFP < 200 ng/mL, the diagnosis is not reliable without a biopsy sample, as recommended in the joint EASL/AASLD/JSH guidelines[21].

Even though the specificity and positive predictive value of nodule biopsy is high, its negative predictive value is low. Considering that there is a 10% risk of false negativity, the presence of HCC cannot be excluded when a biopsy is negative for HCC. Moreover, it has been suggested that a second biopsy performed immediately after the first one has a limited chance of success, with a gain in diagnosis of HCC of only approximately 5%[44,48,75]. In patients with HCC < 2 cm in whom the first nodule biopsy is negative, repeated imaging is preferred to performing a second biopsy. Whether the risk of false-negative findings is far higher in patients with small nodules (< 2 cm in size), compared with larger nodules, has not been yet clearly demonstrated. However, this is likely, as optimal placement of the needle in smaller nodules is more difficult. Indeed a study of the technical aspects of biopsying nodules $\leq$ 1 cm has not been published. However, independent of the size of nodules, the risk of false-negative is higher for those located on the posterior and superior segments of the liver[76].

Percutaneous biopsy of HCC carries a potential risk of tumor seeding along the needle tract. The median risk of seeding is 1%-2%, and is higher if performed alone, compared to combining biopsy with percutaneous ablative techniques[77,78]. Seeding can become manifest after liver transplantation[77,78]. However, if biopsy is necessary, with an indeterminate lesion, it should be combined with percutaneous ablation[77,78]. Another technical variant that may help prevent seeding is the use of a coaxial 17-gauge introducer needle before a 18-gauge biopsy needle, to isolate liver parenchyma during the run of biopsy needle[79]. This should be subject to a comparative study.

Ultrasound-guided fine-needle aspiration (FNA) cytology has been used as an alternative to biopsy because samples can be obtained with smaller needles (23 gauge). Although the specificity and positive predictive value of FNA examination for focal liver lesions is reasonable, the sensitivity ranges between 67% and 93% and thus diagnostic accuracy is less than for histology[77], and risk of seeding is not reduced. Fine-needle aspiration cytology is not recommended for diagnosis.

Histologically diagnosing borderline lesions represents the "Gordian knot" in HCC diagnostics. The three criteria listed from the International Working Party to differentiate HCC from an high grade dysplastic nodule and well-differentiated HCC are: cellular density more than twice normal, irregular nuclear contour of lesion cells and invasion of stroma or portal tract[80]. Tissue

Table 4 Immunohistochemistry for HCC

Marker	Staining pattern	Diagnostic use	Diagnostic value	Ref.
AFP	Specific for HCC Cytoplasm	Expressed in HCC cells cytoplasm but also in: fetal liver, hepatoid tumors, germ cells tumors	Sensitivity 17%-68% Specificity 97% For HCC	[89-104]
GP-3	Specific for HCC Cytoplasm	Expressed in HCC cell cytoplasm (less so if fibrolamellar or sarcomatoid variants) but also in: fetal liver, hepatoblastoma, melanoma	Sensitivity 49%-91% Specificity 89%-100% For HCC	[81,109-115]
CD-34	Endothelium	Surface of normal endothelium and HCC trabeculae or acini but also in: myelodysplasia in transformation, GI stromal tumors (high coexpression with bcl-2)	HCC positivity 82%	[117]
p-CEA	Biliary canalicula	Identifies biliary glycoprotein 1 on hepatocyte canalicular pole and cholangiocyte. Useful for differential diagnosis *vs* cholangiocarcinoma, other adenocarcinoma	HCC positivity 24%-90%	[94,98,100,118-128]
CD-10	Biliary canalicula	Surface of biliary tract cells and in HCC, but also positive in: B cell lymphomas, renal cells carcinoma, melanoma, prostate and pancreas adenocarcinoma. Useful for differential diagnosis *vs* cholangiocarcinoma, other adenocarcinomas	HCC positivity 28%-86%	[94,98,100,119,120,122-124,126,128]
Ki67 HepPar1	Cell proliferation marker HCC & normal Hepatocyte citoplasm	Assessing cell proliferation rate, correlates with tumor grade and clinical course. Useful to differentiate between HCC and hepatic adenoma	HCC positivity rate 10%-50%	[129]
		Expressed in HCC and in normal liver cells, but also in hepatoblastoma. useful for differential diagnosis *vs* cholangiocarcinoma and metastases	HCC positivity 66%-100%	[130-136]
Cytokeratins	Epithelial cells	Useful for differential diagnosis *vs* cholangiocarcinoma. HCC profile: CK7/CK19/CK8/CK18 = - / - / + / +	HCC positivity 76%-96%	[97,101,104,137-140]

markers of HCC might provide a more standardized diagnosis especially for early/well-differentiated HCC and may give information about the probable phenotypic behaviour and thus guide therapy.

Tissue markers

Studies using genome-wide DNA microarray or quantitative real time reverse transcriptase polymerase chain reactions have been done to identify tissue markers of early HCC, in particular to distinguish HCC from dysplastic nodules: Glypican-3 (GPC-3)[81], TGF-b1[82,83], heat shock proteins (HSP) HSP-70 and HSP-27 are the most studied as they inhibit apoptosis[84].

Proteomic studies on liver tissue have traditionally utilized a combination of two-dimensional gel electrophoresis and mass spectrometry analysis[85]. Zeindl-Eberhart *et al*[86] demonstrated the immunoreactivity of aldose reductase-like protein variants (h-ARLP) in cells of HCC and their absence or low signal in normal and cirrhotic livers, fibrolamellar carcinoma or hepatic adenomas. In a more recent study Li *et al*[87] evaluated patients with HCC in HBV-related liver cirrhosis and found 80 proteins with differential expression in HCC. Among these, only two proteins (proliferating cell antigen and stathmin 1) were confirmed by Western blotting analysis.

A 3-gene set (GP3, LYVE1 and survivin) has been proposed as molecular diagnosis of early HCC with accuracy rates of 85%-95% in training and validation sets of more than 70 samples[88].

Current markers available for routine immunochemistry, show different ranges of sensitivity, and few of these are specific to HCC (as are AFP and GP-3).

Although there are always false positives and negatives, other markers can be useful for differential diagnosis of HCC *versus* benign nodule or non-HCC cancers, if the specific HCC markers are negative and/or the experience of the pathologist suggests their use. Trying to classify these immunostaining patterns which are useful to build an HCC diagnostic panel, there are five groups of markers (Table 4): 1. specific HCC products, including, AFP and GP-3; 2. vascular pattern markers, such as CD34; 3. canalicular pattern markers, including polyclonal carcinoembryonic antigen (p-CEA) and CD10; 4. markers of cell proliferation such as Ki67; 5. other hepatocellular products as HepPar1 and cytokeratins.

AFP

AFP is a fetal-specific glycoprotein with a molecular weight of 70 kDa, whose synthesis declines rapidly after birth. The immunohistochemical use of AFP staining has very low sensitivity as it detects just 17%-68% of malignant hepatocellular lesions when used alone[89-103]. In a study by Murakata *et al*[104] there was no staining of clear-cell HCC. However, the specificity of AFP is high with an average value of 97%[89,92,93,95,96,99,101,104].

GP-3

GP-3 is a member of the heparan sulfate proteoglycan family, linked to the cell surface through glycosylphosphatidyl inositol, which may also be found in a secreted form. In patients with HCC, GP-3 is over expressed in neoplastic liver tissue and elevated in the serum but is undetectable in normal liver. Melanoma[105], testicular germ cell tumors[106], Wilms tumor, hepatoblastoma[107], among non-HCC malignant neoplasms, and focal

nodular hyperplasia, regenerative and dysplastic cirrhotic nodules, among liver benign and pre-malignant lesions, may be positive for GPC-3[108-110].

Immunostaining for GP-3 can be focal and so it not surprising that there is a lower sensitivity in studies using needle core biopsy[111,112] and FNA[113]. Wang *et al*[109] using 1-mm tissue microarray also demonstrated a sub-optimal sensitivity of GP-3 (70%). In a recent study using a fine-needle aspiration specimen GP-3 had a sensitivity of only 56.8%. The author suggested that the HCC specimen fixation in alcohol rather than in formalin reduced the positivity rate for GP-3[113], although this has not been confirmed. In the literature the GPC-3 sensitivity and specificity ranges from 49% to 90.5% and from 88.5% to 100% respectively[81,109-115]. Dysplastic nodules show a weak positivity of 0%-25% when low grade[109,110,114] and of 20%-75% when high grade[109,110,114].

CD34

CD34 is a 110 kDa transmembrane glycoprotein founded on normal endothelium. It is absent in normal liver sinusoids. The immunostain highlights regions of sinusoids, both in the vicinity of portal tracts in normal liver, and in areas of capillarization such as occur in the periphery of cirrhotic regenerative nodules[116]. In HCC samples an encircling pattern showing the endothelial cells investing the trabeculae and acini or a fine sinusoidal pattern, may be present in 82.5% of cases[117]. Overall specificity of this immunostaining is poor. However, the gradual increase in CD34 expression in HCC, reflecting the progressive arterialization due to hepatocarcinogenesis, eventually results in a complete CD34 immunostaining pattern, so that specificity becomes acceptable[115].

p-CEA

Polyclonal antibodies against carcinoembryonic antigen cross-react with biliary glycoprotein 1 expressed on cholangiocyte surfaces and on hepatocyte canalicular poles. In HCC, biliary glycoprotein 1 co-localisation may show a typical canalicular pattern. This is useful immunostaining for the differential diagnosis of HCC when it is a sclerosing or acinar variant (versus cholangiocarcinoma) or where there is pseudoglandular formation and/or clear cell changes in HCC (usually poorly differentiated HCC which is useful *versus* the differential of epithelial metastases). Immunostaining for p-CEA is reported positive in 24%-90% of HCC[94,98,100,118-128].

CD10

The diagnostic usefulness of CD10 immunostaining is analogous of that of p-CEA, with a lower sensitivity (28%-86%)[94,98,100,119,120,122-124,126,128], but with a specificity of 100%[119,122,123,126,128].

Ki67

Ki67 is a monoclonal antibody which reacts with nuclear proteins expressed in the G1, G2, M and S phases of cell cycle[129]. Evaluating the rate of proliferation is particularly useful for evaluating HCC grade and in differentiating between liver cell adenoma and HCC.

HepPar1

HepPar1 is an antibody which, on paraffin-embedded tissue, links mitochondrial antigens from both malignant and non-malignant hepatic cells, giving a granular cytoplasmic pattern on immunostaining. Is an excellent marker for HCC diagnosis, distinguishing between HCC and liver metastasis or cholangiocarcinoma. It has a similar accuracy to core needle biopsy and in FNA sampling, with a sensitivity of 66%-100% and a specificity of 70.8%-100%[130-136].

Cytokeratins

A subclass of the intermediate filaments family, the cytokeratins (CKs) are expressed in human liver with a characteristic distribution. CK8 and CK18 stain hepatocytes, CK8, CK7, CK18, and CK19 are present in bile duct cells. CK7 and CK19 are not usually present in HCC (76%-96% immunonegative)[97,101,104,137-140], but they exist in many adenocarcinomas, including, cholangiocarcinoma.

DIAGNOSIS-SERUM MOLECULAR MARKERS

Serum tumor markers have several potential uses: for early diagnosis of HCC in high risk patients, in determining prognosis, to estimate tumor volume as well as to monitor therapeutic response and detect recurrence[141].

AFP

The increase of serum AFP may relate to hepatocarcinogenesis but also to hepatic regeneration in chronic liver disease, and may also occur in embryonic carcinomas, gastric and lung cancers[142-147]. Moreover the positive predictive value for the diagnosis of HCC varies with the cut-off value used, ethnicity of the patients, treatment and tumor stage. An AFP with a cut off value of 20 ng/mL has a sensitivity and specificity for HCC diagnosis of 41%-65% and 80%-94% respectively[146]. About 42% of HCC are diagnosed in the absence of a raised AFP[29]. However, AFP confirms the diagnosis of HCC in cirrhosis when the value is over 200 ng/mL when the suspected nodule is larger than 2 cm[21]. A value of $\geq$ 400 ng/mL is often associated with the large volume of the tumor mass and/or the carcinomatous involvement of the portal vein, and with a poor median survival rate[145]. In a recent study about 61% of patients with AFP level of > 1000 ng/mL presented with vascular invasion[148].

Alpha-Fetoprotein-L3 (AFP-L3)

Studies on a fucosylated variant of the AFP glycoprotein

which has a high affinity to the sugar chain of Lens culinaris showed increased activities in patients with chronic liver disease and HCC. However, sensitivity and specificity range from 36.5% to 71% and from 63% to 91.6%, respectively[149,150]. When compared with AFP, AFP-L3 showed a higher specificity but similar sensitivity. The disease specificity of this marker is limited as non-tumoral, extrahepatic disease (diabetes, pancreatitis and hypothyroidism) are associated with increased serum levels.

Des-g-carboxy prothrombin (DCP)

DCP also known as a protein induced by the absence of vitamin K or antagonist Ⅱ (PIVKA-Ⅱ) was reported firstly by Liebman *et al*[151] as a candidate biomarker for the diagnosis of HCC. Recent case-control studies documented a sensitivity and specificity ranging from 58% to 89% and 93% to 97% respectively[152-154], and one of these studies found that DCP had a higher sensitivity in the diagnosis of small HCC when compared to AFP and AFP-L3[154].

GPC-3

GPC-3 has been mainly evaluated at the tissue level, although some studies report that GPC-3 can be found in the serum in about 55% of patients with HCC. On the other hand GPC-3 is not detectable in healthy subjects and in patients with liver cirrhosis without HCC[114]. The sensitivity and specificity of serum GPC-3 in diagnosing HCC was reported to be 51% and 90% respectively[155].

GP73

GP73, a Golgi glycoprotein is overexpressed in viral-related HCC[156,157]. Total circulating GP73 can be positive in AFP negative cancer[158].

Other studies have evaluated hepatocyte growth factor[85,159], insulin growth factor[160,161], vascular endothelial growth factor[162] (which correlates with venous invasion), transforming growth factor TGFb1[163-165], IL-6 and IL-10[166,167], hepatoma-specific g-glutamyl-transferase GGTII[168], human cervical cancer oncogene (HCCR)[169] (HCC < 2 cm the positive predictive rate in HCC was 69.2%) and tumor-derived autoantibodies (TAA)[170,171].

Although numerous biomarkers with potential diagnostic or prognostic significance for HCC have been identified there are currently only two FDA-approved tumor markers (AFP and AFP-L3). It is likely that panels of 2 or 3 serum and tissue tumor markers will be used in routine clinical practice in the near future. A standardized approach is required to assess panels of tumor markers and validation is needed in large patient cohorts preferably from multiple centres. In the future, new markers may help not only to diagnose indeterminate lesions based on today's criteria but also to distinguish between HCC with worse or better prognosis.

Diagnosis of HCC lesions in patients with cirrhosis requires a multidisciplinary approach. It follows that specialized centres in which patients may find skilled opera-

tors and the most recent diagnostic tools are necessary to optimise diagnosis, particularly with smaller lesions.

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