Review

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Molecular and histological correlations in liver cancer

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Summary

Hepatocellular carcinoma (HCC) is a highly heterogeneous cancer, both at the molecular and histological level. High-throughput sequencing and gene expression profiling have identified distinct transcriptomic subclasses and numerous recurrent genetic alterations; several HCC subtypes characterised by histological features have also been identified. HCC phenotype appears to be closely related to particular gene mutations, tumour subgroups and/or oncogenic pathways. Non-proliferative tumours display a well-differentiated phenotype. Among this molecular subgroup, *CTNNB1*-mutated HCCs constitute a homogeneous subtype, exhibiting cholestasis and microtrabecular and pseudoglandular architectural patterns. Another non-proliferative subtype has a gene expression pattern similar to that of mature hepatocytes (G4) and displays a steatohepatitic phenotype. In contrast, proliferative HCCs are most often poorly differentiated, and notably include tumours with progenitor features. A novel morphological variant of proliferative HCC – designated "macrotrabecular-massive" – was recently shown to be associated with angiogenesis activation and poor prognosis. Altogether, these findings may help to translate our knowledge of HCC biology into clinical practice, resulting in improved precision medicine for patients with this highly aggressive malignancy. This manuscript reviews the most recent data in this exciting field, discussing future directions and challenges.

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Introduction

Liver cancer is the fifth most frequent cancer worldwide and the fourth highest cause of cancer-related death (https://gco.iarc.fr/). Hepatocellular carcinoma (HCC) is the most common primary malignant liver tumour, usually developing in the context of chronic liver disease, which is mainly associated with hepatitis B (HBV) or C (HCV) virus infection, alcohol intake or the metabolic syndrome.[1,2] Clinical outcomes remain poor, with only approximately one-third of patients eligible for curative treatments such as percutaneous ablation, surgical resection or liver transplantation.[2]

Unlike virtually all other human malignancies, HCC diagnosis does not, in most cases, rely on pathological examination. Indeed, particular features on computerised tomography or magnetic resonance imaging have been shown to display high sensitivity and specificity for HCC detection in cirrhotic patients, enabling clinicians to avoid using invasive biopsies.[3,4] However, pathology remains a cornerstone in the clinical care of patients with cancers, as it allows for a definitive diagnosis and provides prognostic information.

Distinct morphological phenotypes of HCC have recently been shown to be associated with the different genetic defects and biological pathways that drive tumour progression.[5–7] The establishment of a classification of HCC that integrates morphology and molecular alterations is thus of critical importance as it may allow us to i) better understand the natural history and the mechanisms of carcinogenesis, ii) improve diagnosis and prognostication, and finally iii) facilitate the development of personalised medicine by identifying tumour entities that may respond to specific targeted therapies. In the present review, we will provide an overview of our current knowledge of how HCC histology relates to its underlying biology, as well as discussing the role of a combined histological and molecular classification.

Pathology of HCC

In normal liver, hepatocytes are arranged in thin cell plates lined by fenestrated endothelial cells and separated by sinusoids (Fig. 1).[8] They do not show any significant atypia.

The vast majority of patients who develop HCC are cirrhotic, and liver carcinogenesis in this clinical context is considered a multistep process. Preneoplastic lesions are indeed characterised by the sequential accumulation of both molecular and morphological abnormalities.[8] The main histological features associated with the early steps of malignant transformation are increased cell density and nuclear-to-cytoplasmic ratio, unpaired arteries and pseudogland formation (Fig. 1).[8] The sequence of carcinogenesis is well-established and early lesions include low-grade macronodule, high-grade macronodule, early HCC and small and progressed HCC.[9,10] Full blown HCC is further characterised by a combination of architectural abnormalities (loss of sinusoidal lining, pseudoglandular formations, stromal invasion) and cytological changes (higher cell density and atypia).

Histological subtyping of HCC is a complex and quickly moving field. Classical HCCs are described according to their architectural growth patterns (microtrabecular, macrotrabecular, pseudoglandular, compact) and cytological aspects (clear cell, fatty change, cholestasis, pleiomorphic cells, spindle cells), with frequent co-existence of several features. HCC differentiation is also graded into 4 categories according to the Edmondson and Steiner classification and into 3 subclasses according to the recommendations of the World Health Organization of Tumours.[9,11] Given this high degree of phenotypical heterogeneity, it is not surprising that a wide array of molecular alterations have also been identified in HCC.[12] They are discussed in the following section.

HCC molecular alterations and transcriptomic subclasses

The recent development of high-throughput sequencing technologies has enabled comprehensive genetic landscaping of most human malignancies, including HCC.[12–17] Each tumour displays a unique combination of somatic mutations, and the major pathways involved in HCC development and progression comprise telomere maintenance, Wnt/β-catenin signalling and cell cycle regulation. The most frequent alterations identified so far are *TERT* promoter, *CTNNB1* and *TP53* mutations.[13,15,17]

Studies based on gene expression profiling also led to the identification of several HCC subclasses. Regardless of the nomenclature used by the different authors, HCC can be schematically divided into 2 major subgroups (Fig. 2).[12,18–24] The first group retains the expression of markers of hepatocellular differentiation (low-proliferation class) and shows chromosomal stability.[12,18,19] Consistently, these tumours are associated with a well-differentiated phenotype (Fig. 2). Among this class, HCCs with activation of the Wnt/β-catenin pathway define a homogeneous subgroup of tumours with a microtrabecular and pseudoglandular pattern. The remaining tumours of this low-proliferation class (G4 subgroup) display a gene expression profile that closely resembles that of normal, non-tumour liver – they are usually small, without satellite nodules and vascular invasion (Fig. 2).[5,19]

The second major class of HCC shows activation of signalling pathways involved in cell cycle progression and is associated with a more aggressive phenotype (high-proliferation class) (Fig. 2).[12,18,19] The main molecular features of this class are chromosomal instability, *TP53* mutations, overexpression of genes involved in the cell cycle and survival, and activation of PI3K (phosphatidylinositol-3-kinase)/AKT and/or MAPK (mitogen-activated protein kinase) signalling pathways.[12,18,19] Patients with proliferative HCC usually have higher alpha-fetoprotein serum levels and an adverse clinical outcome.[12,18] These tumours are also most often poorly differentiated (Fig. 2).[5] Besides the link between molecular subclasses and overall differentiation, associations with more subtle histological features have been reported, leading to the identification of several HCC morpho-molecular entities.[8,9]

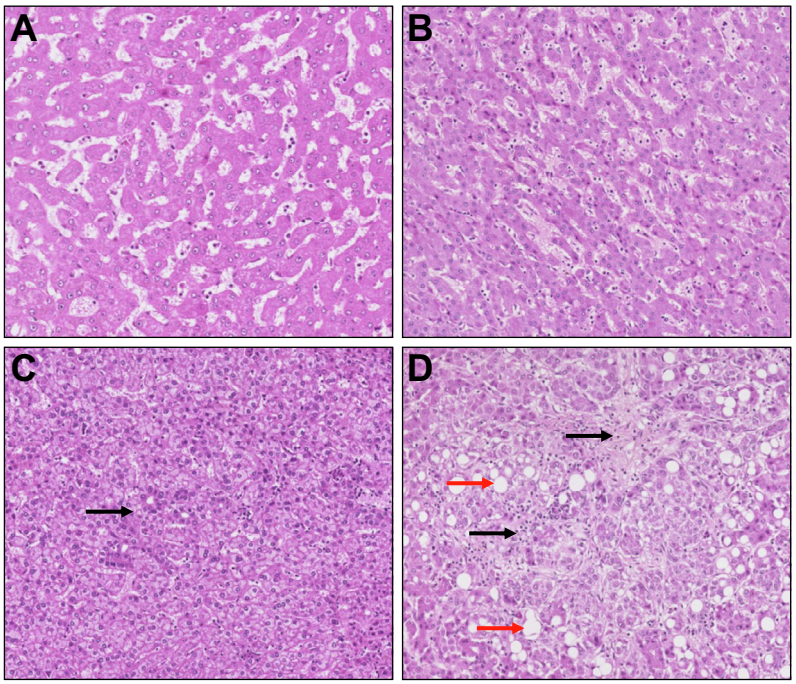

Fig. 1. Histology of normal liver and early lesions. (A) Hepatocytes of normal, non-tumour liver are arranged in thin cell plates lined by endothelial cells and separated by vascular spaces (sinusoids) (HES, ×160). (B) A moderate increase in cell density is observed in this low-grade macronodule (HES, ×100). (C) High cellular density and pseudogland formations (arrows) can be observed in high-grade macronodules (HES, ×100). (D) This nodule underwent full transformation with complete destruction of the sinusoidal architectural pattern, along with a fibrotic stroma (black arrows) and steatosis (red arrows) (HES, ×100). HES, hematein-eosin-saffron.

Key point

Hepatocellular carcinoma displays a high degree of molecular and histological heterogeneity.

HCC morpho-molecular entities

Integrative studies with comprehensive genetic and histological characterisations reported distinct HCC subtypes with unique morphological and molecular features. We will now introduce the main HCC variants identified so far by combining both morphological and molecular features.

CTNNB1 mutated HCC

CTNNB1 encodes β-catenin, a key intracellular transducer of the Wnt signalling pathway that regulates liver physiology and zonation.[25,26] When the pathway is inhibited, β-catenin is phosphorylated at specific serine and threonine residues, leading to its degradation by the proteasome. Mutations result in its stabilisation and subsequent nuclear accumulation, where it interacts with various transcription factors that enhance cell proliferation and

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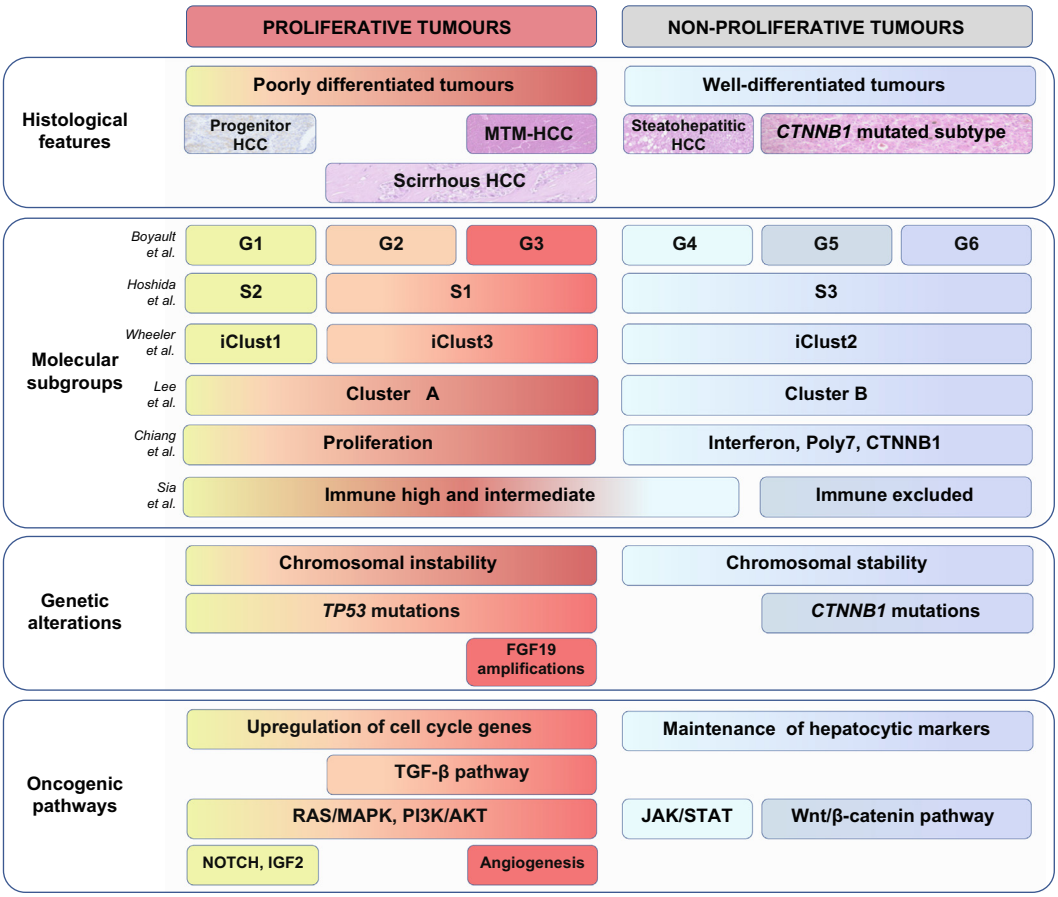

Fig. 2. Integration of HCC histological features, transcriptomic classification and genetic alterations. Non-proliferative tumours are characterised by chromosomal stability and maintenance of expression of hepatocytic markers. They display a well-differentiated phenotype, and, among this subgroup, *CTNNB1*-mutated HCC constitute a homogeneous subtype with cholestasis and microtrabecular and pseudoglandular architectural patterns.[5–7,18–21,24] These tumours are noticeably less infiltrated by immune cells.[5,46] Another non-proliferative variant has a gene expression pattern close to that of mature hepatocytes (G4) and displays a steatohepatitic phenotype.[5] Proliferative HCC are associated with chromosomal instability and *TP53* mutations.[19–24] They are most often poorly differentiated, and include tumours with progenitor features.[18–21,24] The novel morphological variant of proliferative HCC, MTM-HCC, is associated with the G3 subgroup, angiogenesis activation, *TP53* mutations and *FGF19* amplifications.[5,28] HCC, hepatocellular carcinoma; MTM-HCC, macrotrabecular-massive HCC.

survival.[25] Several studies have shown that HCCs with mutations in *CTNNB1* display a particular phenotype with well-differentiated tumours, microtrabecular, pseudoglandular architectural patterns, intratumour cholestasis and lack of immune infiltration (Figs. 3 and 4).[5,7] Consistently, these tumours show retained expression of various genes involved in hepatocellular differentiation and function, such as *APOB, ALB, HNF1A* or *HNF4A*.[5] A major dysregulation of bile salt transporters expression was also observed in these tumours and may, at least in part, contribute to their cholestatic phenotype (Fig. 3).[5] One of these transporters, *SLCO1B3*, is notably responsible for the uptake of the magnetic resonance imaging contrast agent gadoxetic acid.[27]

Macrotrabecular-massive HCC

A systematic review of more than 340 HCCs led to the identification of a novel subtype, designated as macrotrabecular-massive (MTM-HCC). Representing 10–20% of all cases of HCC, it is defined, on surgical specimens, by a predominant (>50% of the tumour area) macrotrabecular (>6 cells thick) architectural pattern, regardless of the associated cytological features (Figs. 4 and 5).[5,28] On biopsy samples, cases are classified MTM-HCC if at least 1 foci of macrotrabecular pattern is identified, without taking into account the percentage. It more frequently occurs in patients infected by HBV and with high alpha-fetoprotein serum levels (Figs. 4 and 5).[5] MTM-HCC is robustly identified by pathologists, with good inter-observer agreements. It exhibits a very aggressive phenotype, with frequent satellite nodules and macrovascular and/or microvascular invasion (Fig. 5).[5,28]

Gene expression profiling demonstrated that angiogenesis activation is a hallmark feature of MTM-HCC, with both angiopoietin 2 and vascular endothelial growth factor A (VEGFA) overexpression.[5] Angiopoietin 2 is responsible for the destabilisation of established blood vessels and

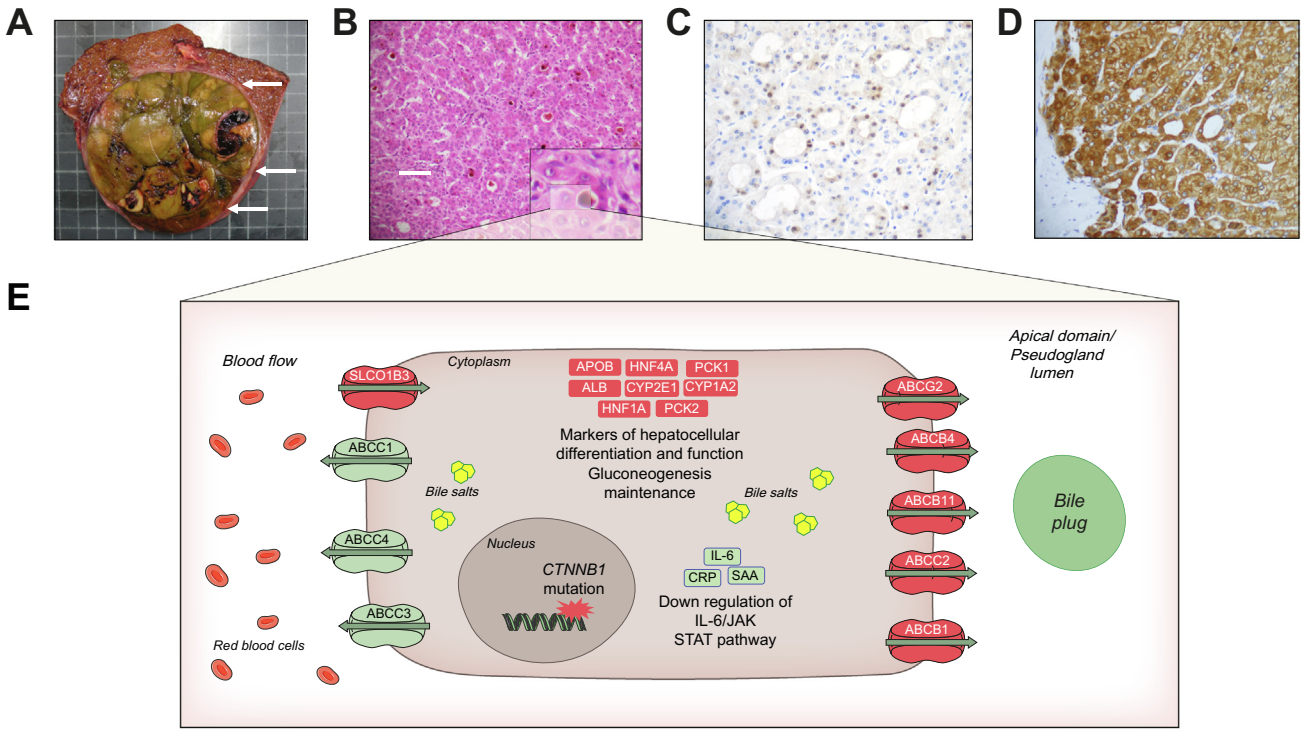

Fig. 3. Pathological features of *CTNNB1*-mutated HCC subtype. (A) Typical macroscopic appearance of *CTNNB1*-mutated HCCs, with a fibrous capsule surrounding the tumour (arrows) and a green colour due to bile production. (B) Microscopic examination shows a well-differentiated tumour with a lack of immune infiltration and micro-trabecular, pseudoglandular architectural patterns and cholestasis (HES, ×100). (C) Immunohistochemistry reveals nuclear accumulation of β-catenin. (D) A strong and diffuse overexpression of glutamine synthetase, a target gene of the Wnt/β-catenin pathway, is usually observed. (E) Schematic representation of the neoplastic cell framed in the lower right corner of panel B: there is a massive dysregulation of bile salts transporters that contributes to the formation of a bile plug in the lumen of the pseudogland. *CTNNB1*-mutated tumours also show retained expression of markers of hepatocellular differentiation and function, along with gluconeogenesis maintenance and downregulation of the IL6/JAK STAT pathway, consistent with the lack of inflammatory infiltrates (genes in red boxes: upregulated, green boxes: downregulated; transporters in red: upregulated, in green: downregulated). HCC, hepatocellular carcinoma; HES, hematein-eosin-saffron.

subsequent vascular sprouting.[29,30] It also disrupts interactions between endothelial and peri-endothelial cells, which results in an increased sensitivity to VEGFA. A strong association with the G3 transcriptomic subgroup, a subclass linked to cell cycle activation and chromosomal instability, was also observed.[5] At the genetic level, MTM-HCC often harbours *TP53* mutations and/or *FGF19* amplifications (Fig. 4).[5]

Scirrhous subtype

The scirrhous subtype is characterised by an abundant, dense fibrous stroma in which clusters of neoplastic cells are embedded (Fig. 6). It is thought to represent approximately 5% of resected HCCs (Fig. 4).[9,31] Expression of various progenitor or cancer stem cell genes, including *CK7 (KRT7), CK19 (KRT19), THY1,* or *CD133 (PROM1)*, has been reported in this variant and we hypothesise that scirrhous HCCs harbour intermediate molecular traits, between HCC and cholangiocarcinoma.[31] Consistent with its histological appearance, gene expression studies showed that scirrhous HCC features activation of transforming growth factor beta (TGF-β) pathway/epithelial-to-mesenchymal transition, with overexpression of *VIM, SNAIL (SNAI1), SMAD4* and *TWIST* (Fig. 4).[5,31]

Steatohepatitic subtype

First identified by Salomao *et al.*, this distinctive subtype is defined by hallmark histological features of alcoholic or non-alcoholic steatohepatitis, namely inflammatory infiltrates, cell ballooning, peri-cellular fibrosis and Mallory-Denk bodies (Fig. 6).[32] Interestingly, several authors have reported that steatohepatitic HCC more frequently develops in patients with non-alcoholic steatohepatitis, reflecting a potential sensitivity of neoplastic cells to systemic metabolic dysregulations.[32,33] These tumours are most often well-differentiated and, consistently, they were shown to be associated with the G4 subclass, which is known to share a gene expression profile similar to that of non-tumour liver (Fig. 4).[5] In a recent study, Lee and collaborators investigated the microenvironment of steatohepatitic HCC and showed that, compared to classical HCC, cancer-associated fibroblasts were characterised by upregulation of interleukin-6, a key regulator of the JAK/STAT pathway.[34] In this context, overexpression of C-reactive protein – a target gene of JAK/STAT signalling – by neoplastic cells was identified using immunohistochemistry.[5] Gene sequencing and immunohistochemical studies have also revealed that this variant very rarely

Key point

Macrotrabecular-massive and progenitor subtypes are linked to adverse clinical outcomes.

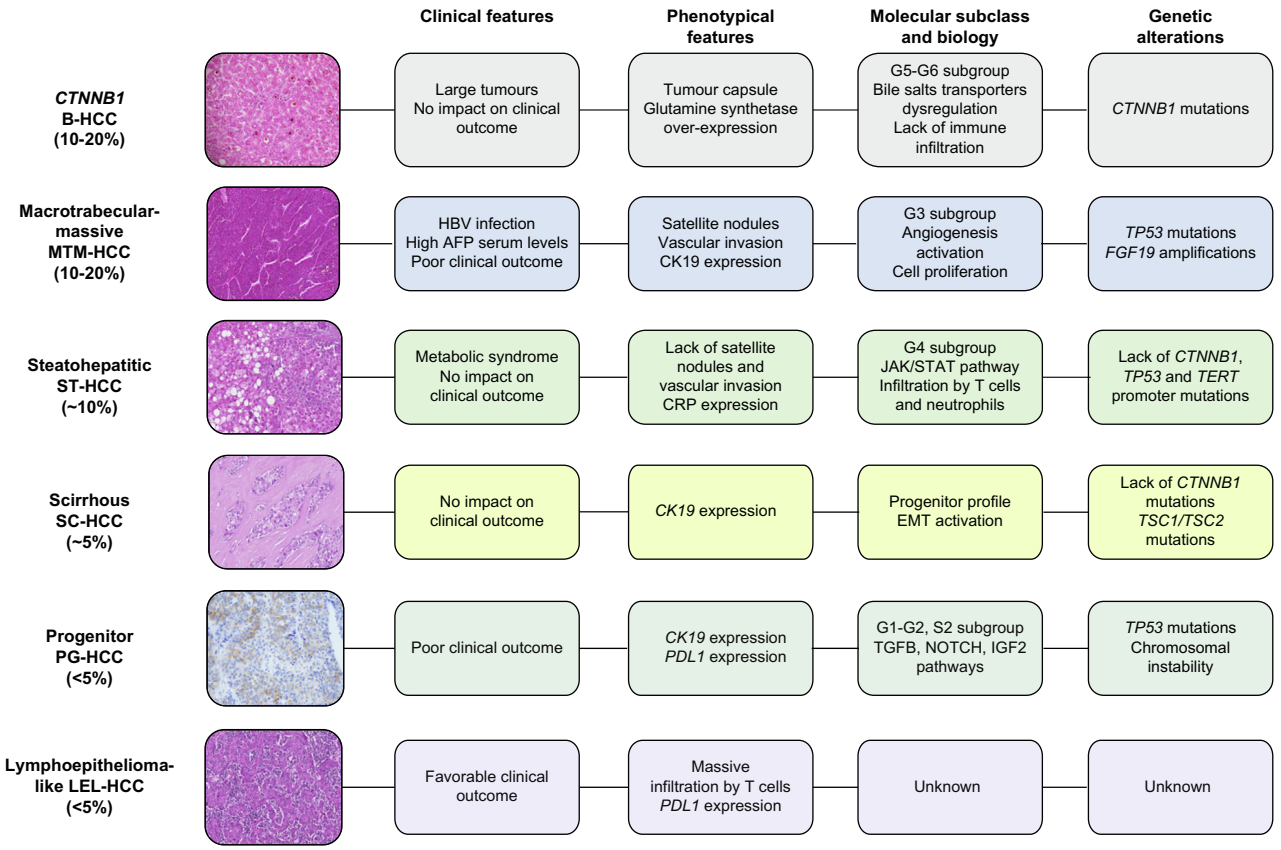

Fig. 4. Clinical, biological, pathological and molecular features of main HCC subtypes. HCC, hepatocellular carcinoma.

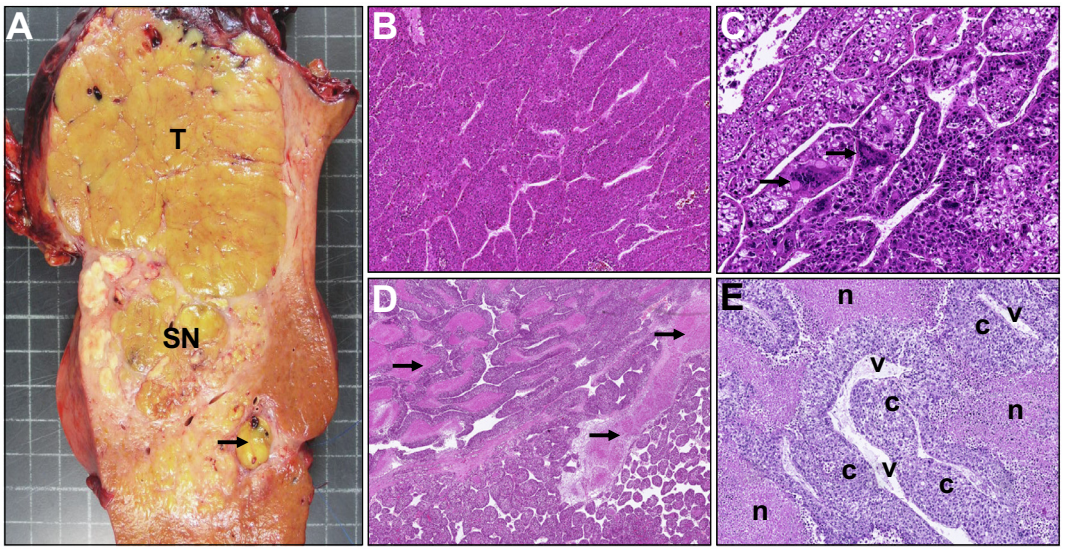

Fig. 5. Macrotrabecular-massive HCC. (A) Gross appearance of an MTM-HCC: satellite nodules (SN) are identified near the main nodule (T), along with a tumour thrombi in a branch of the portal vein (arrow). (B) Neoplastic cells of MTM-HCC are arranged in thick trabeculae surrounded by vascular spaces (HES, ×50). (C) This case of MTM-HCC displayed a high degree of atypia, with multinucleated cells (arrows, HES, ×100). (D) Numerous necrotic foci are observed in this case (black arrows, HES, ×12.5). (E) At high magnification, viable neoplastic cells (c) are located next to the vascular spaces (v). Areas far from vessels are necrotic (n) (HES, ×50). HCC, hepatocellular carcinoma; HES, hematein-eosin-saffron; MTM-HCC, macrotrabecular-massive HCC.

harbours Wnt/β-catenin pathway activation (Fig. 4).[5,35]

Lymphoepithelioma-like subtype

Lymphoepithelioma-like carcinoma (LEL-C) is a term that refers to a particular group of human malignancies with massive lymphocytic infiltration that bear histological resemblance to lymphoepithelioma, a type of poorly differentiated nasopharyngeal tumour characterised by a prominent immune stroma/microenvironment (Figs. 4 and 6).[36–38] LEL-Cs have been reported in various organs, such as the colon, stomach, liver, breast, lung, skin and urinary tract.[39] Association with the Epstein-Barr Virus has been identified in a significant fraction of LEL-C, however this pathogen does not seem to be involved in the development of lymphoepithelioma-like HCC (LEL-HCC).[36–38]

This rare variant (<5%) of HCC has been associated with improved overall survival relative to other subtypes, supporting the hypothesis that the dense lymphocytic infiltrate reflects effective antitumour immunity (Figs. 4 and 6).[38] Several studies have investigated the immunophenotype of the infiltrating immune cells and shown a predominance of cytotoxic CD8+ lymphocytes, along with increased programmed cell death 1 ligand 1 (PD-L1) and programmed cell death 1 (PD1) expression.[38,40] Lymphocytic infiltration has been linked with microsatellite instability and/or high mutational burden in various tumours, such as colon or lung adenocarcinomas.[41–45] However, this does not seem to be the case for HCC, as i) none of the LEL-HCC cases investigated by Chan *et al.* were microsatellite unstable, and ii) the immune subclass of HCC reported by Sia *et al.* was not associated with a higher number of somatic mutations.[38,46]

Due to the rarity of LEL-HCC, there is no data regarding its association with HCC transcriptomic subclasses or gene mutations. However, this variant probably represents a unique model of an effective, *in situ,* antitumour immune response. Additional investigations may provide useful insights for future therapeutic strategies based on immune system modulation. A consensus definition of LEL-HCC, with a cut-off value for intratumour lymphocyte density, is currently lacking.

Progenitor HCC

The so-called progenitor subtype of HCC is not *per se* a morphological variant, as it is defined by the immunohistochemical expression of cytokeratin 19, a marker of biliary lineage, in more than 5% of neoplastic cells (Fig. 7).[47,48] This particular phenotype may be the result of a dedifferentiation of neoplastic hepatocytes or reflect the malignant transformation of hepatic progenitor cells.[48] Indeed, there is growing evidence that progenitor cells, activated during acute and chronic liver diseases, can directly give rise to HCC.[49] This phenotype is associated with *TP53* mutations and particular subclasses of HCC (G1-G3, S2) (Fig. 4).[5,31,48]

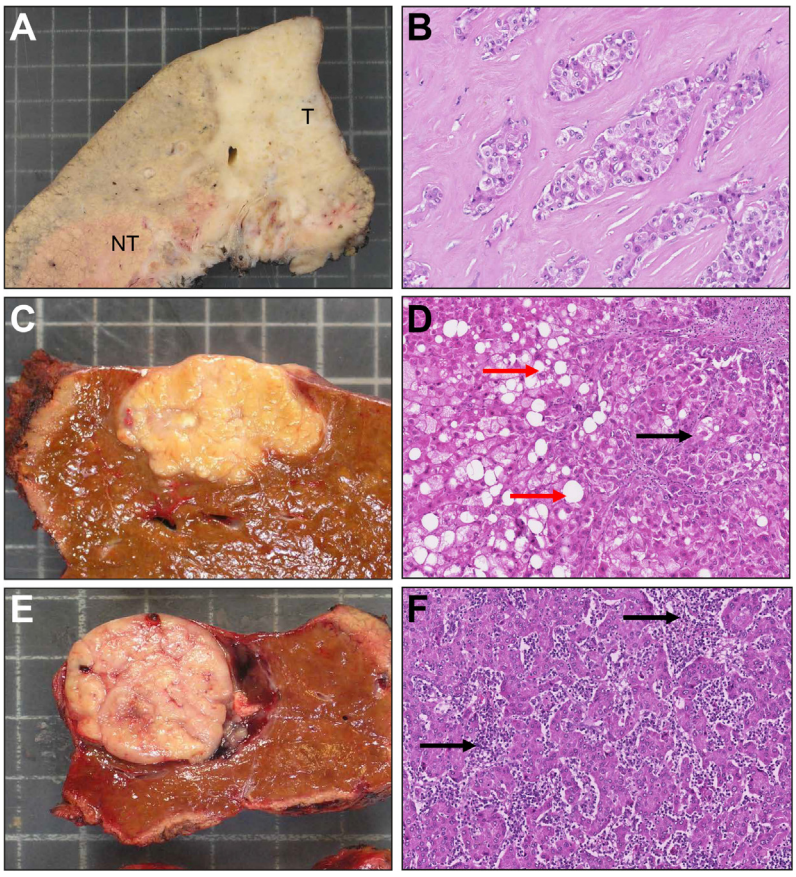

Fig. 6. Scirrhous, steatohepatitic and lymphoepithelioma-like HCC subtypes. (A) Gross examination of this scirrhous HCC reveals a firm mass, with a white colour due to massive intratumour fibrosis. (B) The typical morphological appearance of scirrhous HCC consists clusters of neoplastic cells embedded in a dense, fibrous stroma (HES, ×200). (C) This case of steatohepatitic HCC has a yellow colour due to intratumour steatosis. (D) Steatohepatitic HCC shows hallmark features of alcoholic or non-alcoholic steatohepatitis, with ballooned cells (black arrows), Mallory-Denk bodies and steatosis (red arrows) (HES, ×100). (E) Macroscopic examination of this lymphoepithelioma-like HCC shows a tan, well-circumscribed nodule. The adjacent parenchyma is non-fibrotic. (F) Lymphoepithelioma-like HCC are massively infiltrated by immune cells (arrows) (HES, ×120). HCC, hepatocellular carcinoma; HES, hematein-eosin-saffron.

Key point

Morphological subtypes of hepatocellular carcinoma are strongly associated with tumour subclasses and gene mutations.

The acquisition of a progenitor profile has been reported after locoregional therapies. Lai *et al.* analysed 40 residual/recurrent tumours previously treated by transarterial chemoembolisation, and observed frequent immunohistochemical expression of cytokeratin 19 and CA9 (carbonic anhydrase 9), a marker of hypoxia.[50] Interestingly, double staining demonstrated co-localisation of both proteins in most cases.[50] Zeng *et al.* also reported increased expression of 2 progenitor/cancer stem cell markers, EpCAM (epithelial cell adhesion molecule) and CD133, in HCC previously treated by chemoembolisation.[51] In this context, a high rate of combined hepatocellular-cholangiocarcinomas was observed after local ablation therapies.[52] Mechanistic studies evaluating the underlying biological pathways involved in the induction of this phenotype after treatment are sparse. Yoshida and collaborators

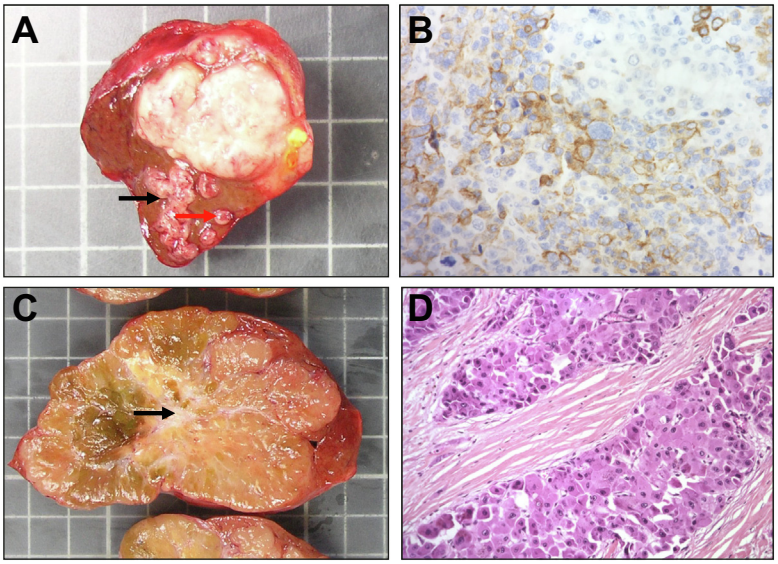

Fig. 7. Progenitor HCC and fibrolamellar carcinoma. (A) Macroscopic examination of this case of progenitor HCC showed satellite nodules (red arrow) and vascular invasion (black arrow). (B) Neoplastic cells of progenitor HCC are characterised by CK19 expression (×400). (C) A central scar can be observed in this fibrolamellar carcinoma. The adjacent liver parenchyma is non-fibrotic. (D) Microscopic examination of fibrolamellar carcinoma shows compact clusters of cells embedded in a lamellar, hyaline and fibrotic stroma (×200). HCC, hepatocellular carcinoma; HES, hematein-eosin-saffron.

showed that a sub-lethal heat treatment of HCC cell lines, simulating the margin zones of radiofrequency ablation, was able to promote epithelial-to-mesenchymal transition, progenitor marker expression and cell cycle activation.[53]

However, large-scale studies with appropriate controls (untreated tumours matched on disease stage) are necessary to confirm the role of locoregional therapies in the induction of this particular variant.

Fibrolamellar carcinoma

First described by Edmondson in 1956 based on its histological features, fibrolamellar carcinoma (FLC) is a well-established and rare (<1%) subtype of HCC with unique clinical, histological and molecular features.[8,9] Its aetiological risk factors remain completely unknown. It is typically diagnosed in young patients with no history of liver disease and without significant liver fibrosis.[8,9] Histologically, FLC consists of clusters of large eosinophilic neoplastic cells embedded in a dense fibrous stroma (Fig. 7). Intracytoplasmic pale or hyaline bodies may also be observed. In contrast to HCC, lymph node metastasis is a common finding.[8,9]

By performing whole-transcriptome and whole-genome sequencing, Honeyman *et al.* identified a large deletion that produces an in-frame fusion of *DNAJB1* (encodes a subunit of the heat shock factor 40 complex) with *PRKACA* (encodes the catalytic subunit of protein kinase A) in all FLC cases investigated (15/15). The resulting *DNAJB1-PRKACA* chimera, highly expressed in tumour samples, was shown to retain its catalytic activity, and functional studies further validated that it is a key oncogenic driver of FLC.[54–56]

Morphological diagnosis of FLC may be challenging, as classical HCC arising in the context of chronic liver disease may have foci with typical FLC morphological features. A consensus cut-off value that defines the percentage of tumour area with this morphology required for the diagnosis of FLC has yet to be established. Indeed, distinction of pure FLC (FLC appearance throughout the entire tumour) and mixed FLC (tumours with both areas typical of FLC and classical HCC) is important as they appear to be 2 distinct entities with the *DNAJB1-PRKACA* fusion mostly occurring in pure FLC.[57–59] Pure FLC also harbours a unique gene expression profile, with glycolytic activation, overexpression of *ERBB2* and various neuroendocrine genes including *PCSK1, NTS* and *CALCA*.[59] Interestingly, differences in clinical features and prognosis were also identified between pure and mixed FLC: in the study by Maalouf *et al.*, patients with pure FLC were younger and had lower alpha-fetoprotein serum levels, higher rates of lymph node metastasis and improved overall survival after surgical resection compared to patients with mixed FLC.[58]

Immunohistochemical experiments showed that FLC displays a specific phenotype with co-expression of cytokeratin 7 (biliary lineage) and hepatocyte paraffin 1 (hepatocytic lineage).[8] Consistently, mucin production, a feature usually observed in cholangiocarcinoma or combined tumours, has been reported in a subset of FLC.[8] Oikawa and collaborators observed that the gene expression profile of FLC resembles that of the biliary tree stem cells, a newly discovered subpopulation of multipotent stem cells located throughout the biliary tree and considered to be precursors of both hepatic and pancreatic tissues.[60] They further hypothesised that FLC may arise from these cells, which is consistent with the mixed hepatocytic, cholangiocytic and endocrine profile of these tumours.[60]

Combined hepatocellular-cholangiocarcinoma

Combined hepatocellular-cholangiocarcinoma displays phenotypic features suggestive of both hepatocytic and biliary differentiation. This subtype is rare (<5%), but it is probably under-recognised by pathologists because of the lack of clear diagnostic criteria on histology. The former classification was complex and reported different hepatocellular-cholangiocarcinoma subclasses associated with distinct morphological features (stem cell typical, stem cell intermediate, cholangiocellular). However, most cases are heterogeneous, and distinct areas within the same tumour may belong to different subclasses. Moeini and collaborators also showed that cholangiolocellular carcinoma is a biliary-derived tumour

and should therefore be classified as a subtype of cholangiocarcinoma.[61]

Molecular studies of hepatocellular-cholangiocarcinoma are sparse. This entity appears to have a distinct mutational profile from that of conventional HCC, with the occurrence of molecular events usually observed in intrahepatic cholangiocarcinoma, such as *IDH* mutations or *FGFR2* fusions.[52] Importantly, it was shown that both components are likely to derive from the same transformed cell.[62]

Other rare and/or provisional subtypes

Besides the main HCC variants, additional rare and/or provisional subtypes of HCC have been reported. Sarcomatoid HCC is characterised by a predominance of spindle cells.[9] Although most cases feature a classical HCC component, differential diagnosis with sarcoma may be challenging and immunohistochemical markers (hepatocyte antigen, glypican 3) are usually performed for the identification of the hepatocytic lineage. Molecular studies of sarcomatoid HCC are lacking. Some authors have reported that a sarcomatoid phenotype is induced after locoregional therapies such as transarterial chemoembolisation.[63,64] However the level of evidence is low and studies with appropriate control groups are needed to confirm this hypothesis.

The candidate subtype "chromophobe with abrupt anaplasia HCC" was proposed by Wood *et al.*[65] It is defined by neoplastic cells with a pale chromophobic cytoplasm, focal nuclear anaplasia and scattered pseudocysts.[65] This variant is associated with the alternative lengthening of telomere phenotype, as detected by telomere fluorescence *in situ* hybridisation.[65] The alternative lengthening of telomere is a telomerase-independent mechanism that allows telomere length maintenance.[66] It has been identified in various human cancers, and is linked to somatic mutations of *ATRX, H3F3A* and *DAXX*.[67,68] However, none of these genes were mutated in the 2 cases investigated by Wood *et al.*[65] Additional studies are needed to validate this variant and identify the underlying molecular alterations responsible for this particular phenotype.

Several reports of granulocyte-colony stimulating factor (G-CSF) HCC have been published.[69–71] These tumours are characterised by the production of G-CSF, which leads to intratumour infiltration by neutrophils. G-CSF expression by neoplastic cells has been reported in various cancers and promotes tumour growth through an autocrine mechanism. Studies assessing large numbers of G-CSF HCC are needed to determine the clinical, biological and molecular characteristics of this subset of tumours. The definition of G-CSF HCC is also vague, with a lack of consensus on other morphological features required, and no available cut-off value for intratumour neutrophil density.

Perspective for precision medicine

The molecular and phenotypical characterisation of large series of tumours have provided a massive amount of information. Yet, tumour subtypes are only useful for therapeutic algorithms if they are able to demonstrate a significant clinical impact. In the following section, we will discuss the potential role of HCC morpho-molecular classification for the prediction of prognosis and response to currently available therapies and/or immunomodulating agents.

Combined histological and molecular classification of HCC for prognosis prediction

In human malignancies other than HCC, the development of pathological and molecular tumour classifications has refined prognostication. Ongoing trials are currently assessing different systemic therapies in sarcomatoid renal carcinoma (NCT03483883 and NCT01164228) – a histological variant of kidney cancer defined by the presence of elongated spindle cells – which is associated with a lower frequency of *VHL* loss and high rates of *TP53* and *PTEN* mutations.[72] In bladder carcinoma, efforts are currently being made to tailor therapeutic strategies according to the prognostic significance of tumour subtypes.[73,74]

In HCC, besides classical pathological indicators such as vascular invasion or satellite nodules, MTM-HCC has been shown, in several independent series of patients treated by surgery, to be a key determinant of patient outcomes, with shorter disease-free and overall survival.[5,28] Its value is retained even after stratification according to common clinical, biological or pathological prognostic features.[5,28] Interestingly, the identification of MTM-HCC on tumour biopsies performed during percutaneous ablation procedures for Barcelona Clinic Liver Cancer stage 0/A HCC was the only predictive factor of both early and overall relapse in a large series of 284 patients.[28] These findings demonstrate that biopsy samples are able to provide significant information on prognosis and outcome. Further studies will need to assess whether MTM-HCC should be implemented in therapeutic algorithms.

The progenitor subtype of HCC is also linked to an adverse clinical outcome.[47] Durnez *et al.* indeed showed that patients with HCC expressing cytokeratin 19 were at an increased risk of recurrence after transplantation, and this prognostic impact has also been demonstrated after percutaneous ablation or liver resection.[75–77] By interrogating a large cancer database of more than 5,000 patients, Liao and collaborators were recently able to identify 40 patients with sarcomatoid HCC and show that this variant is an independent predictor of shorter disease-free and overall survival.[78] This interesting finding needs to be further validated.

Over the last decade, the type, location and density of intratumour immune cells has emerged as a critical determinant of patient outcomes.

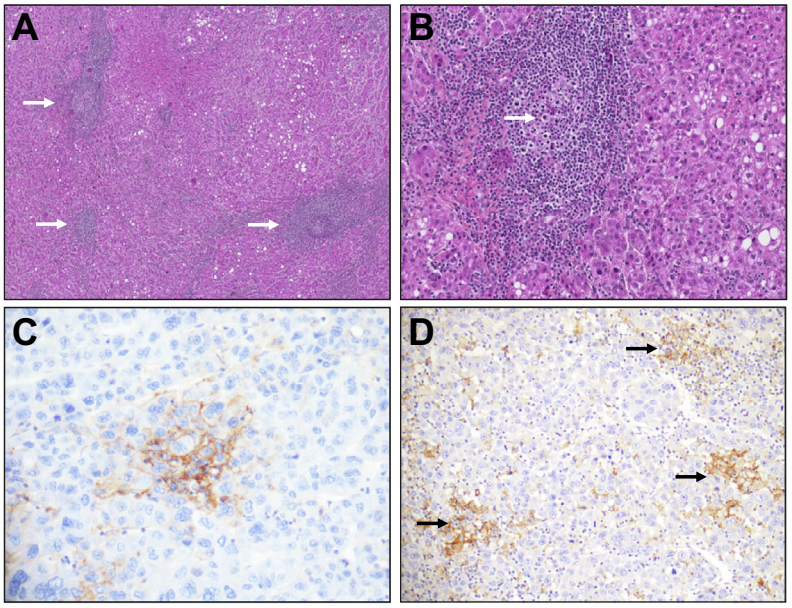

Fig. 8. Morphological features of HCC immune microenvironment. (A) This conventional HCC is infiltrated by numerous TLSs (white arrows, HES, ×35). (B) High magnification of a TLS harbouring a germinal centre (HES, ×130), an area where B cells proliferate, differentiate and undergo mutational processes involving antibody genes (×200). (C) A focal, membranous expression of PD-L1 by neoplastic cells is observed in this progenitor HCC (×400). (D) This lymphoepithelioma-like HCC is infiltrated by numerous PD-L1 expressing inflammatory cells (black arrows, ×200). HCC, hepatocellular carcinoma; HES, hematein-eosin-saffron; TLS, tertiary lymphoid structure.

Cytotoxic CD8+ lymphocytes have the ability, after stimulation by antigen-presenting cells, to release various enzymes (including perforin and granzyme) that lead to neoplastic cell death.[79,80] As expected, patients with HCC deeply infiltrated by CD8+ T cells were shown to have a decreased risk of relapse.[46,81,82] In an elegant large-scale study, Kurebayashi *et al.* used immunohistochemistry to quantify intratumour immune cells and identified 3 main subclasses of HCC: immune-high, immune-mid and immune-low.[82] The immune-high subclass was characterised by increased B-/plasma-cell and T-cell infiltration and was associated with a favourable outcome. Interestingly, the immune-high subtype included HCCs with a progenitor phenotype that had a better prognosis. These results demonstrate that the characterisation of infiltrating immune cells may improve tumour staging.[82]

Intratumour tertiary lymphoid structures (TLSs) were also recently shown to be associated with a low risk of early relapse after surgical resection for HCC (Fig. 8).[83] TLSs are defined as lymphoid aggregates that form in non-haematopoietic organs in response to chronic inflammatory processes. Their role is to stimulate the *in situ* immunity by generating plasma cells and effector T cells. Interestingly, the impact on outcome was linked to the type of TLSs, with a lower risk of relapse for tumours that bear fully functional, mature TLSs (primary/secondary follicles) (Fig. 8).[83]

Key point

The development of a morphomolecular classification of hepatocellular carcinoma is likely to improve precision medicine for patients with this highly aggressive malignancy.

In summary, there is accumulating evidence that HCC morpho-molecular subtypes have an important impact on clinical outcomes, and their implementation in pre-treatment work-up is likely to facilitate more personalised therapeutic strategies. Patients with highly aggressive subtypes (MTM-HCC, Progenitor) may for example benefit from adjuvant therapies and/or upfront registration on liver transplant waiting lists after resection or percutaneous ablation.

Targeted therapies and oncogenic pathways

To promote the implementation of HCC morpho-molecular subtypes into clinical practice, it is also important to demonstrate their value for the prediction of response to currently approved/available therapeutic approaches.

The negative impact of MTM-HCC on clinical outcomes after surgical resection and percutaneous ablation has been documented in large series of patients, however the value of HCC variants for predicting responses to other treatments remains unknown. Unfortunately, characterisation of histological subtypes was not included in recent studies that aimed to identify biomarkers of response to sorafenib or regorafenib.[84,85] In the vast majority of cases, the diagnosis of HCC is performed using imaging procedures, hampering our understanding of the links between HCC subtypes and sensitivity to currently available treatments, including locoregional and systemic therapies. We may hypothesise that the lack of stratification according to HCC subclasses may have contributed, at least in part, to the numerous failures of phase III trials.[86–88] Notably, the biomarker-based METIV-HCC trial, testing the MET-inhibitor tivantinib in patients with high immunohistochemical expression of MET, was also negative, but the selective nature of the drug has been challenged, and additional trials using other drugs in patients with high-MET HCC may be considered.[89]

Targeting particular subsets of tumours remains a promising approach, as shown by the recent success of the REACH-2 trial that assessed the efficacy of ramucirumab, an anti-angiogenic drug, in patients with elevated alpha-fetoprotein serum levels.[90] Interestingly, MTM-HCC is characterised by increased levels of alpha-fetoprotein and activation of angiogenesis; the assessment of the antitumour effect of ramucirumab or other anti-angiogenic drugs in patients with this subset of HCC should be considered.[5]

Enrolment of patients in current trials most often requires tumour biopsy, thus, the value of morpho-molecular subtypes will have to be prospectively assessed.

Immunotherapy

Most HCCs lack druggable genetic alterations. Therefore, as in other human malignancies, immunomodulating therapies are likely to play a significant role in HCC treatment in the near

future.[91,92] However, objective responses are observed in a limited number of patients and the identification of patients that may benefit from immunotherapy is a critical issue.[92,93] Various biomarkers of response have been identified in other tumours, including intratumour immune cell infiltration and/or PD-L1 immunohistochemical expression.[94,95]

An integrative pathological and molecular study showed that HCCs with mutations in *CTNNB1* displayed less infiltration by inflammatory cells.[5] Consistently, by investigating the gene expression profile of more than 900 HCCs, Sia *et al.* also identified an immune subclass of HCC that is characterised by a lack of *CTNNB1* mutations.[46] An association between Wnt/β-catenin pathway activation and a lack of immune infiltration has also been reported in human malignancies other than HCC.[96] Moreover, in melanoma, a mechanistic study showed that tumour-intrinsic β-catenin signalling results in T-cell exclusion from the microenvironment and resistance to anti-PD-L1 or anti-CTLA-4 (cytotoxic T-lymphocyte associated protein 4) monoclonal antibodies.[96–98] Thus, we speculate that patients with *CTNNB1* mutations are not optimal candidates for immunotherapeutic approaches.

PD-L1 immunohistochemistry has been proposed to better select patients that may benefit from PD-L1/PD-1 blocking agents.[99,100] Indeed, PD-L1 expression by neoplastic cells is associated with increased rates of response in various tumours, including lung and bladder cancers.[94,101] In HCC, PD-L1 expression by neoplastic and immune cells is observed in approximately 15% and 75% of the tumours, respectively (Fig. 8).[40] It is also related to particular histological subtypes (progenitor and LEL-HCC) (Fig. 8).[40] Liu *et al.* showed in a large series of 453 tumours that the infiltrating immune cells that express PD-L1 are mainly macrophages.[102] They observed that the PD-L1 expression pattern has an important prognostic impact (adverse clinical outcome if PD-L1 is expressed by both neoplastic cells and macrophages). They also showed that PD-L1 expression by macrophages was characterised by an active immune microenvironment, with higher densities of intratumour CD8 T cells and increased mRNA levels of interferon-gamma, perforin, and granzyme B.[102] The impact of the level and pattern of PD-L1 expression on the sensitivity of tumours to immunomodulating agents remains to be determined.

The type of *in situ* antitumour immune response is very likely to differ among HCC morphological subtypes. LEL-HCC are deeply infiltrated by activated cytotoxic T cells; we hypothesise that patients with this variant will respond to immunotherapeutic strategies.[36–38,40] In contrast, the microenvironment of scirrhous HCC and MTM-HCC may not allow for the development of an effective antitumour immunity: the former is characterised by activation of the immunosuppressive TGF-β pathway and the latter display a lack of TLSs.[5,83]

In conclusion, as observed for oncogenic pathways, tumour subclasses and gene mutations, we believe that there are quantitative and qualitative differences in the immune microenvironments of the reported HCC subtypes. The pathological review of biopsy samples obtained during trials testing immunomodulating agents is necessary to confirm these hypotheses.

Challenges, future directions, and perspectives

Although our growing understanding of how HCC morphology relates to its underlying molecular alterations holds promise for future precision medicine, several points need to be addressed to translate recent research into clinical practice. We will now review these different challenges/issues.

First, studies were mainly performed in tumours that were surgically resected and thus do not represent the full spectrum of the disease. Indeed, most patients present with advanced disease not eligible for resection, and the correlations will have to be validated in this clinical setting where only biopsy can be performed. This remains challenging since diagnosis, in most patients, is performed using imaging procedures (computerised tomography or magnetic resonance imaging). Although this non-invasive approach was initially seen as a major achievement, the lack of tissue for tumour phenotyping and molecular testing has limited the identification of tumour biomarkers and/or subclasses associated with improved sensitivity to current systemic therapies used in patients with HCC.[103] Other malignancies, such as colorectal adenocarcinoma, non-small cell lung cancer or melanoma, have witnessed impressive progress in diagnosis and treatment because of the development of predictive tests and innovative therapies.[104–108]

Due to the existence of intratumour heterogeneity, whether biopsy samples can accurately capture the complex biology of HCC has been questioned.[109] However, it must be said that regarding the main oncogenic drivers and molecular subclasses, this heterogeneity seems rather limited.[24,110,111] Phenotypic and molecular analyses are also widely performed on biopsy samples in various other heterogeneous cancers, such as colon, lung or breast carcinomas.[108]

Another critical point is the definition of a consensus morpho-molecular classification of HCC. An international agreement on common tumour subclasses is needed before they can be applied to clinical care. Efforts in the development of surrogate immunohistochemical markers of HCC subgroups should also be encouraged.

The major challenge is identifying the most relevant characteristics of HCC for personalised medicine: gene mutations/alterations, transcriptomic subclasses, or histological/phenotypical subtypes? Lessons learned from other malignancies show that genetic alterations are key determinants of tumours' sensitivity to targeted therapies. For example, mutations of *KRAS* and *NRAS* predict resistance to anti-EGFR (epidermal growth factor receptor) antibodies in colorectal cancers, and agents targeting tumours that harbour *EGFR* mutations or *ALK* rearrangements have shown impressive efficacy in patients with lung adenocarcinoma.[104,105,112] The overall mutational burden, by promoting the generation of neoantigens, is also predictive of response to immune checkpoint inhibitors in various tumours.[45,113–116]

Transcriptomic subclasses are less frequently implemented in clinical care. Indeed, their use requires expertise in bioinformatics and standardisation, often with poor quality of RNA extracted from formalin-fixed, paraffin-embedded samples. However, several trials have shown that gene expression profiling on such material is feasible and may impact therapeutic strategies in different human tumours.[117,118] Indeed, several gene signatures that could predict breast cancer recurrence and avoid unnecessary adjuvant chemotherapy are currently being assessed, and transcriptomic subtypes of bladder carcinoma were reported to be associated with different rates of response to atezolizumab, a humanised monoclonal antibody that selectively binds to PD-L1.[118–120] For HCC, Nault *et al.* identified a 5-gene score able to predict survival after surgical resection that may be of interest for the stratification of patients in upcoming trials testing adjuvant therapies.[121]

The phenotype of cancers is linked to different prognoses and molecular backgrounds, and it is not surprising that immunohistochemical biomarkers are playing a growing role in diagnostic/pre-treatment work-ups. PD-L1, HER2, and oestrogen and progesterone receptor immunostainings are commonly performed for treatment allocation in different tumours.[122–124] The immunoscore, which assesses the density and location of immune infiltrates of colorectal cancer, has recently been validated in a large cohort of more than 2,500 patients from 13 countries, and is expected to play a critical role in tumour staging in the near future.[95]

Morphological subtypes of tumours also have a significant impact on therapeutic strategies and/or trial design in various tumours such as kidney or bladder cancer.[74,125–128] For HCC, development of particular therapeutic strategies and/or trials focusing on particularly aggressive variants, including MTM-HCC or progenitor HCC, may be considered. One limitation of histological variants is the subjective nature of pathology and the non-optimal inter-observer agreement between pathologists. This issue may be overcome in the near future with the emergence of high-throughput image analysis technologies.[129] Indeed, dedicated software facilitates the acquisition of thousands of morphological features (cell density, size and shape of nuclei, pixel intensity…), and may thus ease the establishment of histology-based classifications. In a pioneering study, Yu *et al.* developed a fully automated pipeline that extracts a series of prognostic image characteristics from lung cancer slides stained with haematoxylin and eosin.[130] The top features that facilitated classification of survival outcomes included nuclei and cytoplasm texture and shape, and were validated in an external series of slides stained in other laboratories.[130]

Finally, deep-learning approaches enable the extraction of a significant amount of "hidden" data from digital histological slides.[131–134] For example, convolutional neural networks were recently shown to be able to predict genetic alterations and overall survival of patients with lung and brain tumours, respectively.[131,135] After the rise of gene expression arrays and next-generation sequencing technologies, computational pathology is thus likely to be one of the next major revolutions in medicine.[132]

Conclusion

Our understanding of HCC biology has drastically improved during the last 2 decades. The main genetic alterations and tumour subclasses are now well established, and we are beginning to understand how they relate to HCC phenotype and histological features. Unfortunately, unlike in other malignancies such as lung or colorectal cancer, this increasing knowledge has not yet resulted in biomarker discovery and improved clinical care. Integrative pathological and molecular studies should be encouraged with the aim of defining a consensus HCC morpho-molecular classification that could be used in ongoing therapeutic trials. Artificial intelligence and automated computerised image analysis are also likely to provide a unique opportunity to achieve this goal in the near future.

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Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

JC and JZR: Drafting the manuscript. All authors: Critical revision of the manuscript.

Supplementary data

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Author names in bold designate shared co-first authorship

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