EDITOR'S PICK

In this next article, Luca Quagliata of the Institute of Pathology, University Hospital of Basel, Basel, Switzerland, discusses the integration of the histological, clinical, and molecular classifications of hepatocellular carcinoma. This is a field of ongoing research without any final conclusions, and thus the data is difficult to interpret. This article not only provides a useful overview of these differing systems but also addresses the difficulty of applying them in a dynamic combination, shedding light on an important avenue for future research.

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CLINICAL, HISTOLOGICAL, AND MOLECULAR CLASSIFICATION OF HEPATOCELLULAR CARCINOMA: HOW DO THEY GET ALONG?

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ABSTRACT

Hepatocellular carcinoma (HCC) will soon become a prominent part of the medical and economic burden on many Western countries' healthcare systems. This review will discuss some emerging scenarios concerning the different classifications of HCC from the clinical, histological, and molecular perspective and to what extent they are integrated with each other. Beginning with an overview of the current numbers and facts regarding HCC, it touches upon the latest development of the epidemiological scenario. It is noteworthy that besides viral hepatitis infection, the fast growing rate of individuals affected by metabolic syndromes represents an additional influential factor on the rising incidence of HCC. However, despite recognised epidemiological evidence, too little is known about the molecular mechanisms that favour HCC development and progression. For instance, long non-coding RNAs playing a major role in the HCC carcinogenesis process have only recently been recognised. Although high cure rates are achieved for clinically asymptomatic patients when small tumours are detected, HCC is typically silent with few severe symptoms until its advanced stages. Patients with severe clinical signs are seldom good candidates for any type of curative therapy. Microscopically, HCC cells resemble normal liver cells to a variable degree, depending on the tumour differentiation status. Pathologists often use a panel of markers to assist HCC differential diagnosis. From a molecular perspective, HCC presents as a highly heterogeneous tumour entity. Despite considerable research efforts, to date no molecular classification has been introduced in clinical practice. A number of classifications have been suggested to stratify HCC patients by the likelihood of survival, with the aim of identifying those with the best chance of being successfully treated. These different systems do not seem to work well in conjunction and the various involved disciplines have so far failed to achieve their common goal. Co-ordinated initiatives involving clinicians, pathologists, biologists, and bioinformaticians are needed to achieve a comprehensive classification of HCC.

Keywords: Hepatocellular carcinoma (HCC), molecular classification, sorafenib, long non-coding RNA (lncRNA).

HEPATOCELLULAR CARCINOMA: AN OVERVIEW OF THE NUMBERS AND FACTS

Unlike most malignancies, mortality from liver cancer has increased significantly over the past 20 years.[1,2] Hepatocellular carcinoma (HCC) accounts for up to 85% of liver tumours.[3] HCC has one of the widest variations in incidence in different parts of the world amongst all tumour types.[4-6] In fact, while HCC is the fifth most common cancer in men and the seventh in women worldwide, it represents the most common cause of death from cancer in East Asia and Sub-Saharan Africa, which have the greatest number of cases.[6,7] HCC incidence in the USA and Europe has doubled over the past two decades.[8] Furthermore, epidemiological evidence anticipates that in Western populations the HCC burden will continuously increase over the next 20 years.[3] This is mostly due to the mounting number of patients with advanced hepatitis C virus (HCV) and/or non-alcoholic steatohepatitis.[9] Although it is clear that HCC will soon become a prominent part of the medical and economic burden on the healthcare systems of Western countries, too little is currently being done to efficiently stem this alarming phenomenon.[1,10,11] Conversely, the incidence of HCC has substantially decreased in other areas, such as China and Hong Kong.[12] This phenomenon can be explained by looking at the epidemiological fluctuation in risk factor exposure, such as the decline of the hepatitis B virus (HBV) infection rate due to vaccination, and a marked reduction of exposure to aflatoxins from grains as a result of the introduction of improved hygiene standards. Aflatoxins are a family of mycotoxins produced by fungi of the *Aspergillus* genus known to be powerful experimental carcinogens. Aflatoxin B contamination of food, predominantly grains and peanuts, is most common in China and Southern Africa.

Nowadays, it is estimated that globally up to 80% of HCC is associated with HBV or HCV infection.[6,8] The risk of developing HCC is increased 5 to 15-fold in chronic HBV carriers and up to 17-fold in HCV infected patients.[3] Though the viruses display a similar tropism, they are different, being implicated in the alteration of distinctly different molecular pathways, and the precise mechanisms by which they can cause HCC onset are not yet fully defined. However, many new lines of evidence now suggest that the pathogenesis of HCC is immune-mediated, as an indirect result of the cycle of inflammation-necrosis-regeneration that is typical of chronic hepatitis.[13]

Metabolic syndromes represent a growing issue influencing HCC incidence. Nearly 30% of new cases of HCC in the USA have no identified aetiological agent and recent data seems to point to non-alcoholic fatty liver disease related cirrhosis as the main predisposing factor.[9] In Western populations, heavy alcohol consumption and alcohol related liver disease is the second most common HCC risk factor after HCV infection.[3] Finally, hereditary diseases also contribute to the HCC burden, with hereditary haemochromatosis, tyrosinaemia, and α1-antitrypsin deficiency playing a major role.[14]

Irrespective of geographical location, HCC occurs more frequently in men than women; it is also worth noting that age at diagnosis can be significantly different depending on the geographical area.[6]

In conclusion, despite the well-known risk factors briefly described above, such as gender, age, viral infection, alcohol intake, diabetes, obesity, ethnicity, and portal hypertension,[2,4,7,15] there is little known about the mechanisms that favour HCC development and progression.[4]

CLINICAL FACTS AND CLASSIFICATION: COMPREHENSIVE OR LIMITED?

HCC is typically a silent disease with few clinically severe symptoms until its advanced stages.[16] Upper abdominal pain along with weight loss and hepatomegaly, with signs of decompensated liver disease like jaundice or ascites, are very common at presentation.[3] Given that most patients live with underlying liver cirrhosis, complications such as hepatic decompensation in the form of accumulating ascites, hepatic encephalopathy, or obstructive jaundice occur at a significant rate.[3]

Radiographic imaging is still the most frequently used approach to evaluate patients with a suspected severe liver tumour.[17] Once symptomatic, HCC is easily detectable by ultrasound, computed tomography (CT) scan, magnetic resonance imaging (MRI), or angiography. Ultrasonography-based investigation for surveillance/detection of early stage tumours has become the method of choice, easily detecting nodules with diameters of 2–3 cm in size.[17] HCC nodules are exclusively supported by an arterial blood supply, typically

present as a hypervascular lesion with washout in the venous phase. A number of widely used contrast agents can be employed in combination with ultrasound, CT, or MRI to depict blood flow in lesions <1 cm.[17] According to the guidelines of the European Association for the Study of the Liver (EASL),[2] and those of the American Association for the Study of Liver Disease (AASLD), the detection of a typical vascular profile with at least one dynamic imaging technique is already sufficient for a diagnosis of HCC for lesions >2 cm in size.[2] Thus, no pathological examination of tissue for these cases is required. Conversely, for lesions that range between 1 and 2 cm, a minimum of two dynamic imaging techniques are necessary, and there is a possibility of having to perform a biopsy for radiologically atypical lesions.[2]

In the early stages of HCC, the majority of tumour nodules appear to grow within an encapsulated mass. Once enlarged, they tend to infiltrate, damage, and destroy the adjacent tissue by substantially replacing the normal parenchyma of the liver and generating a number of characteristic satellite nodules.[18] Microvascular invasion and/or intrahepatic metastases are very common events observed in up to 60% of tumours <5 cm in diameter and >95% for those >5 cm.[16] Thrombosis of the portal vein along with its branches appears in 65–75% of advanced tumours, and in the hepatic veins in about 20–25% of cases. Invasion of the large bile ducts with obstructive jaundice can also occur, but it is a comparatively rare event (~5% of cases).[3] Although metastases are common in advanced HCC, with almost half of patients having at least regional lymph node and lung involvement, most patients eventually die as a result of liver failure. In advanced stages of the disease, HCC patients have a median survival time of 1–3 months. Although longer, survival expectations in patients with earlier stages who are not eligible for resection are still extremely low: ~20% at 1 year and ~8% at 2 years.[19] These numbers demonstrate the harsh reality of HCC.

The tumour, nodes, metastases (TNM) system, laid out by the International Union against Cancer (UICC), is available for HCC. Often however, different staging systems are combined to more accurately define HCC status, for example by integrating features of the underlying liver disease, the functional state of the liver, and the size of the tumour mass. Studies comparing different staging systems suggest that the Barcelona Clinic Liver Cancer Classification (BCLC),[20] which incorporates tumour extent, liver function, and overall patient performance status, represents the best option to identify treatable patients as well as to predict survival.[20]

HCC management has advanced considerably in the past 20 years.[16] High cure rates are achieved for patients with small tumours that are detected whilst clinically asymptomatic;[21,22] however, most patients only present in the advanced stages of the disease. HCC patients with severe clinical signs are rarely good candidates for any type of curative therapy.[16] Some improvements in the direction of palliative therapy have recently been achieved, though most efforts have been correctly pointed towards surveillance and early diagnosis in high-risk populations. Current BCLC recommendations for HCC therapy suggest that Stage 0 and A (very early and early HCC, respectively) patients should receive curative treatment; surgical resection is optimal for Stage 0 and A1, and transplantation or ablation for Stage A2, A3, or A4. Surgical resection is mostly effective for small tumours in patients with no underlying liver disease. For patients with multiple small tumours and compensated cirrhosis, liver transplantation is the best option for curing the underlying liver disease as well as the tumour.[23] Patients selected according to the Milan criteria (i.e. solitary HCC ≤5 cm or up to three tumours each ≤3 cm) have a 1-year survival of 81% and 5-year survival of 51%.[24] Transplantation is clearly not always available, thus percutaneous tumour ablation (ethanol or radiofrequency) has become the most widely used treatment for early but unresectable tumours.[25] At the same time, since HCC receives its blood supply from the hepatic artery rather than the portal vein, angiographic embolisation of the artery has been used to produce tumour necrosis and prolong survival. Conversely, palliative therapy is recommended for Stage B and C (intermediate or advanced HCC, respectively), with chemoembolisation for Stage B and sorafenib for Stage C. Sorafenib is a multiple tyrosine kinase inhibitor that effectively blocks several receptors' activity, such as vascular endothelial growth factor receptor, platelet-derived growth factor receptor, and the RAF serine/threonine kinases along the RAF/MEK/ERK pathway.[26] Nevertheless, sorafenib has shown a consistent but limited survival benefit in HCC (10–12 weeks increased survival) accompanied by a number of moderate-to-severe

side effects.[26,27] Finally, patients with Stage D (end-stage HCC) receive symptomatic treatment. This makes HCC somewhat unique among cancers, having no standard cytotoxic therapy.[16] Overall, it is imperative to identify new therapeutic targets as well as biomarkers to predict response to therapy.

HISTOLOGICAL CLASSIFICATION: THE OLD, THE NEW, AND THE UNKNOWN

Microscopically, HCC cells resemble normal liver cells to a variable degree, mostly depending on the tumour differentiation status.[18] Nuclei are often clear and prominent, with a concomitant high nuclear–cytoplasmic ratio. Commonly, light hyperchromatism and nuclear irregularity are observed. HCC cells typically have distinct cell membranes and a modest amount of eosinophilic, finely granular cytoplasm.[18] In the earliest stages, HCC-transformed cells simply grow within pre-existing liver cell plates.[18,28] In this case, they retain the reticulin framework, and not infrequently preserve the portal tracts.[18] Nonetheless, the cells of such well-differentiated tumours have high nuclear–cytoplasmic ratios, generating the typical nuclear crowding appearance. By further proliferating, these tumours produce abnormal structural patterns with thin trabeculae and/or pseudoglands.[29]

In 1954, Edmondson and Steiner suggested grading HCC on a scale from I–IV, having increasing nuclear irregularity, hyperchromatism, and nuclear–cytoplasmic ratio, accompanied by diminished differentiation status.[30] This system holds substantial importance to both the amount and appearance of the cytoplasm, and the nuclear–cytoplasmic ratio. Thus, Grade 4 tumours have very scant cytoplasm even if the nuclei might be minimally anaplastic. The correlation between the Edmondson–Steiner grade and HCC prognosis is still disputed, but generally tumour grade is a weak independent predictor of the clinical course, providing very little prognostic information.[18] Finally, the Edmondson–Steiner grading system is highly subjective and relies heavily on the pathologist's expertise. A univocal system to comprehensively grade HCC has not yet been established.

Several markers are currently used to assist the differential diagnosis of HCC. Glycoprotein I is the most useful and is frequently employed in distinguishing HCC from other malignancies.[31] Hepatocyte paraffin 1 stains for urea cycle enzyme carbamoyl phosphate synthetase 1 in liver mitochondria, which is positive in about 90% of all HCC cases, showing a typical granular pattern in most liver specimens (however it is not specific to the hepatocytes). Additional markers that are useful in the diagnosis of HCC include heat shock protein 70, the glutamine synthetase, annexin A2, and arginase-1.[18,31] None of these markers alone are sufficient for a definitive diagnosis and a combination of multiple markers is normally the best approach.

MOLECULAR CLASSIFICATION: MUCH EFFORT, LITTLE HELP, BUT HIGH HOPES

From a molecular point of view, HCC presents as a highly heterogeneous tumour entity.[32] This is not entirely surprising due to the wide range of its aetiologically associated factors.[7,33] Several molecular approaches such as coding-gene expression profiling (either by microarray or massive RNA sequencing), along with deep DNA sequencing analysis and array comparative genomic hybridisation (CGH) have identified the key alterations favouring the onset of HCC.[32]

A number of genomic rearrangements are consistently observed in HCC samples independent of their aetiology. For example, the amplification of the chromosome 6p21 (also containing the *VEGFA* gene) is observed in approximately 6–8% of all HCC cases.[34] Interestingly, patients with *VEGFA*-amplified HCCs show an improved survival compared with non-amplified cases under sorafenib treatment.[35] A meta-analysis of several sets of independently performed CGH experiments, including a total of 169 HCC samples, highlighted that overall, chromosomal gains are most abundant in numerous specific large (i.e. 1q, 6p, 8q, 17q, and 10q) and two narrow (5p15.33 and 9q34.2–34.3) genomic regions, while as many as 88 significant losses are repeatedly present in the 4q, 6q, 8p, 9p, 13q, 14q, 16q, and 17p18 regions.[36] Performing whole exome and/or genome sequencing, several studies have attempted to combine copy number variation with single nucleotide variation data and/or gene expressing profile, with the aim of establishing a molecular classification of HCCs.[15,33,37-39] Such classifications effectively enable scientists to group HCCs on the basis of specific dysregulation of a limited set of molecular pathways. A recent HCC classification has established a molecular signature based on the combined evaluation of as few as

five genes including *HN1, RAN, RAMP3, KRT19*, and *TAF9*. This 5-gene signature can independently define any other clinical and pathological tumour features to predict HCC patients' outcome when treated by surgical resection.[40] Additionally, a recent meta-analysis of HCC data, comprising several hundred HCC tumours, identified two main subclasses: S1-S2 and S3.[41] The S1-S2 subgroup is characterised by: more aggressive HCCs presenting with severe genetic instability; the impairment of tumour suppressor *TP53*; the activation of pro-survival signals controlled by *E2F1* and *MET* pathways; *KRT19* positivity; a high rate of cellular proliferation; a larger tumour mass; low differentiation status; higher incidence of tumour recurrence, and a poorer prognosis overall.[41] Further subclassification highlights that the S1 group shows the activation of the transforming growth factor β pathway while the S2 group shows positivity for stemness markers, such as *EpCAM, AFP*, and *GPC3*, in addition to the insulin-like growth factor 2 pathway activation. Those patients in the S3 class are characterised by less aggressive features, such as recurrent somatic mutations in exon 3 of *CTNNB1* along with the expression of specific genes, such as *GLUL, LGR5*, and *SLC1A2*, but little alteration of canonical WNT pathway genes. They also have smaller and more differentiated tumours. All of these features partially preserve the normal hepatocyte function in S3 patients; this results in an overall better prognosis.[41]

It should be noted that all of the previously cited HCC molecular classifications were established using resected tumours, introducing a selection bias towards patients who have no liver cirrhosis and are at an early stage of the disease. A recent study set out to develop a molecular classification system using liver biopsy instead of resection specimens, thus removing the biases associated with a given stage of HCC (Makowska et al., accepted). This study has challenged all previously reported data, suggesting that clear-cut differences in HCC might be missed by merely looking at the gene expression profile or mutation spectrum.

In conclusion, despite considerable research efforts, to date no molecular classification has been introduced in clinical practice, and currently all HCC cases are treated according to their stage rather than their molecular subtype.

LONG NON-CODING RNAS: THE DARK SIDE OF THE LIVER

The unprecedented fast progress of deep sequencing technology, along with the improvement of bioinformatics tools to conduct complex whole genome data analysis,[42] has revealed that while >70% of the human genome is transcribed into RNA,[43] only as little as 2–5% of the RNA produced is eventually translated into proteins.[44] The next challenge is to unravel the biological functions of the vast amount of non-coding RNA (ncRNA) transcripts and to define their impact on cell physiology.[44]

ncRNAs are broadly grouped into two major classes: 1) transcripts shorter than 200 nucleotides, namely small ncRNAs, mainly including Piwi-interacting RNAs, small interfering RNAs, and microRNAs; and 2) long non-coding RNAs (lncRNAs) ranging in length from 200 nt to ~100,000 kb, with an mRNA-like transcript structure and a very low conservation rate across species. Nevertheless, the definition of ncRNAs, far from being complete, remains a topic of debate as our understanding of their functions grows.[45-47] For most of the predicted lncRNAs the potential functions and mechanisms of action are still undetermined; one significant discovery is that most lncRNAs show a tissue-specific pattern of expression.[47]

One major contribution, aiming to ameliorate the current molecular classification of HCC, may occur with the integration of lncRNA expression profiles with existing data sets. Lately, a growing critical mass of researchers have started to focus their activities on the implications of lncRNA alterations in pathophysiology.[48,49] To date, a number of lncRNAs have been proven to be associated with HCC disease development and progression.[42,43,50] Plasma HULC (Highly Upregulated in Liver Cancer) was one of the first lncRNAs to be examined in HCC, isincreased in a consistent proportion of HCC plasma[51] and tissue samples, and is associated with histological grade and HBV infection.[52] Such findings envision the use of lncRNAs as non-invasive novel diagnostic and/or prognostic biomarkers, which may also allow monitoring of disease progression. Other compelling examples include metastasis-associated lung adenocarcinoma transcript 1, reported to be associated with metastasis formation and HCC recurrence.[53] The HOXA transcript at the distal tip was found to be

highly upregulated in HCC and was able to predict both disease progression and patient outcome.[54]

Defining ncRNA functions, expression patterns, and regulatory mechanisms will be critical to fully appreciate their biological relevance in controlling liver functionality and pathological conditions, such as HCC.

COMPREHENSIVE CLASSIFICATION OF HEPATOCELLULAR CARCINOMA: A MAJOR UNMET NEED

A number of staging systems have been recommended to stratify HCC patients by the likelihood of survival and to identify those with the best chance of being successfully treated. This review has attempted to assess different perspectives on defining some of the important characteristics of HCC. Far from being exhaustive, the aim of this paper was to underline that despite the efforts of numerous researchers in recent years with relation to the definition of HCC pathophysiological features, the different disciplines involved have so far failed to achieve their common goal. This is due, on one hand, to the high complexity of the studied subject, but also to the lack of co-ordinated initiatives involving clinicians, pathologists, biologists, and bioinformaticians simultaneously. Such co-operative attitudes have only very recently emerged[16] and will represent a fundamental step in achieving a comprehensive classification of HCC. One might think of a common data-sharing platform for the HCC research community where all relevant information could be readily available for researchers to use. Often such platforms are only curated for one specific aspect (e.g. molecular features, such as DNA sequencing data) and are poorly annotated for the others (clinical and histological).

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