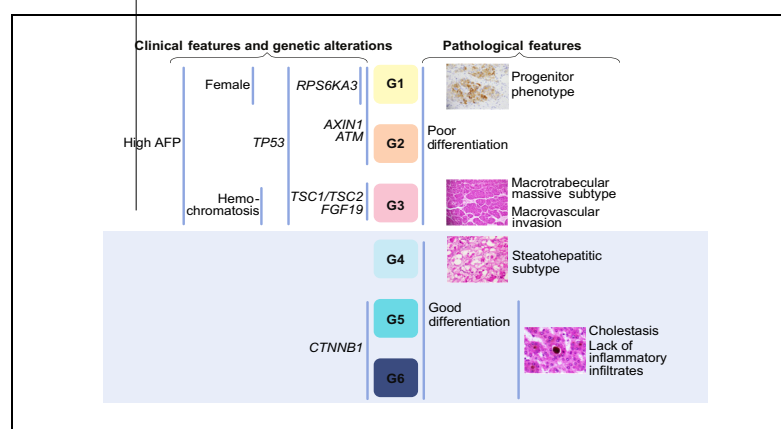


Histological subtypes of hepatocellular carcinoma are related to gene mutations and molecular tumour classification

Graphical abstract



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Lay summary

HCC is a very heterogeneous tumour, both at the pathological and molecular levels. We show here that HCC phenotype is tightly associated to its molecular alterations and underlying oncogenic pathways.

Highlights

- *CTNNB1* and *TP53* mutations define two distinct tumour phenotypes.
- Histological subtypes of HCC are associated with clinical and molecular features.
- The novel macrotrabecular-massive subtype has clinical and biological relevance.
- Relationships between HCC phenotype and biology has translational implications.

Histological subtypes of hepatocellular carcinoma are related to gene mutations and molecular tumour classification[☆]

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Background & Aims: Our increasing understanding of hepatocellular carcinoma (HCC) biology holds promise for personalized care, however its translation into clinical practice requires a precise knowledge of its relationship to tumour phenotype.

Methods: We aimed at investigating molecular-phenotypic correlations in a large series of HCC. To this purpose, 343 surgically resected HCC samples were investigated by pathological review, immunohistochemistry, gene expression profiling and sequencing.

Results: *CTNNB1* (40%) and *TP53* (21%) mutations were mutually exclusive and defined two major groups of HCC characterized by distinct phenotypes. *CTNNB1* mutated tumours were large ($p = 0.002$), well-differentiated ($p < 0.001$), cholestatic ($p < 0.001$), with microtrabecular ($p < 0.001$) and pseudoglandular ($p < 0.001$) patterns and without inflammatory infiltrates ($p < 0.001$). *TP53* mutated tumours were poorly differentiated ($p < 0.001$) with a compact pattern ($p = 0.02$), multinucleated ($p = 0.01$) and pleomorphic ($p = 0.02$) cells and frequent vascular invasion ($p = 0.02$). World Health Organization (WHO) classification of histological subtypes were also strongly related to molecular features. The scirrhous subtype was associated with *TSC1/TSC2* mutations ($p = 0.005$), epithelial-to-mesenchymal transition and a progenitor expression profile. The steatohepatic subtype showed frequent IL-6/JAK/STAT activation without *CTNNB1*, *TERT* and *TP53* pathway alterations ($p = 0.01$). Pathological review

identified a novel subtype, designated as “macrotrabecular-massive” associated with poor survival ($p < 0.001$), high alpha-fetoprotein serum level ($p = 0.02$), vascular invasion ($p < 0.001$), *TP53* mutations ($p < 0.001$) and *FGF19* amplifications ($p = 0.02$), features also validated in The Cancer Genome Atlas (TCGA) data. Finally, integration of HCC pathological characteristics with its transcriptomic classification showed phenotypically distinct tumour subclasses closely related to G1-G6 subgroups.

Conclusion: HCC phenotypes are tightly associated with gene mutations and transcriptomic classification. These findings may help in translating our knowledge of HCC biology into clinical practice.

Lay summary: HCC is a very heterogeneous tumour, both at the pathological and molecular levels. We show here that HCC phenotype is tightly associated to its molecular alterations and underlying oncogenic pathways.

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Introduction

Malignant primary liver tumours are the second leading cause of cancer-related death worldwide, with an increasing incidence in almost all countries.¹ They are mainly represented by hepatocellular carcinoma (HCC) that results from the malignant transformation of hepatocytes. The most frequent risk factors of HCC comprise of alcohol intake, infection by hepatitis B (HBV) or hepatitis C (HCV) viruses, and metabolic syndrome.² Although surveillance protocols of patients at-risk to develop HCC have significantly improved, clinical outcome remains poor with a majority of patients presenting with advanced disease not eligible for curative therapy.³

Keywords: Histopathology; Hepatocellular carcinoma; Mutations; Molecular carcinogenesis.

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At the pathological level, HCC is a morphologically heterogeneous tumour.^{4,5} Although HCC neoplastic cells most often grow in cords of variable thickness lined by endothelial cells mimicking the trabeculae and sinusoids of normal liver, other architectural patterns are frequently observed and numerous cytological variants are also recognized, comprising clear, pleomorphic, or bile producing cells.^{4–6} Moreover, several HCC subtypes defined by distinctive and easily recognizable histological features have been identified.^{4,5,7} In the last decade, there has been a growing interest for a particular subtype of HCC with so-called “progenitor features”. Thought to derive from progenitor cells, this tumour subtype shares an overlapping gene expression profile with oval cells and foetal hepatoblasts.⁸ It is characterized by an adverse clinical outcome. Immunohistochemical expression of cytokeratin 19 (CK19) by more than 5% of neoplastic cells is usually considered as a surrogate marker of this variant.⁹

In parallel to this pathological heterogeneity, gene expression profiling has allowed the establishment of several HCC transcriptomic classifications.^{10–14} Our group proposed a classification comprising of six subclasses associated with clinical and genetic features.¹¹ Groups G1 to G3 are characterized by chromosomal instability, cell cycle activation, *TP53* mutations (G2–G3) and HBV infection (G1–G2), while groups G4 to G6 are associated with chromosomal stability and Wnt/ β -catenin pathway activation mainly due to activating *CTNNB1* mutations (G5–G6).¹¹

High-throughput sequencing techniques have also defined the comprehensive mutational landscape of HCC. The most frequent mutations and chromosome alterations were identified in *TERT* promoter, *CTNNB1*, *TP53*, *AXIN1*, *ARID1A*, *NFE2L2*, *ARID2* and *RPS6KA3*.^{15–20}

Although this increasing understanding of HCC biology holds promise for future targeted therapies and personalized care, its translation into clinical practice will require a precise knowledge of its relationship to tumour phenotype. Here, we aimed to determine how HCC molecular features relate to its phenotype by combining comprehensive pathological analyses, gene expression profiling and gene sequencing in a series of 343 resected tumours developed in patients with various underlying liver diseases.

Material and methods

Patients and samples

A total of 343 unselected HCC developed in 339 patients treated by surgical resection in two French university hospitals (Henri Mondor, Créteil and Pellegrin, Bordeaux) were included in the study (“InsermU1162 Cohort”) (the overall study design and flow chart is provided in the [Supplementary material and methods](#)). Tumour and non-tumour liver samples were frozen immediately after surgery and stored at -80°C . All patients were treatment naïve before surgery and informed consent was obtained in all cases. The study was approved by institutional review board committees (CCPRB Paris Saint Louis, 1997, 2004, 2010 and 2016, approval number 2016/57NICB; Bordeaux 2010-A00498–31).

The following clinical and biological features were systematically recorded: age (recoded as >60 or ≤ 60 years), gender, liver disease aetiology (infection by HBV or HCV, alcohol intake, metabolic syndrome, hereditary haemochromatosis or undetermined), Barcelona Clinic of Liver Cancer (BCLC) stage, pre-operative alpha-fetoprotein (AFP) serum levels (recoded as >100 ng/ml or ≤ 100 ng/ml) and follow-up (early relapse and disease-specific survival).

Pathological examination

For each case, all available histological slides were reviewed by two expert pathologists (JC and PBS) specialized in liver disease and blinded to the clinical and molecular characteristics. A training set of 60 tumours was first used to determine

the reviewing and quotation method ([Table S1](#)), then all features were recorded by the two pathologists during common review sessions. Cases with a disagreement among the pathologists were discussed and a consensus was reached.

The following histological features were systematically assessed: tumour differentiation (according to Edmondson–Steiner grade), satellite nodules, macrovascular or microvascular invasion, tumour capsule, microtrabecular, macrotrabecular, pseudoglandular or compact pattern, pleomorphic cells, cholestasis, multinucleated cells, clear cells, sarcomatous changes, hyaline bodies, inflammatory infiltrates, steatohepatic, scirrhous, lymphoepithelioma-like, and sarcomatoid subtypes ([Table S1](#)).^{5,7,21,22} To reflect the co-existence of several histological features within the same tumour, architectural patterns and cytological variants identified in at least 20% of the tumour were considered present ([Table S1](#)).

During the review of the training set of tumours, we observed cases with a striking predominant macrotrabecular architecture involving more than 50% of the tumour. Their microscopic appearance indeed mainly consisted of neoplastic cells arranged in thick trabeculae coated by endothelial cells and surrounded by dilated vascular spaces. Trabeculae observed in cross sections also appeared as endothelium-coated tumour clusters. A link between the macrotrabecular architecture and poor clinical outcome has been previously suggested,²³ and we further aimed to assess if those cases may constitute a novel HCC subtype. This novel candidate subtype was further designated as “macrotrabecular-massive HCC” (MTM-HCC).

Validation of the relevance of this subtype was further performed using the public database from The Cancer Genome Atlas (TCGA) Research Network, which consists of a large series of HCC samples with available clinical annotations, digital slides, and molecular data.^{24,25} The website (www.cbioportal.org) was accessed on December 2016. One digital slide was available for each case, and, after the exclusion of cases with histological features suggestive of either cholangiocarcinoma ($n=8$) or hepatocholangiocarcinoma ($n=3$), a series of 359 HCC with molecular data was finally selected for further analysis (“TCGA Cohort”). For survival and early relapse studies, patients with a prior history of anti-tumour treatment and/or incomplete surgical resection (R1 or R2) were excluded. Survival and relapse status were available in 313 and 277 cases, respectively.

Immunohistochemistry

Expression of glutamine synthetase (GS), β -catenin, CK19, epithelial cell adhesion molecule (EPCAM), C-reactive protein (CRP), *ARID1A*, phospho-S6 protein, and phospho-ERK was investigated on whole tumour sections to avoid sampling heterogeneity inherent to tissue-micro-array techniques. Staining's were performed manually or using Leica Bondmax or Dako autostainers (a detailed description of quotation methods, antibodies used and staining protocols is provided in [Table S1](#), [Supplementary material and methods](#) and the [CTAT](#) table).

Transcriptomic classification of HCC samples and gene expression

Total RNAs were extracted with the RNeasy kit (Qiagen®), and quality assessment was performed as previously described.²⁶ Five hundred nanograms of RNA were further reverse-transcribed using the High Capacity Transcription kit (Life technologies®). After pre-amplification of the specific targets, Real-Time Polymerase Chain Reaction (PCR) was performed using the high-throughput BioMark Real-Time PCR system (Fluidigm®, South San Francisco, CA) according to manufacturer's instructions, using Fluidigm 96.96 dynamic arrays and TaqMan predesigned assays ([Supplementary material and methods](#)). Ct values were calculated using the Fluidigm Real-Time PCR Analysis software (4.1.3). Gene expression was normalized to ribosomal 18S mRNA, and the expression level of the tumour samples was compared with the mean level of the corresponding gene expression in normal liver tissues ($n=5$), expressed as an n-fold ratio. The relative amount of RNA was calculated with the 2-delta delta CT method.

Transcriptomic classification of HCC samples was performed in 265 cases using a combination of 16 genes, as previously described.¹¹ In addition, the expression of the following genes was investigated in a subset of 186 tumours: *ALB*, *AFP*, *APOB*, *CYP11A1*, *CYP11A2*, *CYP3A4*, *CYP2E1*, *CYP2C9*, *HNF4A*, *HNF1A* (hepatocellular differentiation and function), *GCK*, *GCKR*, *GPI* (glycolysis), *FBP1*, *PKC1*, *PKC2* (gluconeogenesis), *ACACA*, *ACLY*, *FADS1*, *FADS2*, *FASN*, *ME1* (fatty acid synthesis), *ABCB11*, *ABCG2*, *ABCB4*, *ABCC2*, *ABCB1*, *SLCO1B3* (transporters involved in bile salts efflux into canaliculi), *ABCC1*, *ABCC3*, *ABCC4* (transporters involved in bile salts efflux into blood flow), *GLUL*, *LGR5*, *TBX3* (Wnt/ β -catenin pathway target gene), *MKI67*, *RRM2*, *AURKA*, *EZH2* (cell proliferation), *ANGPT1*, *ANGPT2*, *VEGFA* (angiogenesis), *KRT19*, *EPCAM*, *SOX9*, *SALL4*, *ALDH1A*, *CD44*, *CD24*, *KRT7*, *CD133*, *THY1* (progenitor/cancer stem cell markers), and *TGFBI*, *TGF β RI*, *SMAD4* and *VIM* (TGF β pathway/epithelial-to-mesenchymal transition) ([Supplementary material and methods](#)).

Gene sequencing

For 129 cases, we extracted data from our former whole-exome sequencing study and selected genes mutated or altered in at least 5% of the tumours (*TERT* promoter, *CTNNB1* exon 3, *TP53*, *ALB*, *ARID1A*, *AXIN1*, *APOB*, *NFE2L2*, *ARID2*, *TSC1/TSC2*, *ATM*, *CDKN2A*, *ACVR2A*, *RPS6KA3*, *FGF19*).¹⁶ For additional cases, genomic DNA was extracted using a Promega Maxwell[®] Instrument and samples were further sequenced for *TERT* promoter, *CTNNB1*, *TP53*, *AXIN1*, *ALB*, *APOB*, *ARID1A*, *ARID2*, *NFE2L2*, *ATM*, *TSC1/TSC2* and *RPS6KA3*, using a MiSeq instrument (Illumina, San Diego, CA, USA) (Supplementary material and methods), or direct Sanger sequencing, as previously described.¹⁵ It has been formerly demonstrated that HCC display a relatively modest degree of intra-tumour heterogeneity regarding driver genetic alterations,^{27–29} and one frozen sample was available and analyzed for each case.

Statistical analysis

Statistical analysis was performed using R software (3.2.2). Comparison between qualitative and quantitative data was performed using Chi-square and Fisher exact tests and Mann-Whitney *U* and Kruskal-Wallis tests, respectively. Monte-Carlo method was used to adjust the *p* value for multiple tests. Patients with curative (R0) resection (*n* = 278) and features with more than 10% infrequency were included in survival analysis. Variables associated with disease-specific survival were identified using univariate and multivariate Cox proportional hazards regression models (Wald test), using the *survival*, *rms* and *Publish* packages. Prior to the multivariate analysis, collinear variables (BCLC B-C and macrovascular invasion; macrotrabecular histological pattern and macrotrabecular-massive subtype) were first tested in a bivariate analysis and the variable with the highest odds ratio (OR) was further included in the multivariate analysis. Kaplan-Meier plots were used to describe survival rates among all cases. All reported *p* values were two-tailed and differences were considered significant when the *p* value was under 0.05.

For further details regarding the materials used see Supplementary material and methods and refer to the CTAT table.

Results

Patients and pathological examination

The clinical features of our series were common for Western patients with HCC treated by liver resection (Table 1). There was a strong male predominance (85%), with main risk factors being alcohol intake (41%), followed by HCV infection (18%) and HBV infection (17%). Mixed aetiologies were observed in 15% of the patients. Different disease stages were included with very early/early (BCLC 0-A, 67%) as well as intermediate/advanced (BCLC B-C, 33%) tumours. High AFP protein levels (>100 ng/ml) were detected in 28% of the patients. As expected in a series of surgically resected HCC, we identified a high rate of non-cirrhotic patients (F0-F1 38%, F2-F3 33%, F4 29%). Early relapse and disease-related death occurred in 41% (115/278) and 33% (92/278) of the patients, respectively.

Tumours were frequently large (61% with diameter >50 mm), with satellite nodules (46%), and macrovascular (18%) or microvascular (60%) invasion (Table 1). A complete fibrous capsule surrounding the tumour was identified in 11% of the cases. We observed a slight predominance of poorly differentiated HCC (Edmondson grade III-IV, 57%). A high intra-tumour heterogeneity was observed, with a median of four architectural patterns/cytological variants co-existing within the same tumour. Areas with microtrabecular, macrotrabecular, pseudoglandular and compact histological architectural patterns were identified in 73%, 52%, 53% and 45% of the tumours, respectively (Table 1). Among the cytological variants, we observed foci of clear cells and cholestasis in 31% and 25% of

the cases, respectively. Sarcomatous changes, multinucleated cells and hyaline bodies were observed in 10–25% (Table 1). Identified in 6% of the tumours, pleomorphic cells were a less frequent cytological variant.

Following the WHO Classification of Tumours definitions, several HCC subtypes were identified: scirrhous (5%), steatohepatic (6%), and lymphoepithelioma-like (2%) (Table 1). No sarcomatoid HCC was observed. The novel macrotrabecular-massive subtype candidate represented 10% of the tumours.

Molecular features

Transcriptomic subgroups were determined using qRT-PCR and the 16-gene signature previously described.¹¹ Tumours were assigned to proliferative (G1 5%, G2 6%, G3 20%) or less proliferative (G4 34%, G5 23%, G6 12%) HCC subclasses (Table 1). We next searched for alterations of major oncogenes/tumour suppressor genes and identified mutations in *TERT* promoter (65%, *n* = 208/322), *CTNNB1* (40%, *n* = 130/332), *TP53* (21%, *n* = 72/341), *ALB* (16%, *n* = 27/170), *ARID1A* (14%, *n* = 45/313), *AXIN1* (11%, *n* = 34/322), *APOB* (11%, *n* = 18/171), *ARID2* (7%, *n* = 23/313), *NFE2L2* (6%, *n* = 19/308), *TSC1/TSC2* (6%, 15/239), *ATM* (6%, 15/238), *RPS6KA3* (5%, *n* = 16/311) and *ACVR2A* (5%, *n* = 6/129) (Table 1; Table S2). In addition, whole-exome sequencing analysis revealed *CDKN2A* homozygous deletions or mutations (5%, *n* = 7/129) and *FGF19* amplifications (5%, *n* = 6/129).

Associations between molecular alterations and HCC phenotype

CTNNB1 mutations define a specific cholestatic well-differentiated subtype of HCC

We further searched for correlations between gene mutations/alterations and pathological features. Interestingly, the major correlations were identified with *CTNNB1* exon 3 and *TP53* mutations, which showed, as formerly reported, a significant tendency towards mutual exclusivity (*p* < 0.001) (Table S3).³⁰ *CTNNB1* mutated tumours represented 40% of the series and were frequently larger (odds ratio (OR) 2.14, *p* = 0.002), well-differentiated (OR 2.46, *p* < 0.001), with intact tumour capsule (OR 2.54, *p* = 0.02), areas of microtrabecular (OR 4.32, *p* < 0.001) and pseudoglandular (OR 3.27, *p* < 0.001) histological patterns, tumour cholestasis (OR 6.53, *p* < 0.001), and a lack of inflammatory infiltrates (OR 3.41, *p* < 0.001) (Table 2, Fig. 1A–C). There were no associations of *CTNNB1* mutations with the degree of intra-tumour heterogeneity (median number of architectural patterns/cytological variants per tumour: *CTNNB1* mutated 4 vs. *CTNNB1* non-mutated 4). As expected, elevated mRNA levels of Wnt/β-catenin pathway targets and surrogate immunohistochemical markers of its activation (β-catenin nuclear staining and high GS expression) were also associated with *CTNNB1* mutations (*p* < 0.001) (Fig. 1A, D and E; Table S3). Consistent with the observed lack of inflammatory infiltrates in *CTNNB1* mutated HCC, these tumours also exhibited a downregulation of the IL6/JAK/STAT pathway, with low *IL6*, *CRP* and *SAA2* mRNA levels and low CRP immunohistochemical expression (*p* < 0.001) (Fig. 1A and E). Lack of *ARID1A* immunohistochemical expression was also frequently observed (Fig. 1A).

Gene expression experiments showed that *CTNNB1* mutations were associated with the maintenance of various markers of hepatocellular differentiation and function (*APOB*, *CYP1A1*, *CYP1A2*, *CYP3A4*, *CYP2E1*, *CYP2C9*, *HNFI1A*, *HNF4A*), low AFP expression

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Table 1. Clinical, phenotypic and molecular features of the series.

Variables	Available data (n)	n (%)
Age (>60 years)	343	234 (68%)
Gender (Male/female)	343	293 (85%)/50 (15%)
Aetiology		
Alcohol/HBV/HCV/haemochromatosis	337	138 (41%)/58 (17%)/61 (18%)/28 (8%)
MS/undetermined	335	58 (17%)/39 (8%)
BCLC (0/A/B/C)	343	12 (3%)/220 (64%)/57 (17%)/54 (16%)
Pre-operative serum alpha-fetoprotein >100 ng/ml	278	76 (28%)
Histological and gross features of the tumors		
Tumour size >50 mm	343	208 (61%)
Intact tumour capsule	342	38 (11%)
Satellite nodules	343	157 (46%)
Macrovascular invasion, microvascular invasion	343	56 (18%)/205 (60%)
Edmonson grade: I-II/III-IV	343	147 (43%)/196 (57%)
Architectural pattern: Microtrabecular/macrotrabecular/ pseudoglandular/compact	343	252 (73%)/180 (52%)/182 (53%)/156 (45%)
Tumoral cholestasis/clear cells	343	86 (25%)/105 (31%)
Pleomorphic cells/multinucleated cells	343	21 (6%)/76 (22%)
Tumoural steatosis	343	98 (29%)
Sarcomatous changes/hyaline bodies	343	39 (11%)/55 (16%)
Macrotrabecular-massive/steatohepatic subtype	343	36 (10%)/20 (6%)
Scirrhous/lymphoepithelioma-like subtype	343	18 (5%)/6 (2%)
Non-tumour liver fibrosis		
METAVIR F0-F1/F2-F3/F4	343	130 (38%)/113 (33%)/99 (29%)
Immunohistochemical phenotype of the tumours		
β-catenin nuclear expression/GS strong and diffuse staining	218	78 (36%)/84 (39%)
CK19/EPCAM expression	219/218	16 (7%)/30 (14%)
CRP/ARID1A expression	214/216	118 (55%)/153 (71%)
Phospho-S6 (mean% of stained cells, range)	211	32 (0–100)
Phospho-ERK expression	203	21 (10%)
Gene alterations		
TERT promoter	322	208 (65%)
CTNNB1	332	130 (40%)
TP53	341	72 (21%)
ALB	170	27 (16%)
ARID1A	313	45 (14%)
AXIN1	322	34 (11%)
APOB	171	18 (11%)
ARID2	313	23 (7%)
NFE2L2	308	19 (6%)
TSC1/TSC2	239	15 (6%)
ATM	238	15 (6%)
RPS6KA3	311	16 (5%)
CDKN2A	129	7 (5%)
ACVR2A	129	6 (5%)
FGF19 amplifications	129	6 (5%)
Transcriptomic subgroups		
G1/G2/G3/G4/G5/G6	265	13 (5%)/15 (6%)/52 (20%)/91 (34%)/61 (23%)/32 (12%)

and low cell proliferation compared to non-mutated HCC (Fig. 1E). Interestingly, a major deregulation in bile salt transporters expression (upregulation of transporters involved in canalicular bile salt efflux and downregulation of those responsible for bile salts efflux into blood flow) was observed and may contribute to the cholestatic phenotype of these tumours (Fig. 1E, F).

TP53 mutated HCC are poorly differentiated, highly proliferative and macrotrabecular-massive

TP53 mutations, identified in 21% of the tumours, were associated with poor differentiation (OR 6.41, $p < 0.001$), macrovascular (OR

2.57, $p = 0.004$) and microvascular (OR 2.03, $p = 0.02$) invasion, areas of compact histological pattern (OR 1.90, $p = 0.02$), foci of sarcomatous changes (OR 2.67, $p = 0.01$), pleomorphic (OR 3.33, $p = 0.02$) and multinucleated (OR 2.27, $p = 0.01$) cells, and lack of tumour cholestasis (OR 5.75, $p < 0.001$) (Fig. 2A–C; Table S3). No relationship between TP53 mutations and the degree of intra-tumour heterogeneity was observed (median number of architectural patterns/cytological variants per tumour: TP53 mutated 4 vs. TP53 non-mutated 4). Interestingly, TP53 mutated tumours exhibited PI3K/AKT pathway activation, as assessed by an overexpression of phospho-S6 protein by tumour cells using immunohistochemistry (Fig. 2A; Table S3). Gene expression

Table 2. Analysis of clinical, biological and histological features for early recurrence and disease-specific survival.

	Early recurrence				Disease-specific survival			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	OR (CI 95%)	p value	OR (CI 95%)	p value	OR (CI 95%)	p value	OR (CI 95%)	p value
Clinical and biological features								
Age >60 years	1.06 (0.72–1.57)	0.71			0.90 (0.52–1.57)	0.71		
Male sex	1.23 (0.71–2.11)	0.45			1.42 (0.61–3.32)	0.42		
BCLC stage B-C*	2.38 (1.64–3.42)	<0.001			3.25 (1.89–5.58)	<0.001		
Alcohol	0.94 (0.65–1.37)	0.77			1.20 (0.71–2.05)	0.49		
HCV infection	1.32 (0.86–2.04)	0.20			0.91 (0.46–1.81)	0.79		
HBV infection	1.15 (0.72–1.85)	0.56			1.45 (0.76–2.75)	0.26		
Metabolic syndrome	0.69 (0.38–1.26)	0.23			0.65 (0.26–1.64)	0.36		
AFP serum level >100 ng/ml	1.43 (0.95–2.18)	0.09			2.6 (1.47–4.61)	0.001	1.44 (0.77–2.67)	0.25
Pathological features								
Tumour size >50 mm	1.42 (0.97–2.08)	0.08			2.53 (1.30–4.91)	0.004	1.19 (0.56–2.52)	0.65
Intact tumour capsule	0.57 (0.27–1.23)	0.15			0.52 (0.16–1.67)	0.26		
Satellite nodules	3.40 (2.31–5.02)	<0.001	2.02 (1.28–3.18)	0.002	4.60 (2.47–8.60)	<0.001	1.68 (0.77–3.67)	0.19
Macrovascular invasion*	3.54 (2.39–5.26)	<0.001	1.82 (1.17–2.86)	0.008	6.00 (3.51–10.28)	<0.001	2.80 (1.47–5.35)	0.002
Microvascular invasion	2.85 (1.88–4.31)	<0.001	1.60 (0.97–2.62)	0.06	8.24 (3.28–20.7)	<0.001	3.24 (1.04–10.06)	0.04
Edmonson grade III-IV	1.57 (1.08–2.27)	0.004	1.29 (0.86–1.93)	0.21	3.13 (1.65–5.94)	<0.001	1.59 (0.79–3.20)	0.19
Macrotrabecular pattern	0.99 (0.66–1.49)	0.63			0.65 (0.38–1.15)	0.14		
Macrotrabecular pattern*	1.79 (1.23–2.62)	0.002			3.29 (1.73–6.25)	<0.001		
Compact pattern	0.88 (0.61–1.29)	0.54			0.88 (0.51–1.53)	0.66		
Pseudoglandular pattern	0.82 (0.57–1.19)	0.30			0.74 (0.43–1.26)	0.26		
Inflammatory infiltrates	0.64 (0.42–0.99)	0.02	0.65 (0.41–1.02)	0.06	0.57 (0.29–1.11)	0.09		
Cholestasis	1.19 (0.79–1.79)	0.70			0.84 (0.44–1.61)	0.61		
Clear cells	0.80 (0.53–1.21)	0.42			0.97 (0.53–1.76)	0.92		
Multinucleated cells	0.87 (0.54–1.39)	0.47			1.49 (0.82–2.71)	0.18		
Tumour steatosis	0.78 (0.52–1.18)	0.16			0.71 (0.47–1.07)	0.09		
Sarcomatous changes	0.78 (0.43–1.43)	0.48			0.80 (0.32–2.01)	0.63		
Hyaline bodies	0.94 (0.59–1.50)	0.68			0.88 (0.43–1.81)	0.73		
Macrotrabecular-massive subtype*	2.88 (1.71–4.83)	<0.001	1.70 (0.99–2.91)	0.05	5.68 (3.07–10.47)	<0.001	2.19 (1.06–4.50)	0.03
METAVIR F0-F1	0.94 (0.65–1.36)	0.66			0.79 (0.45–1.40)	0.42		
METAVIR F2-F3	0.90 (0.61–1.32)	0.48			0.88 (0.50–1.57)	0.67		
METAVIR F4	1.21 (0.82–1.79)	0.21			1.46 (0.83–2.55)	0.18		

InsermU1162 Cohort, n = 278, only features with more than 10% infrequency were selected. Statistical analysis was performed using univariate and multivariate Cox proportional hazards regression models (Wald test).

OR, odds ratio; CI, confidence interval; AFP, alpha-fetoprotein.

* For collinear variables (BCLC B-C and macrovascular invasion; macrotrabecular histological pattern and macrotrabecular-massive subtype), a bivariate analysis was performed prior to the multivariate analysis. The variable with the highest OR was further included in the multivariate analysis.

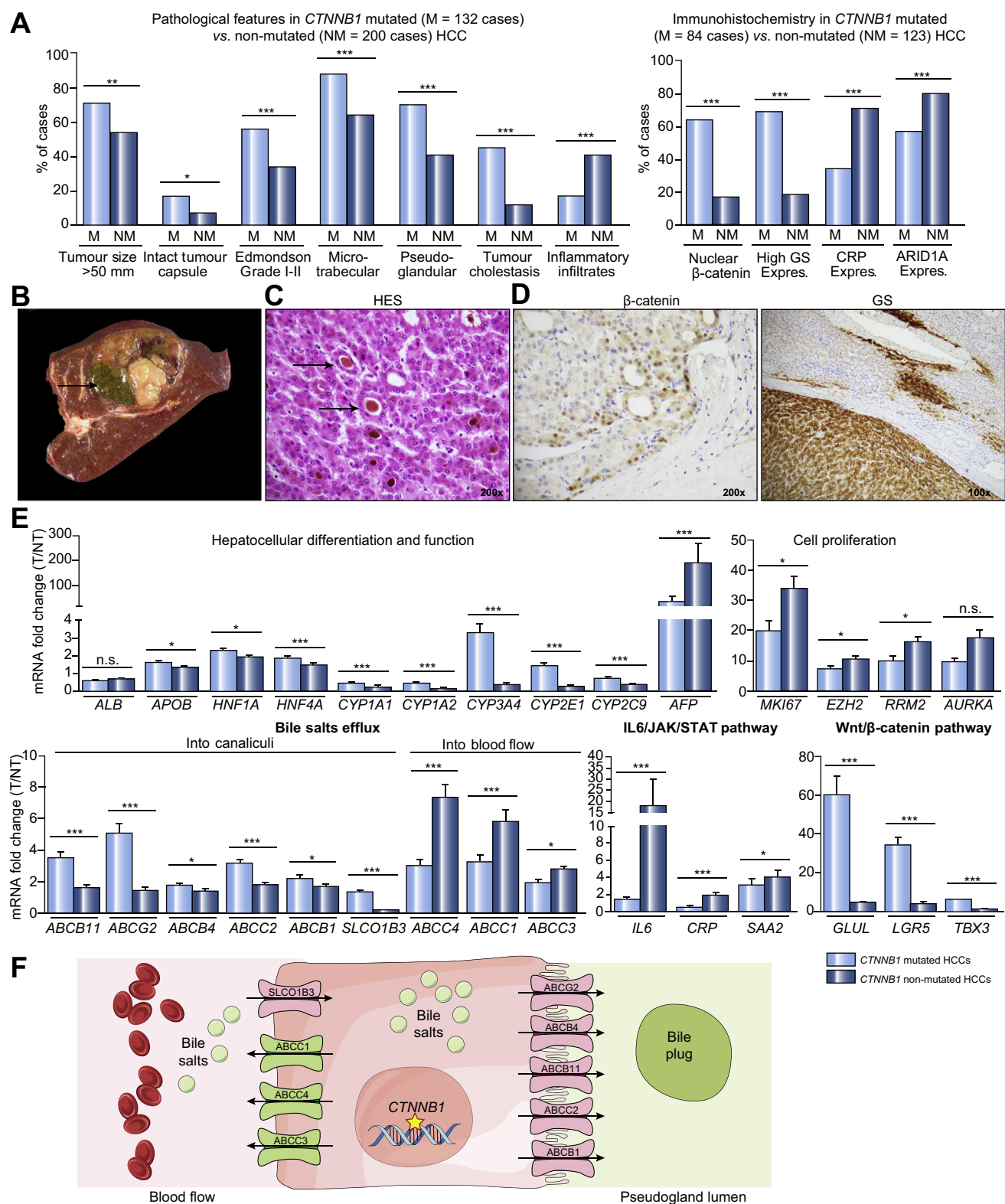
experiments showed an association of *TP53* mutations with increased cell proliferation, epithelial-to-mesenchymal transition and angiogenesis activation (Fig. 2D).

A heat-map graphical representation of the main pathological and immunohistochemical features linked to *CTNNB1* mutated (tumour cholestasis, β -catenin nuclear staining and high GS expression), *TP53* mutated (compact pattern, macrotrabecular-massive subtype, Edmondson-Steiner III-IV and microvascular invasion) and non-mutated (inflammatory infiltrates and CRP expression) HCC cases is provided in Fig. 2E.

Noticeably, *TP53* mutations were also strongly correlated to the novel macrotrabecular-massive subtype of HCC (MTM-HCC) identified during the pathological review ($p < 0.001$) (Figs. 2A and 3A–D). This subtype, representing 10% of all tumours and characterized by a predominant (>50%) macrotrabecular growth pattern, was more frequent in HBV infected patients ($p = 0.007$), with high AFP serum levels ($p = 0.02$) and histological features of aggressiveness (satellite nodules $p < 0.001$, macrovascular $p < 0.001$ and microvascular $p < 0.001$ invasion) (Fig. 3E; Table S4). Regarding molecular alterations and gene expression, we observed an association of MTM-HCC

with G3 subgroup ($p < 0.001$), *TP53* ($p < 0.001$) and *ATM* mutations ($p = 0.03$), *FGF19* amplifications ($p = 0.02$), and angiogenesis activation (upregulation of *ANGPT2* $p = 0.004$ and an almost significant trend towards *VEGFA* overexpression $p = 0.07$) (Fig. 3E and Table S5). This subtype also frequently demonstrated a progenitor phenotype (CK19 immunohistochemical expression $p = 0.02$) (Fig. 3E). Finally, MTM-HCC was significantly associated, in multivariate analysis, with early tumour relapse ($p = 0.05$) and poor overall survival ($p = 0.03$) (Table 2 and Fig. 3F). Moreover, it retained a prognostic value even when patients were stratified according to the existence of microvascular invasion and satellite nodules ($p < 0.001$).

We took advantage of the TCGA public database to validate the relevance of this novel subtype. Available digital slides were reviewed without knowledge of clinical, biological and molecular features. We identified 61 tumours (17%, 61/358) with histological features of MTM-HCC (Table S6). We were able to validate the associations of MTM-HCC with high AFP serum levels ($p = 0.001$), macrovascular ($p = 0.002$) and microvascular ($p = 0.003$) invasion, *TP53* mutations ($p < 0.001$), *FGF19* amplifications ($p = 0.007$) and activation of angiogenesis with *ANGPT2* upregulation (z-scores



0.55 vs. -0.18, $p < 0.001$) (Fig. 3E). MTM-HCC was also associated, in univariate analysis, with early tumour relapse ($p = 0.002$) and poor overall survival ($p = 0.02$) (Fig. 3F).

Associations of other HCC subtypes with clinical and molecular features

We further aimed to determine the potential clinical and/or biological implications of other recognized HCC subtypes:

- The scirrhous HCC subtype, defined by marked stromal fibrosis, was characterized by *TSC1/TSC2* ($p = 0.005$), lack of *CTNNB1* ($p < 0.001$) mutations and CK19 expression ($p < 0.001$) (Fig. 4A–C; Table S4). In this line, RT-PCR experiments revealed an upregulation of progenitor/cancer stem cell genes (*CD24*, *KRT19*, *THY1*, *CD133*), and epithelial-to-mesenchymal transition activation (*TGFβ*, *VIM*) (Fig. 4D; Table S5). A trend of increased phospho-S6 protein expression was observed (50% vs. 31%, $p = 0.2$).
- The steatohepatic subtype of HCC was not related to specific clinical features, but displayed a less aggressive histological phenotype with a lack of satellite nodules ($p = 0.02$) and microvascular invasion ($p = 0.04$) (Fig. 4E, F; Table S4). At the molecular level, it was associated with the G4 transcriptional subgroup and to a lack of Wnt/β-catenin pathway activation (lack of *CTNNB1* mutations $p = 0.01$ and low GS expression $p = 0.005$) (Fig. 4F). Activation of the IL6/JAK/STAT pathway was frequent with CRP immunohistochemical expression ($p = 0.02$) (Fig. 4F; Table S4). Although histological appearance of this subtype might suggest dysregulation of metabolic processes, no significant changes in genes involved in fatty acid synthesis, glycolysis or neoglucogenesis were observed (Table S5).
- Finally, the lymphoepithelioma-like subtype was not associated with any clinical or molecular feature, however our series only comprised six cases of this rare variant.

Integrative analysis of the pathological phenotype with the molecular HCC classification

For 265 tumours with available classification, we integrated the various histopathological, clinical and genetic features into the G1–G6 transcriptomic classification to obtain a more global view of the molecular and pathological correlations (Fig. 5; Table S7).

Tumours belonging to the transcriptomic G1G2G3 subclasses, known to be characterized by high cell proliferation and chromosomal instability, were associated with several clinico-biological features: high AFP serum levels (G1G2G3, $p < 0.001$), female gender (G1, $p < 0.001$) and haemochromatosis (G3, $p = 0.01$). Gene

sequencing showed enrichment in *TP53* (G1G2G3, $p < 0.001$), *RPS6KA3* (G1, $p = 0.03$), *AXIN1* and *ATM* (G1G2, $p = 0.004$ and 0.01 , respectively) mutations, and *FGF19* and *TSC1/TSC2* alterations (G3, $p = 0.02$ and 0.01 , respectively). Histologically, G1G2G3 HCCs were poorly differentiated (G1G2G3, $p < 0.001$), with frequent macrovascular invasion (G3, $p = 0.01$) foci of clear cells (G1, $p = 0.001$), sarcomatous changes (G1G2, $p = 0.02$), areas of compact (G3, $p = 0.01$) and macrotrabecular (G3, $p < 0.001$) histological patterns, and foci of pleomorphic (G3, $p = 0.04$) and multinucleated (G3, $p < 0.001$) cells. The G1 subtype showed a progenitor phenotype with both CK19 ($p = 0.002$) and EpCAM ($p < 0.001$) immunohistochemical expression. Activation of MAP-Kinase and PI3K/AKT pathways, as assessed by phospho-ERK and phospho-S6 protein expression, were identified in tumours classified as G1 ($p = 0.02$) and G3 ($p = 0.02$), respectively (Fig. 5).

Tumours classified in G4G5G6 subclasses, characterized by low cell proliferation and chromosomal stability, were most often well-differentiated (G4G5G6, $p < 0.001$). G4 HCCs were additionally associated with small tumour size ($p = 0.03$), lack of satellite nodules ($p = 0.004$) and microvascular invasion ($p < 0.001$), steatohepatic subtype ($p = 0.004$), inflammatory infiltrates ($p = 0.04$) and CRP expression ($p = 0.01$). They were also characterized by a lack of *CTNNB1* ($p < 0.001$) and *TP53* ($p = 0.047$) mutations (Fig. 5). G5G6 subclasses, strongly associated with *CTNNB1* activating mutations ($p < 0.001$), were related to the microtrabecular pattern of growth ($p = 0.01$), the presence of tumour cholestasis ($p < 0.001$) and a lack of inflammatory infiltrates ($p = 0.01$). Immunohistochemical experiments showed Wnt/β-catenin pathway activation with β-catenin nuclear staining ($p < 0.001$) and strong GS expression ($p < 0.001$), along with lack of ARID1A expression ($p = 0.002$).

Discussion

The present study revealed strong relationships linking molecular and pathological features in HCC. Two distinct HCC phenotypes were delineated by the mutually exclusive *CTNNB1* and *TP53* mutations that define 57% of all tumours. Other more infrequent molecular defects were also associated with rare pathological features such as *TSC1/TSC2* mutations and the scirrhous subtype.

CTNNB1 mutations activating β-catenin are oncogenic driver mutations associated with a specific HCC subtype present in well-differentiated tumours with cholestasis and microtrabecular and pseudoglandular patterns, as previously reported.³¹ Interestingly, our gene expression experiments provide additional insights into the molecular basis of the particular phenotype of *CTNNB1* mutated tumours: their good differentiation is indeed associated with the maintenance of several markers of hepatocel-

Fig. 1. Pathological features of *CTNNB1* mutated tumours. (A) Gross, microscopic and immunohistochemical features associated with *CTNNB1* mutations. (B) Gross appearance of a *CTNNB1* mutated tumour: the tumour has an intact capsule and a green color due to bile production. (C) Microscopic features of a *CTNNB1* mutated HCC: microtrabecular and pseudoglandular architectural pattern with numerous bile plugs within the lumens of the pseudoglands (black arrows) (#CHC1715T). (D) Nuclear β-catenin staining (left panel, #CHC1715T) with strong GS expression (right panel, #CHC1715T). (E) Gene expression profiling of *CTNNB1* mutated tumours: maintenance of markers of hepatocellular differentiation and function, low cell proliferation, dysregulation in bile salts transporters expression with upregulation of transporters involved in canalicular bile salts efflux and downregulation of transporters responsible for bile salts efflux into blood flow, low IL6/JAK/STAT pathway activation and upregulation of Wnt/β-catenin pathway targets (*CTNNB1* mutated cases $n = 35$; *CTNNB1* non-mutated cases, $n = 149$). (F) Schematic representation of a *CTNNB1* mutated neoplastic cell: the dysregulation of bile salts transporters (upregulation = violet, downregulation = yellow) leads to bile salts uptake from the blood flow and excretion into the canaliculi (pseudogland lumen). HES, Hematein-Eosin-Saffron; Expres., expression; T, tumour; NT, non-tumour. Comparison between qualitative and quantitative data was performed using Chi-square and Fisher exact tests and Mann-Whitney *U* and Kruskal-Wallis tests, respectively. Monte-Carlo method was used to adjust the *p* value for multiple tests.

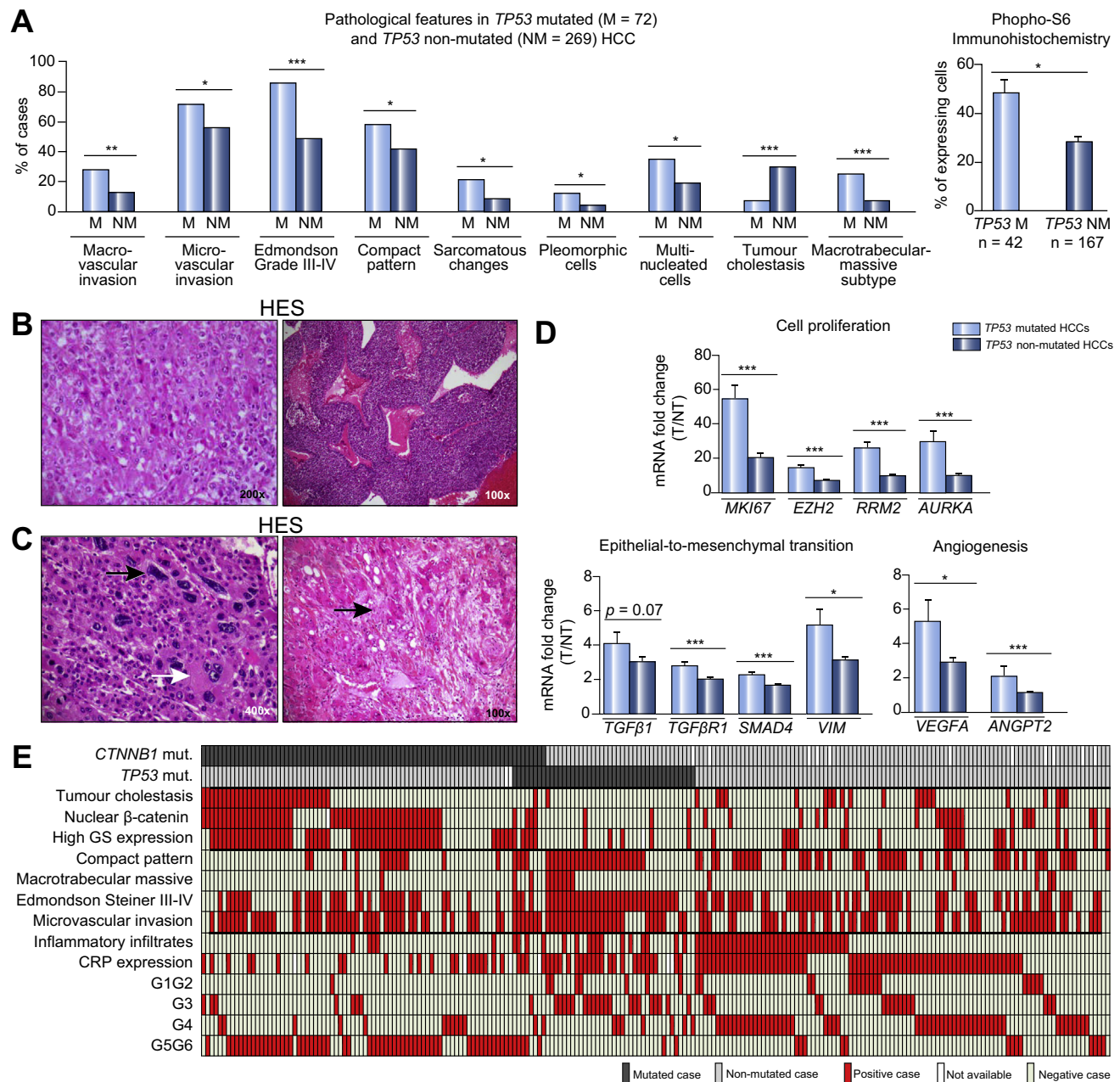


Fig. 2. Pathological features of *TP53* mutated tumours. (A) Gross, microscopic and immunohistochemical features associated with *TP53* mutations. (B) Micrographs of compact (left panel) and macrotrabecular (right panel) architectural patterns. (C) Micrographs of pleomorphic (black arrow) and multinucleated (white arrow) cells (left panel, #CHC1754T), and sarcomatous changes characterized by elongated neoplastic cells embedded in a fibrous stroma (black arrow, right panel, #CHC1182T). (D) RT-PCR experiments showed increased cell proliferation and activation of epithelial-to-mesenchymal transition and angiogenesis (*TP53* mutated HCCs n = 37, *TP53* non-mutated HCCs n = 149). (E) Heat-map graphical representation of the main pathological and immunohistochemical features linked to *CTNNB1* and *TP53* mutational status (series with available immunohistochemistry, n = 219). HES, Hematein-Eosin-Saffron; mut., mutation; M, mutated; NM, non-mutated. Comparison between qualitative and quantitative data was performed using Chi-square and Fisher exact tests and Mann-Whitney *U* and Kruskal-Wallis tests, respectively. Monte-Carlo method was used to adjust the *p* value for multiple tests.

lular differentiation and function, while the dysregulation of bile salt transporters expression may account for their cholestatic appearance. Noticeably, detection of malignant and benign *CTNNB1* mutated hepatocellular tumours by the Magnetic Resonance Imaging contrast agent gadoxetic acid has been demonstrated to rely on the overexpression of one of these transporters, *SLCO1B3*, which is responsible for bile salt uptake from the blood flow.^{32,33} In the present study, we identified an inverse

correlation between intra-tumour inflammatory infiltrates and Wnt/ β -catenin pathway activation. In this line, *CTNNB1* mutated HCC showed a downregulation of several components of the IL6/JAK/STAT pathway. In contrast, activation of the Wnt/ β -catenin signaling pathway has been reported to induce, in the liver of modified mouse models, a specific inflammatory response with an increase of intra-parenchymal inflammatory cells.³⁴ Discrepancies between human HCC and mouse models might

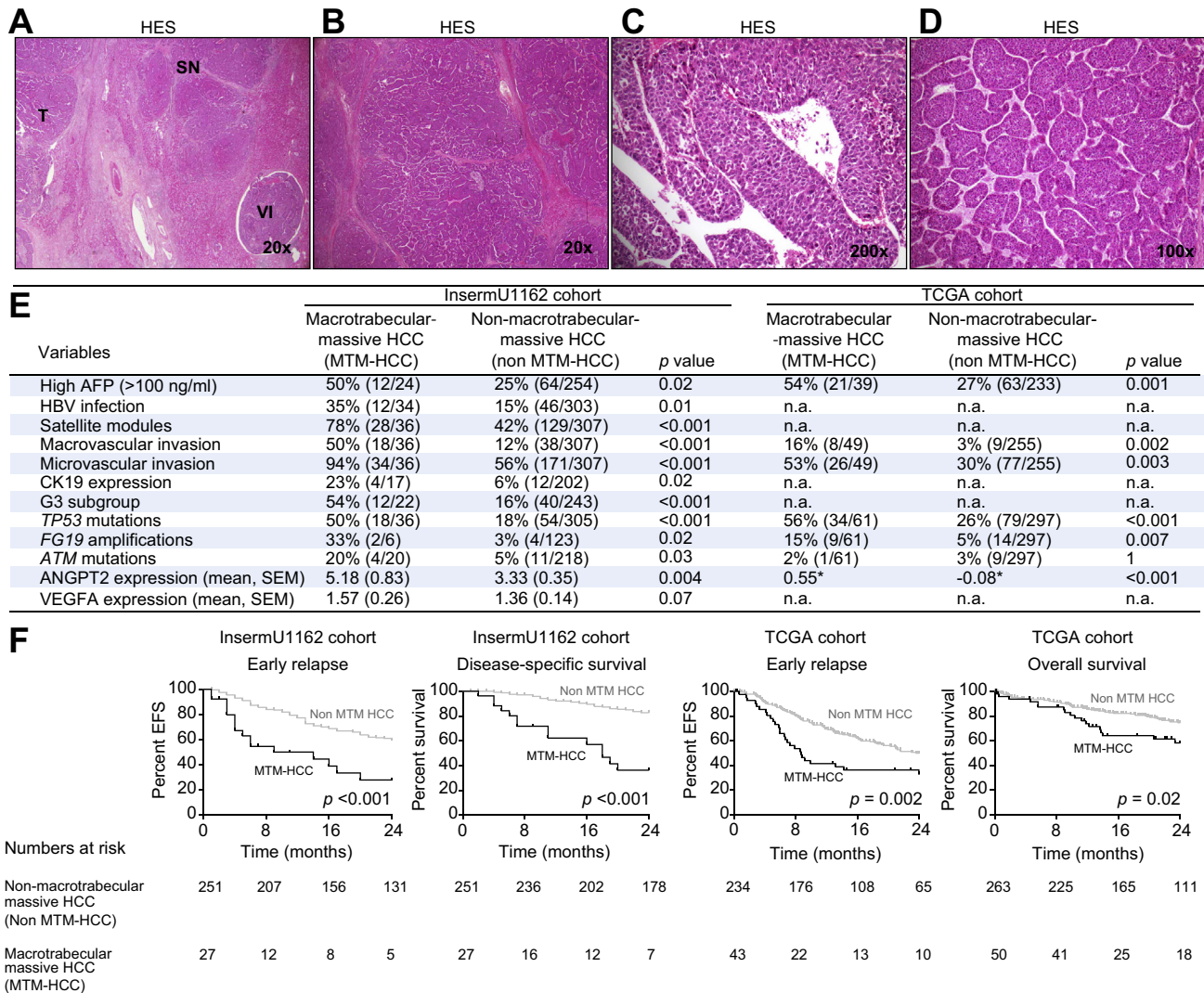


Fig. 3. Clinical, pathological and molecular features of macrotrabecular-massive HCC. (A) Low magnification of a macrotrabecular-massive HCC, with a vascular invasion (VI) and a satellite nodule (SN) located outside the main tumour (T) (#CHC1754T). (B) Identification of a predominant macrotrabecular pattern in this low-power microscopic field (#CHC1754T). (C) Thick macrotrabeculae coated by endothelial cells and surrounded by vascular spaces (#CHC1754T). (D) Trabeculae appearing as tumour clusters when observed in cross sections (#CHC1754T). (E) Associations between macrotrabecular-massive HCC subtype and clinical, pathological and molecular features in both cohorts (for RT-PCR experiments of InsermU1162 cohort MTM-HCC *n* = 12, Non-MTM-HCC *n* = 174; for RNA sequencing experiments of TCGA cohort MTM-HCC *n* = 61, Non-MTM-HCC *n* = 292). (F) Association of macrotrabecular-massive HCC with early relapse and shorter disease-specific survival. HES, Hematein-Eosin-Saffron; EFS, Event-free survival, n.a., not available. Comparison between qualitative and quantitative data was performed using Chi-square and Fisher exact tests and Mann-Whitney *U* and Kruskal-Wallis tests, respectively. Monte-Carlo method was used to adjust the *p* value for multiple tests. *ANGPT2 expression data from the TCGA cohort was compared using the z-scores provided on the TCGA webportal.

indicate that β -catenin activation could act as a pro- and anti-inflammatory signal in mice and human, respectively. Interestingly, anti-inflammatory effects were previously identified in other human tumours such as melanoma, in which Wnt/ β -catenin pathway activation is indeed responsible for T-cell exclusion from the intra-tumour microenvironment.³⁵

TP53 mutations were mostly identified in poorly differentiated tumours with frequent vascular invasion and activation of cell proliferation, angiogenesis and epithelial-to-mesenchymal transition. TP53 inactivation was also noticeably linked to the novel macrotrabecular-massive HCC subtype (MTM-HCC), the relevance of which was further confirmed by additional associations with clinical (HBV infection, early relapse and poor survival), pathological (satellite nodules and vascular invasion)

and molecular (FGF19 amplifications) features. The histological appearance of this variant, consisting of thick trabeculae surrounded by vascular spaces, was reminiscent of a particular architectural pattern first described by Sugino and collaborators in a xenograft model of a mammary tumour cell line.³⁶ Consisting of endothelium-coated cell clusters, this pattern was further demonstrated to promote the *in vivo* metastatic potential of a mouse HCC cell line through the release of tumour clusters into the blood stream in an epithelial-to-mesenchymal transition independent manner.³⁷ Silencing the expression of ANGPT2 growth factor by neoplastic cells was able to disrupt the formation of this particular pattern and reduce both intrahepatic and pulmonary metastases.³⁷ ANGPT2 is known to promote neoangiogenesis and endothelial sprouting in cooperation with

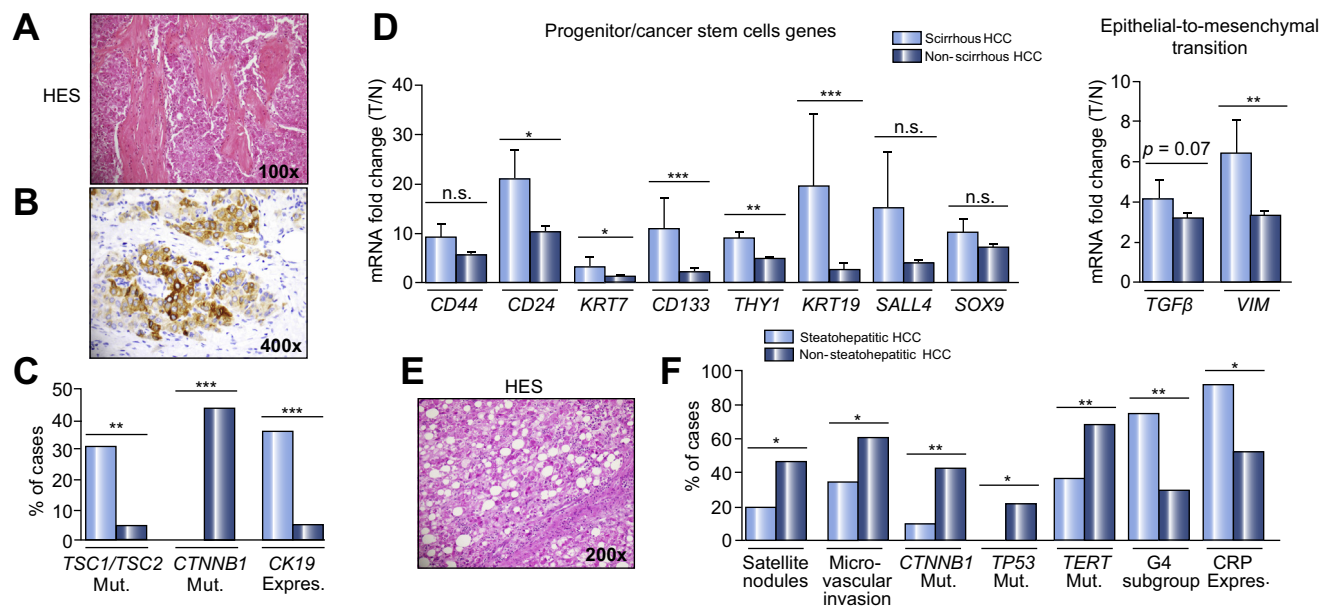


Fig. 4. Pathological and molecular features of scirrhous and steatohepatic HCC. (A) A scirrhous HCC with dense, hyalin stroma surrounding clusters of neoplastic cells (#CHC1198T). (B) Scirrhous HCC with CK19 expression (#CHC1182T). (C) Associations between the scirrhous subtype of HCC and molecular and immunohistochemical features. (D) Upregulation of progenitor/cancer stem cell genes and activation of epithelial-to-mesenchymal transition (Scirrhous HCCs $n = 12$, non-scirrhous HCCs $n = 174$). (E) A case of steatohepatic HCC with cell ballooning, steatosis, Mallory-Denk bodies and inflammation (#CHC1712T). (F) Associations between the steatohepatic subtype of HCC and pathological and molecular features. HES, Hematein-Eosin-Saffron; Mut., mutation; Express., expression. Comparison between qualitative and quantitative data was performed using Chi-square and Fisher exact tests and Mann-Whitney U and Kruskal-Wallis tests, respectively. Monte-Carlo method was used to adjust the p value for multiple tests.

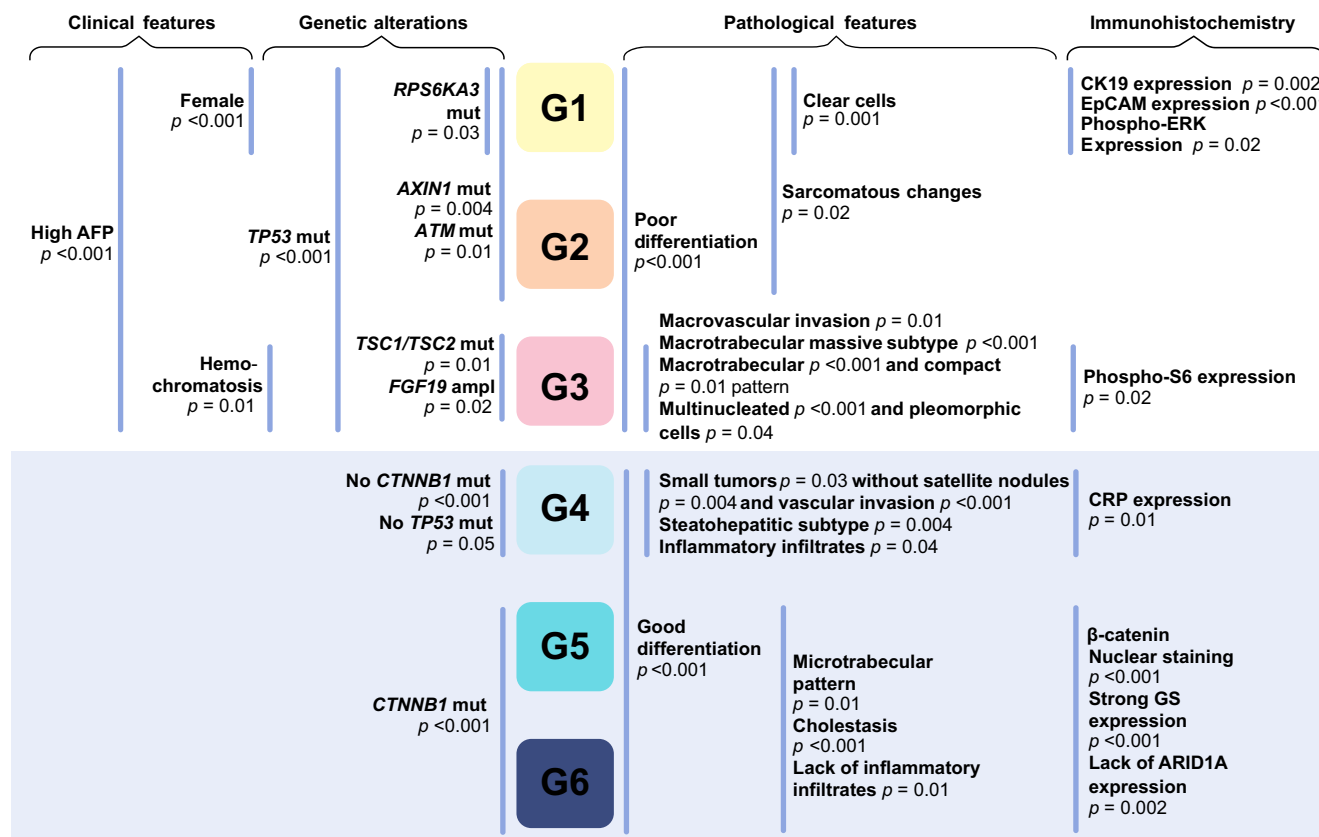


Fig. 5. Integration of clinical, pathological and molecular features into HCC molecular classification. Mut., mutation; ampl, amplification. Comparison between qualitative data was performed using Chi-square and Fisher exact test. Monte-Carlo method was used to adjust the p value for multiple tests.

Please refer to the accompanying [ICMJE disclosure](#) forms for further details.

Authors' contributions

Study design: JC, JZR

Generation of experimental data: JC, GC, JCN

Analysis and interpretation of data: JC, GC, SI, YH, JCN, JZR

Collection of samples and histological and clinical data: JC, GA, DA, CL, JFB, PBS, JZR

Drafting of the manuscript: JC, JCN, JZR

Revision of the manuscript and approval of its final version: JC, GC, SI, GA, JFB, CL, YH, DA, PBS, JCN, JZR

Obtained funding: JZR

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jhep.2017.05.014>.

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VEGFA,³⁸ and consistently, we observed higher *ANGPT2* mRNA levels in MTM-HCC, and a trend towards an increase in *VEGFA* expression. Our results may open novel therapeutic perspectives for this highly aggressive HCC subtype, as inhibitors of both *ANGPT2* and *VEGFA* signaling are available and have shown remarkable *in vivo* anti-tumour efficacy if used in combination.³⁹

Other well-recognized HCC subtypes were also related to clinical and/or molecular characteristics. The so-called steatohepatic HCC is characterized by its resemblance with non-neoplastic steatohepatitis and is considered to preferentially occur in patients with metabolic syndrome.^{7,21} In our series, we did not identify any association of this subtype with liver disease aetiology despite a proportion of 17% of patients with metabolic syndrome. We were however able to demonstrate, in keeping with a previous report by Ando and collaborators,⁴⁰ that steatohepatic HCC display a particular molecular profile mainly defined by the lack of Wnt/ β -catenin pathway activation. The oncogenic mechanisms involved in the progression of this variant thus remain to be identified.

A link between cirrhotic HCC and progenitor traits has been previously shown.⁴¹ We have here confirmed these results, with the association of CK19 expression and the upregulation of various cancer stem cell/progenitor genes. In addition, we demonstrated that this HCC subtype is also related to the activation of the PI3K/AKT pathway, with *TSC1/TSC2* mutations and a trend towards phospho-S6 overexpression. Further studies will have to be done to determine if patients with this particular variant might benefit from PI3K/AKT pathway inhibitors.

Finally, integration of clinical, genetic, and pathological features into HCC classification showed strong associations with tumour subclasses. Different oncogenic pathways drive the progression of HCC subgroups and they thus result in distinct tumour phenotypes. These findings are in keeping with those of Tan *et al.*, who showed that clinicopathological features were associated with Hoshida HCC classification.⁴²

Altogether, our study provides a comprehensive overview of the relationship between HCC phenotype and its molecular characteristics. We have showed that histological features recognized by the WHO Classification of Tumours are related to particular gene mutations and transcriptomic subgroups, and further unraveled a novel histological subtype linked to molecular alterations that may have clinical relevance. Our next challenge is to validate our findings in different clinical settings, for example in advanced tumours with only biopsy samples available. However, our results already provide the tools to translate HCC molecular classification into clinical practice.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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