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Vessels Encapsulating Tumor Clusters (VETC) Is a Powerful Predictor of Aggressive Hepatocellular Carcinoma

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We investigated the clinical significance of a vascular growth pattern of hepatocellular carcinoma (HCC), the vessels that encapsulate tumor clusters (VETC), previously linked to HCC metastatic dissemination. VETC was assessed in a large multi-institutional cohort of 541 resected HCCs from Italy, Korea and Japan, and matched against a full spectrum of clinical and pathological variables. The VETC phenotype (defined as ≥ 55% tumor area by CD34 immunostaining) was easily reproducible and reliably detectable in whole sections and small-sized tissues of tissue microarray. VETC HCCs represented 18.9% of the whole series, the lowest proportion occurring in the cohort with smallest tumors (8.7%, Japanese series). VETC was significantly associated with several clinical and pathological features such as high alfa-fetoprotein (AFP) level, tumor size greater than 5 cm, poor differentiation, macrotrabecular pattern, less compact pattern, less inflammatory infiltrates, and frequent microvascular invasion. VETC was associated with early recurrence (hazard ratio [HR]: 1.52 [1.06-2.19], P = 0.023), disease-free survival (HR: 1.66 [1.21-2.27], P = 0.002), and overall survival (HR: 2.26 [1.37-3.72], P = 0.001) at multivariable analysis. VETC affected the survival in HCC patients stratified for etiology (hepatitis C virus/hepatitis B virus), vascular invasion, and specific molecular phenotypes (β-catenin/GS+). This distinct vascular pattern was enriched in the recently reported macrotrabecular massive HCC subtype, which was seen in 7.8% (42 of 541) of patients and associated with high AFP levels and poor differentiation. *Conclusion:* The VETC pattern was found to be easily detectable in a consistent fraction of HCC and a powerful pathological finding affecting survival. This study suggests that the heterogeneous pattern of angiogenesis is involved in HCC behavior. (Hepatology 2019;0:1-13).

The development of hepatocellular carcinoma (HCC) is a multistep process from precancerous lesions (dysplastic nodule) to early and advanced HCC.[(1)] This has been widely investigated, as most research has been focused on morphological, phenotypical, and molecular features of the neoplastic cells (i.e., hepatocytes), whereas less attention has been dedicated to the dynamic changes of microenvironmental nontumoral supporting cells. Among this heterogeneous population, endothelial cells in HCC are of particular interest because they are strikingly involved in the tumor growth and represent a potential therapeutic target (transarterial chemoembolization, drugs). Intratumoral endothelial cells progressively lose sinusoidal markers, including stabilin-1, stabilin-2, LYVE-1 (lymphatic vessel endothelial hyaluronan receptor 1), CD32 (cluster of differentiation 32/34), and ICAM (intercellular adhesion molecule 1)[(2)]; at

Abbreviations: AFP, alpha-fetoprotein; BCLC, Barcelona Clinic of Liver Cancer; CI, confidence interval; DFS, disease-free survival; GS, glutamine synthetase; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HR, hazard ratio; MTM, macrotrabecular massive; OS, overall survival; TMA, tissue microarray; VETC, vessels completely encapsulating tumor clusters.

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the same time, they gain markers of continuous, nonfenestrated endothelial cells (i.e., capillary), such as CD34.[3,4] Accordingly, the process of pathological angiogenesis in HCC is mostly known as capillarization. We have recently shown that dysplastic nodule and early HCC are characterized by a focal, if any, sinusoidal capillarization/incomplete angiogenesis.[5] In contrast, diffuse capillarization/complete angiogenesis was seen in 33% of small and progressed HCC (pHCC), and in up to 90% of pHCC arisen in the so-called nodule in nodule HCC.[5,6] As in many other solid tumors, once angiogenesis is completed, HCC is more prone to progress and to metastasize. Moreover, a shift from a "dormant" angiogenesis that supports tumor growth, but not metastases, to an "active" more aggressive, pro-metastatic angiogenesis has been postulated in many organs.[7,8] Interestingly, a signature of five genes (angiopoietin-2 [*ANGPT2*], delta-like ligand 4 [*DLL4*], neuropilin/tolloid-like 2 [*NETO2*], endothelial cell-specific molecule-1 [*ESM1*], and nuclear receptor subfamily 4, group A, member 1 [*NR4A1*]), all related to angiogenesis, characterized a subset of highly aggressive and fast-growing HCC at onset.[9] It is therefore likely that vascular pattern heterogeneity can also play a role in tumor progression.[10] Interestingly, Fang et al. reported a distinct pattern of HCC vascularization that predicted rapid tumor dissemination and high recurrence rates.[11] This pattern is characterized by the presence of $CD34^+$ vessels completely encapsulating tumor clusters (VETC).[11] Although conducted in a limited HCC population (hepatitis B virus [HBV]-related and >5 cm), the authors elegantly showed VETC clusters delivering tumor emboli into larger vessels. Moreover, the same authors recently claimed sorafenib therapy to be more effective in VETC HCC.[12]

To lend further support to the hypothesis that a VETC pattern can primarily affect patient prognosis, we collected a large series of surgically resected HCCs with various etiologies from different geographic areas of high HCC incidence, where VETC was evaluated against consolidated and novel prognostic markers.

Materials and Methods

PATIENTS AND SAMPLES

The series included 541 HCCs (541 patients) treated by surgical resection in three different Institutions (Humanitas Research Hospital [Rozzano, Italy], Severance Hospital [Seoul, Korea], and Kurume University Hospital [Kurume, Japan]), consecutively collected from 2006 to 2012 and with available follow-up of not less than 2 years. Only cases staged R0 after surgical resection were included in the study. The clinical outcomes of the patients were obtained retrospectively by reviewing the electronic medical records. The endpoints were defined as follows: Overall survival (OS) was defined by the interval between surgery and death regardless of cause; and disease-free survival (DFS) was defined as the time from surgery to an initial diagnosis of recurrence regardless of location. The mean follow-up duration was 52 months.

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The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in *a priori* approval by the institution's human research committee. This study was approved by the institutional review boards of the three hospitals, and the requirement for informed consent was waived.

The following clinical features were systematically recorded: age, gender, etiology of chronic liver disease (HBV, hepatitis C virus [HCV], alcohol intake, other), and pre-operative alpha-fetoprotein (AFP) serum levels and follow-up. Staging according to the Barcelona Clinic of Liver Cancer (BCLC) system was also available in most cases.

PATHOLOGICAL EXAMINATION

The following features were analyzed regarding the general macroscopic characteristics: tumor size, multiplicity, and macrovascular invasion. Regarding the general microscopic features, the following were analyzed: tumor grade (according to Edmondson-Steiner); pattern of growth (at least 20% of the tumor area: microtrabecular, macrotrabecular, compact, or pseudoglandular); microvascular invasion; tumor capsule; inflammatory infiltrate; and cirrhosis of the surrounding liver parenchyma. Moreover, the following tumoral cytological findings were recorded: cholestasis, clear, multinucleated and pleomorphic cells, sarcomatous changes, and hyaline bodies. The tumoral subtype (scirrhous, lymphoepithelioma-like, sarcomatoid, steatohepatitic, and the recently described macrotrabecular massive [MTM] subtype, the latter in at least 50% tumor area[13,14]) and several phenotypic markers (glutamine synthetase [GS], β-catenin, p53, and CD34) were also recorded.

All available histological slides were reviewed by at least 2 expert liver pathologists (L.D.T., H.Y.W., Y.N.P., M.R., H.Y., and J.A.), who were blinded to the clinical data.

IMMUNOHISTOCHEMISTRY

Tissue microarrays (TMAs; diameter ranging from 1-2 mm) from the surgical specimens were used to investigate the expression of phenotypic markers. Six different tumoral cores were obtained from central (n° 3) and peripheral areas (n° 3); three additional spots were also obtained from the surrounding liver parenchyma.

Immunohistochemical stain was performed using an automated staining system (Discovery XT; Ventana Medical Systems, Oro Valley, AZ) according to the manufacturer's instructions; antibodies, sources, clones, and dilutions are detailed in Supporting Table S1. The quantification of the phenotypic markers was done as follows: positivity was defined as unequivocal immunoreactivity (GS [cytoplasmic], β-catenin [nuclear], and p53 [nuclear]) in at least 50% of the tumor hepatocytes. Regarding CD34 evaluation, unequivocal immunoreactivity of a continuous lining around tumor clusters was defined as VETC, and the area of VETC was semi-quantitatively evaluated from 0% to 100% in 5% of the units. This VETC pattern was considered alternative to the other common capillary pattern, sustained by the growth of small circular vessels. Both patterns are illustrated in Supporting Fig. S1. To check the correlation between the VETC of whole sectioned slides and that of TMAs, an immunohistochemical stain for CD34 was performed in matched whole sections and the TMAs of 96 HCCs.

STATISTICAL ANALYSIS

Categorical variables were compared using the χ^2 test or Fisher's exact test. Kaplan-Meier survival curves and log-rank statistics were used to evaluate the time to early (≤2 years) or late (>2 years) recurrence and OS. Multivariable regression analysis was performed using the Cox proportional hazards model. After we confirmed that VETC is a meaningful prognostic factor as a continuous variable, we used the K-adaptive partitioning algorithm to find the point at which the log-rank statistics are maximized and obtained the optimal cutoff value.

The intraclass correlation coefficient was used to compare the VETC (percentage) of surgical specimens and TMAs in 96 cases, and the Fleiss' kappa value was used to compare the agreement of the 4 reviewers (H.Y.W., Y.N.P., M.R., and L.D.T.) in 60 cases. Both values were considered to be good agreement when it was 0.6 or more.

All statistical analyses were performed using SPSS software (version 21.0; IBM Statistics, Armonk, NY) and R software (version 3.5.1 for Windows; the R foundation for statistical computing, Vienna, Austria). All statistical tests were two-tailed; $P < 0.05$ was considered statistically significant, and $0.05 \leq P < 0.1$ was considered a trend toward significance, to increase the sensitivity to detect potential selection bias.

Results

CLINICO-PATHOLOGIC FEATURES

The clinico-pathologic features of the whole series are reported in Table 1, and those of Italian, Korean, and Japanese cohorts are summarized in Supporting Table S2.

Briefly, there was a strong male predominance (78.2%). Most of the cases were related to HBV (51.8%) or HCV (34.8%) infections, and rarely to alcohol intake (5.5%). Most of the Italian and Korean cases were BCLC 0/A (85.2%). Most HCCs were 5 cm or less (82.4%), and the Japanese cohort showed the smallest HCCs (0.8% > 5 cm). Multiple tumors accounted for 16.3%. Poorly differentiated HCCs (Edmondson grade III-IV) were seen in 46.6%. An

TABLE 1. Clinical, Pathological, and Phenotypical Features of the Whole HCC Series (n = 541)

Variables	n (%)
Clinical Features	
Age (>60 years)	290 (53.6)
Gender (male/female)	423 (78.2)/118 (21.8)
Etiology (HBV/HCV/alcohol/undetermined)	280 (51.8)/188 (34.8)/30 (5.5)/43 (7.9)
BCLC (0/A/B/C)*	42 (10.1)/311 (75.1)/38 (9.2)/23 (5.6)
Pre-operative serum AFP >400 ng/mL	95 (17.6)
Pre-operative treatment	20 (3.7)
General Macroscopic	
Tumor size >5 cm	95 (17.6)
Multiplicity	88 (16.3)
Macrovascular invasion	31 (5.7)
General Microscopic	
Edmondson grade I-II/III-IV	289 (53.4)/252 (46.6)
Microtrabecular/macrotrabecular/pseudoglandular/compact	398 (73.6)/307 (56.7)/195 (36.0)/234 (43.3)
Microvascular invasion	292 (54.0)
Capsule infiltration	297 (54.9)
Inflammatory infiltrates	144 (26.6)
Fibrosis of parenchyma (METAVIR F0-F3/F4)	270 (49.9)/271 (50.1)
Tumoral Cytological Findings	
Tumoral cholestasis/clear cells	93 (17.2)/159 (29.4)
Pleomorphic cells/multinucleated cells	156 (28.8)/148 (27.4)
Sarcomatous changes/hyaline bodies	12 (2.2)/113 (20.9)
Tumoral Variants	
Scirrhous/lymphoepithelioma-like/sarcomatoid/steatohepatitic/ macrotrabecular-massive	8 (1.5)/6 (1.1)/3 (0.6)/38 (7.0)/42 (7.8)
VETC Phenotype	102 (18.9)
Molecular Phenotypes†	
p53+	108 (20.0)
βcatenin/GS+	122 (22.6)
Double positive	11 (2.0)
Double negative	300 (55.5)
Follow-up‡	
Nonsurvivor	96 (18.0)
Recurrence	240 (45.1)
Early recurrence (≤2 years)	174 (32.7)
Late recurrence (>2 years)	66 (12.4)

*Available in 414 cases.
†p53+: p53+ and βcatenin/GS-; βcatenin/GS+: p53- and βcatenin/GS+; double positive: p53+ and βcatenin/GS+; and double negative: p53- and βcatenin/GS-.
‡Available in 532 cases.

average of 3.3 architectural patterns or cytological features were observed in each case. Microtrabecular, macrotrabecular, pseudoglandular, and compact patterns were identified in 73.6%, 56.7%, 36.0%, and 43.3% of the tumors, respectively. MTM HCC was the most common subtype identified (7.8%), followed by steatohepatitic (7%), scirrhous (1.5%), lymphoepithelioma-like (1.1%), and sarcomatoid HCC (0.6%).

HCCs were further classified according to the following molecular phenotypes: p53+ and β-catenin/GS- (briefly: p53+) 20.0%; p53- and β-catenin/GS+ (briefly: β-catenin/GS+) 22.6%; p53+ and β-catenin/GS+ (briefly: double positive) 2.0%; and p53- and β-catenin/GS- (briefly: double negative) 55.5%.

Follow-up data were available for 532 patients: recurrence and death occurred in 45.1% (240 of 532) and 18.0% (96 of 532) of HCC patients, respectively. Early (≤2 years) and late (>2 years) recurrence was observed in 32.7% (174 of 532) and 12.4% (66 of 532) of HCC in the whole series.

VETC PATTERN IS A ROBUST PREDICTOR OF HCC AGGRESSIVENESS, RELATED TO VASCULAR PERMEATION

The degree of VETC positive area ranged from 0% to 100% of the tumor surface. The reliability between VETC of whole sectioned slides and that of TMA was evaluated in 96 matched cases, and it was good (intraclass correlation coefficient [95% confidence interval (CI): 0.642] 0.489-0.733); then VETC was evaluated in a total of 541 HCCs using TMA. At least 5% VETC tumor area was seen in 39.0% (222 of 541) of HCCs, and the median value of VETC was 0% (Q1-Q3, 0%-35%). Multivariable regression analysis with the collected parameters confirmed that VETC, evaluated in 5% unit, was a meaningful prognostic factor. VETC was significantly associated with poorer DFS and OS (DFS HR: 1.02 [1.00-1.05]; $P = 0.019$; OS HR: 1.04 [1.01-1.07]; $P = 0.020$) and with a tendency toward early relapse (HR: 1.02 [1.00-1.05]; $P = 0.094$) at multivariable analysis.

By using K-adaptive partitioning algorithm, we set a value of 55% as the optimal cutoff value of VETC phenotype to predict prognosis. By applying the cutoff value of VETC 55%, HCCs were further divided into VETC-HCC and non-VETC-HCC. The agreement of the VETC phenotype among 4 reviewers (H.Y.W., L.D.T., M.R., and Y.N.P.) was good (Fleiss' kappa value [95% CI]: 0.887 [0.784-0.991]). The VETC phenotype (≥55%) was observed in 18.9% of HCCs (102 of 541) and was found in 23.5% of the Italian cohort, 21.5% of the Korean cohort, and 8.7% of the Japanese cohort. The VETC phenotype was not associated with specific etiologic factors. It was significantly higher in HCC patients with high AFP serum level (>400 ng/mL, $P < 0.001$), larger tumor size (>5 cm, $P < 0.001$), Edmondson grade III-IV ($P < 0.001$), macrotrabecular pattern ($P < 0.001$), MTM subtype ($P = 0.006$), microvascular invasion ($P < 0.001$), and less inflammatory infiltrates ($P = 0.013$). A trend toward significance was found for macrovascular invasion ($P = 0.059$). For molecular subgroups, VETC was enriched in β-catenin/GS+ and in the double-positive HCC ($P = 0.025$ and $P = 0.038$, respectively) and impoverished in double-negative HCC ($P = 0.006$) phenotypes (Fig. 1A,B and Table 2).

VETC HCC revealed earlier recurrence, poorer DFS and OS compared with non-VETC HCC in the whole cohorts (n = 532) ($P < 0.05$ for all) (Fig. 1C). The significant clinical impact of VETC phenotype was confirmed at multivariable analysis for early recurrence (HR 1.52 [1.06-2.19]; $P = 0.023$), DFS (HR 1.66 [1.21-2.27]; $P = 0.002$), and OS (HR 2.26 [1.37-3.72]; $P = 0.001$) (Table 3). The VETC phenotype affected early recurrence, DFS, and OS even when HCC patients were stratified according to etiologies of HBV (n = 280) and HCV (n = 179) ($P < 0.05$ for all) (Supporting Fig. S2A). A predictive value of VETC phenotype on early HCC recurrence, DFS, and OS was also retained for HCCs of up to 5 cm (n = 444; $P < 0.05$ for all); a trend toward significance was present in OS for those greater than 5 cm (n = 88) (Supporting Fig. S2B). It was also associated with early recurrence, DFS, and OS for HCCs with micro/macrovascular invasion (n = 289; $P < 0.05$ for all), and poorer DFS for those without micro/macrovascular invasion (n = 243; $P = 0.038$) (Supporting Fig. S2C). For molecular phenotype, the VETC phenotype remained a significant predictive factor of early recurrence and OS in β-catenin/GS+ (n = 120) and double negative (n = 297; $P < 0.05$ for all), but not in p53+ (n = 104) phenotypes (Supporting Fig. S2D). Among three independent cohorts, the VETC phenotype was a significant predictive factor for early HCC recurrence, DFS, and OS in the Italian and Korean cohorts ($P < 0.05$ for all), but not in the Japanese cohorts (Supporting Fig. S3).

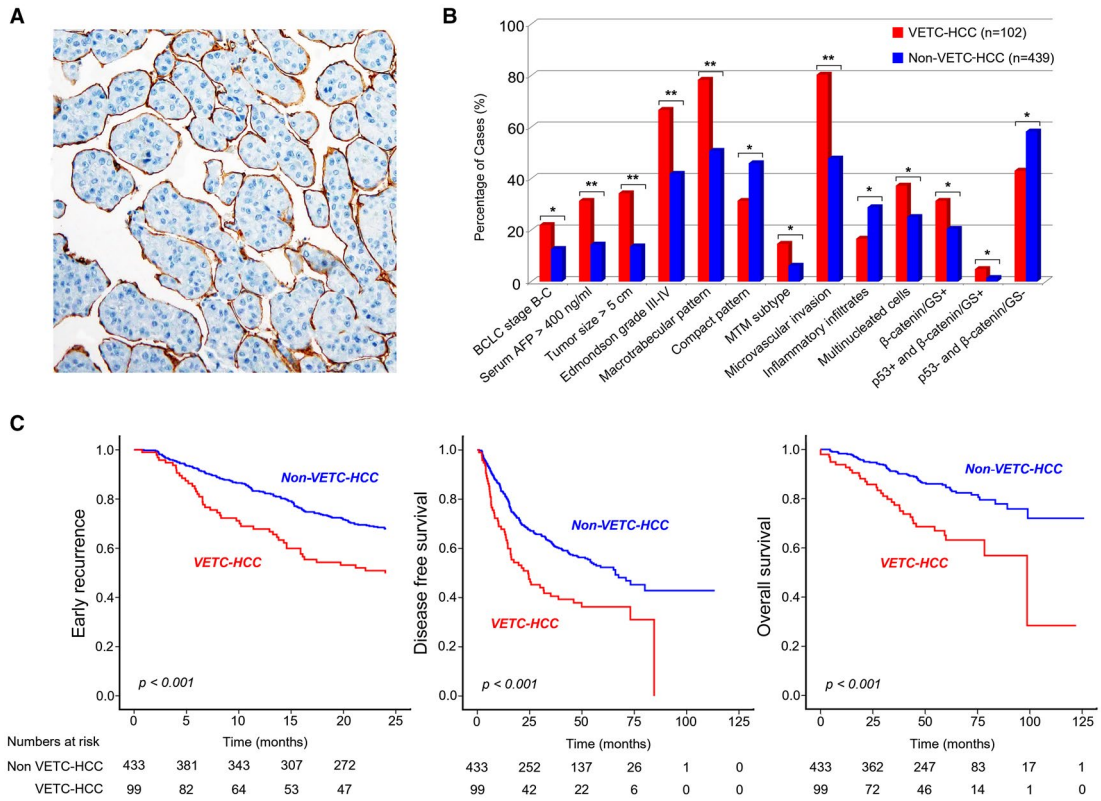

FIG. 1. (A) Morphologic features of VETC HCC. This HCC shows clusters of tumor cells bordered by a complete rim of CD34-positive endothelial cells in more than half of the tumor area (i.e., VETC phenotype). (B) Clinical and pathologic features associated with VETC HCC (*P < 0.05, **P < 0.001; χ^2 test). (C) Impact of VETC phenotype on survival of HCC patients.

VETC IS ENRICHED IN THE MTM SUBTYPE HCC

VETC was significantly enriched in the MTM subtype HCC (P = 0.006). The MTM subtype was seen in 7.8% (42 of 541) of HCCs (Fig. 2A), and it was not associated with specific etiological factors. MTM showed a significant correlation with clinical and pathological features of aggressiveness such as high AFP serum levels (>400 ng/mL, P < 0.001), Edmondson grade III-IV (P = 0.001), and less pseudoglandular pattern (P = 0.019). Interestingly, among molecular phenotypes, MTM was significantly higher in p53+ and double-positive phenotypes (P < 0.001 and P = 0.046, respectively) and lower in β-catenin/GS+ phenotype (P = 0.006) (Fig. 2B and Table 4). MTM showed no significant difference in early recurrence, DFS, and OS in whole series in univariable analysis (Table 3 and Fig. 2C).

IMPACT ON PROGNOSIS OF FEATURES OTHER THAN VETC

Several other clinical and pathological parameters showed an impact on early recurrence, DFS, and OS at multivariable analysis. Focusing on pathologic features, some of them (number and size of tumor, macrovascular invasion, and cirrhosis) can be detected at imaging and are already part of the current staging systems for HCC such as BCLC. Other parameters can be detected only under the microscope. Among the latter, the sarcomatoid variant showed a robust association with DFS (HR 8.72 [1.17-64.76]; P = 0.034) and OS (HR 24.99 [3.05-204.63]; P = 0.003); however, this variant is extremely rare (3 of 541, 0.6%). Microvascular invasion (HR 2.19 [1.31-3.66]; P = 0.003) and hyaline bodies (HR 1.80 [1.13-2.87]; P = 0.013) were associated with shorter OS, whereas microtrabecular pattern was associated with longer OS (HR 0.59

TABLE 2. Clinical and Pathological Features Associated With VETC HCC (n = 541)

	n (%)	Non-VETC HCC (n [%])	VETC HCC (n [%])	*P* Value
Clinical Features				
Age >60 years	290 (53.6)	233 of 439 (53.1)	57 of 102 (55.9)	0.660
Male sex	423 (78.2)	345 of 439 (78.6)	78 of 102 (76.5)	0.690
Alcohol	30 (5.5)	24 of 439 (5.5)	6 of 102 (6.8)	1.000
HCV infection	188 (34.8)	155 of 439 (35.3)	33 of 102 (32.4)	0.645
HBV infection	280 (51.8)	223 of 439 (50.8)	57 of 102 (55.9)	0.380
BCLC stage B-C*	61 (14.7)	41 of 323 (12.7)	20 of 91 (22.0)	0.031
AFP serum level >400 ng/mL	95 (17.6)	63 of 439 (14.4)	32 of 102 (31.4)	<0.001
Pre-operative treatment	20 (3.7)	16 of 439 (3.6)	4 of 102 (3.9)	0.772
General Macroscopic				
Tumor size >5 cm	95 (17.6)	60 of 439 (13.7)	35 of 102 (34.3)	<0.001
Multiplicity	88 (16.3)	68 of 439 (15.5)	20 of 102 (19.6)	0.371
Macrovascular invasion	31 (5.7)	21 of 439 (4.8)	10 of 102 (9.8)	0.059
General Microscopic				
Edmondson grade III-IV	252 (46.6)	184 of 439 (41.9)	68 of 102 (66.7)	<0.001
Microtrabecular pattern	398 (73.6)	318 of 439 (72.4)	80 of 102 (78.4)	0.262
Macrotrabecular pattern	307 (56.7)	227 of 439 (51.7)	80 of 102 (78.4)	<0.001
Pseudoglandular pattern	195 (36.0)	156 of 439 (35.5)	39 of 102 (38.2)	0.647
Compact pattern	234 (43.3)	202 of 439 (46.0)	32 of 102 (31.4)	0.008
Microvascular invasion	292 (54.0)	210 of 439 (47.8)	82 of 102 (80.4)	<0.001
Capsule infiltration	297 (54.9)	233 of 439 (53.1)	64 of 102 (62.7)	0.079
Inflammatory infiltrates	144 (26.6)	127 of 439 (28.9)	17 of 102 (16.7)	0.013
Cirrhosis	271 (50.1)	216 of 439 (49.2)	55 of 102 (53.9)	0.442
Tumor Cytological Findings				
Cholestasis	93 (17.2)	70 of 439 (15.9)	23 of 102 (22.5)	0.144
Clear cells	159 (29.4)	129 of 439 (29.4)	30 of 102 (29.4)	1.000
Pleomorphic cells	156 (28.8)	121 of 439 (27.6)	35 of 102 (34.3)	0.183
Multinucleated cells	148 (27.4)	110 of 439 (25.1)	38 of 102 (37.3)	0.014
Sarcomatous changes	12 (2.2)	10 of 439 (2.3)	2 of 102 (2.0)	1.000
Hyaline bodies	113 (20.9)	92 of 439 (21.0)	21 of 102 (20.6)	1.000
Tumor Variants				
Lymphoepithelioma-like	6 (1.1)	6 of 439 (1.4)	0 of 102 (0.0)	0.600
Sarcomatoid	3 (0.6)	3 of 439 (0.7)	0 of 102 (0.0)	1.000
Scirrhous	8 (1.5)	8 of 439 (1.8)	0 of 102 (0.0)	0.363
Steatohepatitic	38 (7.0)	29 of 439 (6.6)	9 of 102 (8.8)	0.518
Macrotrabecular-massive	42 (7.8)	27 of 439 (6.2)	15 of 102 (14.7)	0.006
Molecular Phenotype†				
p53+	108 (20.0)	87 of 439 (19.8)	21 of 102 (20.6)	0.891
βcatenin/GS+	122 (22.6)	90 of 439 (20.5)	32 of 102 (31.4)	0.025
Double positive	11 (2.0)	6 of 439 (1.4)	5 of 102 (4.9)	0.038
Double negative	300 (55.5)	256 of 439 (58.3)	44 of 102 (43.1)	0.006

*Available in 414 cases.
†p53+: p53+ and βcatenin/GS-; βcatenin/GS+: p53- and βcatenin/GS+; double positive: p53+ and βcatenin/GS+; and double negative: p53- and βcatenin/GS-.

[0.37-0.95]; *P* = 0.029) (Table 3). Although molecular phenotypes had a significant association with several clinical and pathological features (Fig. 3, Supporting Tables S3-S4), they did not show significant association with early recurrence, DFS, and OS in the multivariable model (Table 3).

TABLE 3. Impact of Clinical and Pathological Features on Early Recurrence, DSF, and OS (n = 532)

	Early Recurrence (n = 532)				DSF (n = 532)				OS (n = 532)			
	Univariable Analysis		Multivariable Analysis		Univariable Analysis		Multivariable Analysis		Univariable Analysis		Multivariable Analysis	
	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value
Clinical Features												
Age >60 years	1.44 (1.06-1.95)	0.018	1.47 (1.07-2.02)	0.017	1.38 (1.07-1.78)	0.013	1.26 (0.92-1.73)	0.163	2.25 (1.46-3.46)	<0.001	1.81 (1.08-3.03)	0.026
HCV infection	1.53 (1.13-2.06)	0.006	1.01 (0.59-1.73)	0.976	1.63 (1.26-2.12)	<0.001	1.48 (1.11-1.98)	0.007	2.56 (1.71-3.84)	<0.001	0.97 (0.51-1.83)	0.913
HBV infection	0.71 (0.53-0.96)	0.025	0.97 (0.59-1.61)	0.910	0.72 (0.56-0.93)	0.011	1.09 (0.69-1.72)	0.977	0.37 (0.24-0.57)	<0.001	0.53 (0.31-0.90)	0.019
Serum AFP >400 ng/mL	1.38 (0.96-1.98)	0.079			1.22 (0.89-1.67)	0.229			1.65 (1.04-2.61)	0.035	1.41 (0.79-2.51)	0.314
Pre-operative treatment	4.08 (2.20-7.59)	<0.001	3.22 (1.70-6.09)	<0.001	3.99 (2.15-7.41)	<0.001	2.79 (1.49-5.23)	0.001	5.43 (2.17-13.64)	<0.001	2.78 (1.03-7.50)	0.043
General Macroscopic												
Tumor size >5 cm	2.20 (1.56-3.10)	<0.001	1.68 (1.17-2.42)	0.005	1.94 (1.42-2.64)	<0.001	1.77 (1.27-2.45)	0.001	1.93 (1.18-3.16)	0.009	1.57 (0.90-2.73)	0.094
Multiplicity	2.46 (1.74-3.48)	<0.001	1.98 (1.39-2.82)	<0.001	2.42 (1.77-3.31)	<0.001	2.01 (1.46-2.76)	<0.001	2.92 (1.86-4.60)	<0.001	2.12 (1.30-3.46)	<0.001
Macrovascular invasion	3.61 (2.26-5.76)	<0.001	2.19 (1.37-3.50)	0.001	2.84 (1.81-4.46)	<0.001	1.84 (1.17-2.90)	0.008	7.28 (4.51-11.77)	<0.001	4.19 (2.45-7.16)	<0.001
General Microscopic												
Edmondson grade III-IV	1.75 (1.29-2.36)	<0.001	1.40 (1.01-1.95)	0.046	1.42 (1.11-1.83)	0.006	1.15 (0.85-1.56)	0.291	1.99 (1.32-2.98)	0.001	1.22 (0.73-2.05)	0.647
Microtrabecular pattern	0.69 (0.50-0.95)	0.022	0.95 (0.64-1.39)	0.777	0.74 (0.55-0.98)	0.032	0.88 (0.64-1.21)	0.399	0.35 (0.23-0.52)	<0.001	0.59 (0.37-0.95)	0.029
Macrotrabecular pattern	1.95 (1.42-2.68)	<0.001	1.35 (0.97-1.89)	0.078	1.56 (1.20-2.02)	0.001	1.07 (0.80-1.42)	0.429	2.37 (1.52-3.70)	<0.001	1.10 (0.67-1.80)	0.547
Compact pattern	0.98 (0.73-1.33)	0.904			1.04 (0.81-1.35)	0.749			1.86 (1.24-2.79)	0.003	1.05 (0.64-1.72)	0.860
Microvascular invasion	1.99 (1.45-2.73)	<0.001	1.36 (0.95-1.96)	0.098	1.64 (1.26-2.13)	<0.001	1.34 (1.01-1.78)	0.045	2.76 (1.75-4.35)	<0.001	2.19 (1.31-3.66)	0.003
Capsule infiltration	0.79 (0.59-1.07)	0.125			0.83 (0.64-1.07)	0.152			0.59 (0.39-0.88)	0.010	0.90 (0.57-1.44)	0.669
Inflammatory infiltrates	0.79 (0.56-1.13)	0.193			0.84 (0.63-1.13)	0.253			1.12 (0.72-1.74)	0.619		
Cirrhosis	1.60 (1.18-2.17)	0.003	1.63 (1.19-2.25)	0.003	1.79 (1.38-2.33)	<0.001	1.76 (1.34-2.30)	<0.001	1.80 (1.19-2.73)	0.006	1.71 (1.09-2.68)	0.020
Tumoral Cytological Findings												
Pleomorphic cells	1.33 (0.97-1.83)	0.076			1.24 (0.94-1.63)	0.132			1.57 (1.04-2.38)	0.033	0.99 (0.62-1.58)	0.930
Sarcomatous changes	3.34 (1.64-6.81)	0.001	0.91 (0.31-2.70)	0.862	2.65 (1.36-5.17)	0.004	0.68 (0.26-1.78)	0.435	4.15 (1.81-9.54)	0.001	0.69 (0.20-2.34)	0.552
Hyaline bodies	1.01 (0.69-1.47)	0.967			1.09 (0.80-1.49)	0.589			1.95 (1.26-3.00)	0.003	1.80 (1.13-2.87)	0.013
Tumoral Variants												
Lymphoepithelioma-like	0.95 (0.24-3.84)	0.945			1.19 (0.38-3.71)	0.770			2.15 (0.53-8.76)	0.284		
Sarcomatoid	10.54 (3.29-33.81)	<0.001	5.38 (0.46-63.11)	0.181	10.54 (3.29-33.81)	<0.001	8.72 (1.17-64.76)	0.034	9.97 (1.34-74.32)	0.025	24.99 (3.05-204.63)	0.003
Scirrhous	2.76 (1.22-6.24)	0.015	3.85 (1.67-8.90)	0.002	2.06 (0.91-4.63)	0.082			2.38 (0.75-7.53)	0.140		
Steatohepatitic	1.75 (1.06-2.89)	0.028	1.17 (0.64-2.15)	0.612	1.32 (0.82-2.14)	0.258			1.78 (0.97-3.26)	0.063		
Macrotrabecular-massive	0.94 (0.54-1.66)	0.834			0.98 (0.62-1.57)	0.944			1.28 (0.64-2.54)	0.489		
VETC Phenotype	1.91 (1.38-2.64)	<0.001	1.52 (1.06-2.19)	0.023	1.81 (1.36-2.41)	<0.001	1.66 (1.21-2.27)	0.002	2.35 (1.56-3.54)	<0.001	2.26 (1.37-3.72)	0.001
Molecular Phenotype*												
p53+	1.10 (0.76-1.59)	0.620			1.04 (0.75-1.43)	0.833			0.89 (0.52-1.52)	0.659		
βcatenin/GS+	0.96 (0.67-1.38)	0.837			1.00 (0.74-1.35)	0.977			1.85 (1.21-2.82)	0.004	1.41 (0.75-2.64)	0.177

TABLE 3. *Continued*

	Early Recurrence (n = 532)				DSF (n = 532)				OS (n = 532)			
	Univariable Analysis		Multivariable Analysis		Univariable Analysis		Multivariable Analysis		Univariable Analysis		Multivariable Analysis	
	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value
Double positive	2.36 (1.11-5.03)	0.026	1.28 (0.57-2.89)	0.545	2.41 (1.24-4.69)	0.010	1.54 (0.76-3.14)	0.193	1.51 (0.48-4.76)	0.485		
Double negative	0.88 (0.66-1.19)	0.411			0.90 (0.70-1.16)	0.415			0.63 (0.42-0.94)	0.023	0.99 (0.56-1.73)	0.358

*p53+: p53+ and βcatenin/GS-; βcatenin/GS+: p53- and βcatenin/GS+; double positive: p53+ and βcatenin/GS+; and double negative: p53- and βcatenin/GS-.

Discussion

This paper aimed to evaluate the robustness of the impact on survival of a vascularization pattern of HCC named VETC, in a large cohort of patients from different geographic areas (Italy, Korea, and Japan). VETC HCC accounted for 18.9%, regardless of etiology, a proportion of enough size to capture a consistent and clinically significant fraction of HCC. Our study confirms the prognostic impact of VETC on a large multicenter cohort. Interestingly Fang et al. reported 39% VETC HCC, but in that series the authors did not use the more selective 55% cutoff value for VETC; moreover, 75% of HCCs were greater than 5 cm (versus 17.6% in the present study), suggesting that this specific vascular pattern could be related to the tumor progression.[11] Indeed, in the Japanese cohort of the present series, which included the smallest tumors (>5 cm, 0.8%), the percentage of VETC HCC dropped to 8.7%. Recently, Villa et al. reported a vascular signature (occurring in 25% of HCC cases) that was able to predict an unfavorable prognosis and a fast-growing tumor.[9]

The VETC phenotype we studied was consistently detectable in the eastern and western series. It was also reliably reproducible as shown by the intralaboratory (correlation between TMA and whole section) and interlaboratory (correlation among 4 reviewers) data consistency. We easily found (and then looked for) VETC in tumoral areas where the capillary pattern was largely missing. Notably, the good correlation of VETC occurrence between TMA and whole section suggested that this feature could be reliably detected in small tissue samples as in core needle biopsies. Indeed, one of the main limitations of HCC biopsy is the poor performance in the detection of microvascular invasion, an adverse prognostic feature that was well correlated with the VETC in our series ($P < 0.001$). We also observed VETC to be associated with the macrotrabecular pattern of growth as well as with the recently reported MTM subtype,[13,14] which occurred in 7.8% of our series. Indeed, hematoxylin and eosin staining of tumor areas with thick trabeculae revealed a VETC pattern in a significant percentage of cases, as opposed to a compact pattern.

Most importantly, we provided a morphological tool that is easily incorporable into clinical practice with a cutoff value for VETC HCC of 55% (i.e., most of the tumor area). This cutoff revealed to be a robust prognostic parameter that discriminates aggressive

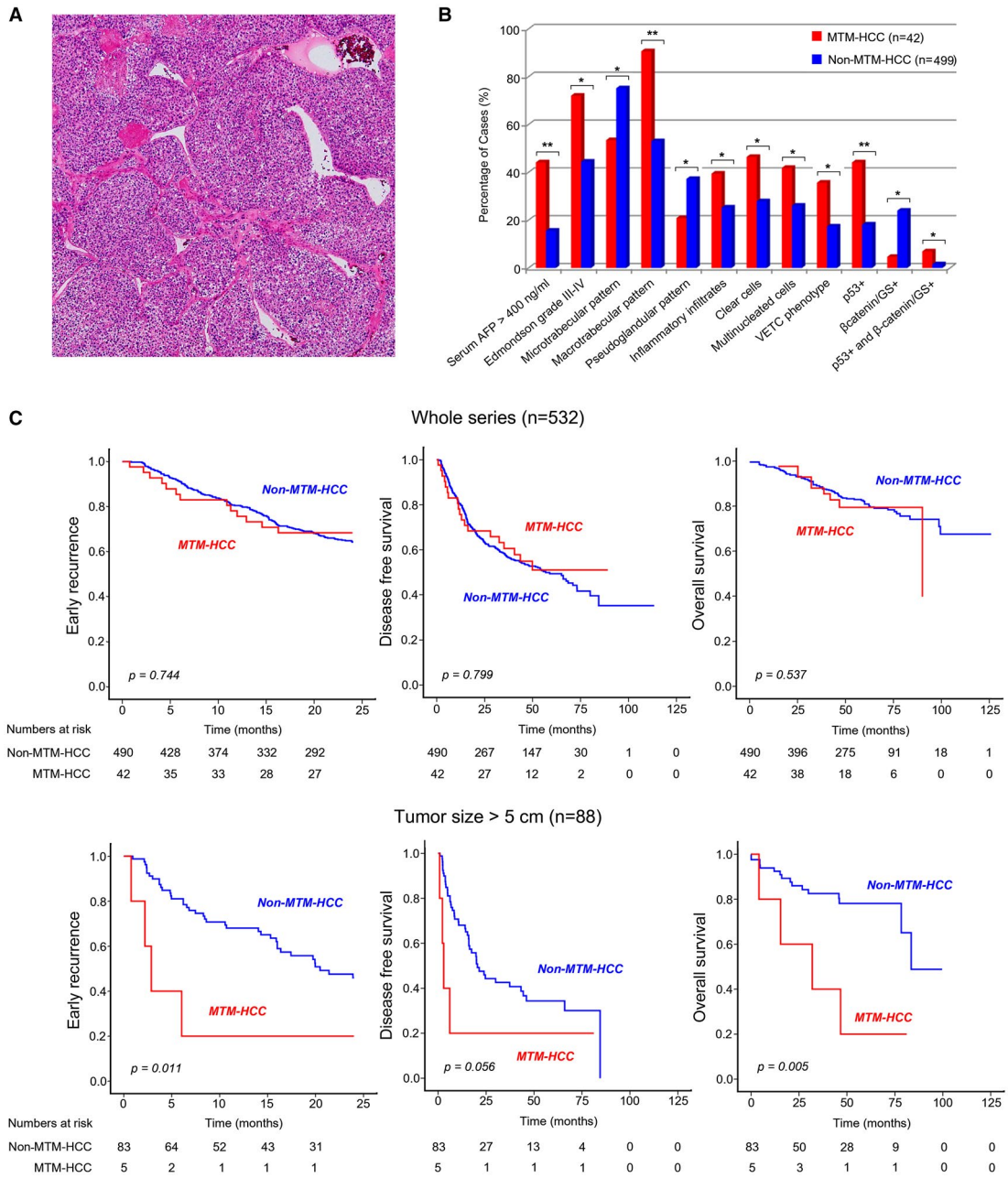

FIG. 2. (A) Morphologic features of a MTM HCC, showing a thick trabecular pattern of growth in more than half of the tumor area. (B) Clinical and pathologic features associated with MTM HCC (*P < 0.05, **P < 0.001; χ^2 test). (C) Impact of MTM on survival of HCC patients.

HCCs with a significant impact on OS, DFS, and early recurrence. Early recurrent HCCs are those showing an intrahepatic/extrahepatic dissemination, well in keeping with the endothelial entrapment of clusters of malignant hepatocytes, robustly sustained by novel vascular connections. Notably, VETC values were independent from HCC etiology and were retained in smaller and earlier tumors (≤5 cm) as well as in HCCs already showing micro/macrovascular invasion. These data suggest the potential value of discerning VETC HCC, to optimize further the individual therapeutic approach. Our results validate and strongly extend the findings of Fang et al. in a large multicentered cohort that includes HCV-related and

TABLE 4. Clinical and Pathological Features Associated With MTM HCC (n = 541)

	n (%)	Non-MTM HCC (n [%])	MTM HCC (n [%])	*P* Value
Clinical Features				
Age >60 years	290 (53.6)	272 of 499 (54.5)	18 of 42 (42.9)	0.151
Male sex	423 (78.2)	392 of 499 (78.6)	31 of 42 (73.8)	0.559
Alcohol	30 (5.5)	29 of 499 (5.8)	1 of 42 (2.4)	0.721
HCV infection	188 (34.8)	177 of 499 (35.5)	11 of 42 (26.2)	0.243
HBV infection	280 (51.8)	253 of 499 (50.7)	27 of 42 (64.3)	0.108
BCLC stage B-C*	61 (14.7)	56 of 376 (14.9)	5 of 38 (13.2)	0.819
AFP serum level >400 ng/mL	95 (17.6)	77 of 499 (15.4)	18 of 42 (42.9)	<0.001
Pre-operative treatment	20 (3.7)	18 of 499 (3.6)	2 of 42 (4.8)	0.663
General Macroscopic				
Tumor size >5 cm	95 (17.6)	90 of 499 (18.0)	5 of 42 (11.9)	0.401
Multiplicity	88 (16.3)	81 of 499 (16.2)	7 of 42 (16.7)	1.000
Macrovascular invasion	31 (5.7)	29 of 499 (5.8)	2 of 42 (4.8)	1.000
General Microscopic				
Edmondson grade III-IV	252 (46.6)	222 of 499 (44.5)	30/42 (71.4)	0.001
Microtrabecular pattern	398 (73.6)	375 of 499 (75.2)	23 of 42 (54.8)	0.006
Macrotrabecular pattern	307 (56.7)	265 of 499 (53.1)	42 of 42 (100.0)	<0.001
Pseudoglandular pattern	195 (36.0)	187 of 499 (37.5)	8 of 42 (19.0)	0.019
Compact pattern	234 (43.3)	217 of 499 (43.5)	17 of 42 (40.5)	0.748
Microvascular invasion	292 (54.0)	264 of 499 (52.9)	28 of 42 (66.7)	0.107
Capsule infiltration	297 (54.9)	270 of 499 (54.1)	27 of 42 (64.3)	0.258
Inflammatory infiltrates	144 (26.6)	127 of 499 (25.5)	17 of 42 (40.5)	0.045
Cirrhosis	271 (50.1)	250 of 499 (50.1)	21 of 42 (50.0)	1.000
Tumor Cytological Findings				
Cholestasis	93 (17.2)	88 of 499 (17.6)	5 of 42 (11.9)	0.403
Clear cells	159 (29.4)	140 of 499 (28.1)	19 of 42 (45.2)	0.022
Pleomorphic cells	156 (28.8)	141 of 499 (28.3)	15 of 42 (35.7)	0.375
Multinucleated cells	148 (27.4)	130 of 499 (26.1)	18 of 42 (42.9)	0.021
Sarcomatous changes	12 (2.2)	11 of 499 (2.2)	1 of 42 (2.4)	1.000
Hyaline bodies	113 (20.9)	101/499 (20.2)	12 of 42 (28.6)	0.234
Tumor Variants				
Lymphoepithelioma-like	6 (1.1)	6 of 499 (1.2)	0 of 42 (0.0)	1.000
Sarcomatoid	3 (0.6)	3 of 499 (0.6)	0 of 42 (0.0)	1.000
Scirrhous	8 (1.5)	8 of 499 (1.6)	0 of 42 (0.0)	1.000
Steatohepatitic	38 (7.0)	35 of 499 (7.0)	3 of 42 (7.1)	1.000
VETC Phenotype	102 (18.9)	87 of 499 (17.4)	15 of 42 (35.7)	0.006
Molecular Phenotype†				
p53+	108 (20.0)	90 of 499 (18.0)	18 of 42 (42.9)	<0.001
βcatenin/GS+	122 (22.6)	120 of 499 (24.0)	2 of 42 (4.8)	0.006
Double positive	11 (2.0)	8 of 499 (1.6)	3 of 42 (7.1)	0.046
Double negative	300 (55.5)	281 of 499 (56.3)	19 of 42 (45.2)	0.196

*Available in 414 cases.
†p53+: p53+ and βcatenin/GS-; βcatenin/GS+: p53- and βcatenin/GS+; double positive: p53+ and βcatenin/GS+; and double negative: p53- and βcatenin/GS-.

HBV-related HCCs, revealing VETC as an independent predictor of shorter recurrence-free survival in 164 HBV-related HCC patients.[11]

Interestingly, VETC also maintained a significant prognostic impact in HCCs unrelated to p53 activation, detected using surrogate immunohistochemical markers (GS and β-catenin) that likely picked up a spectrum of Wnt/β-catenin deregulated cases. We had 122 cases (22.6%) of Wnt/β-catenin deregulated HCC, and we found that 26.2% (32 of 122) of these showed

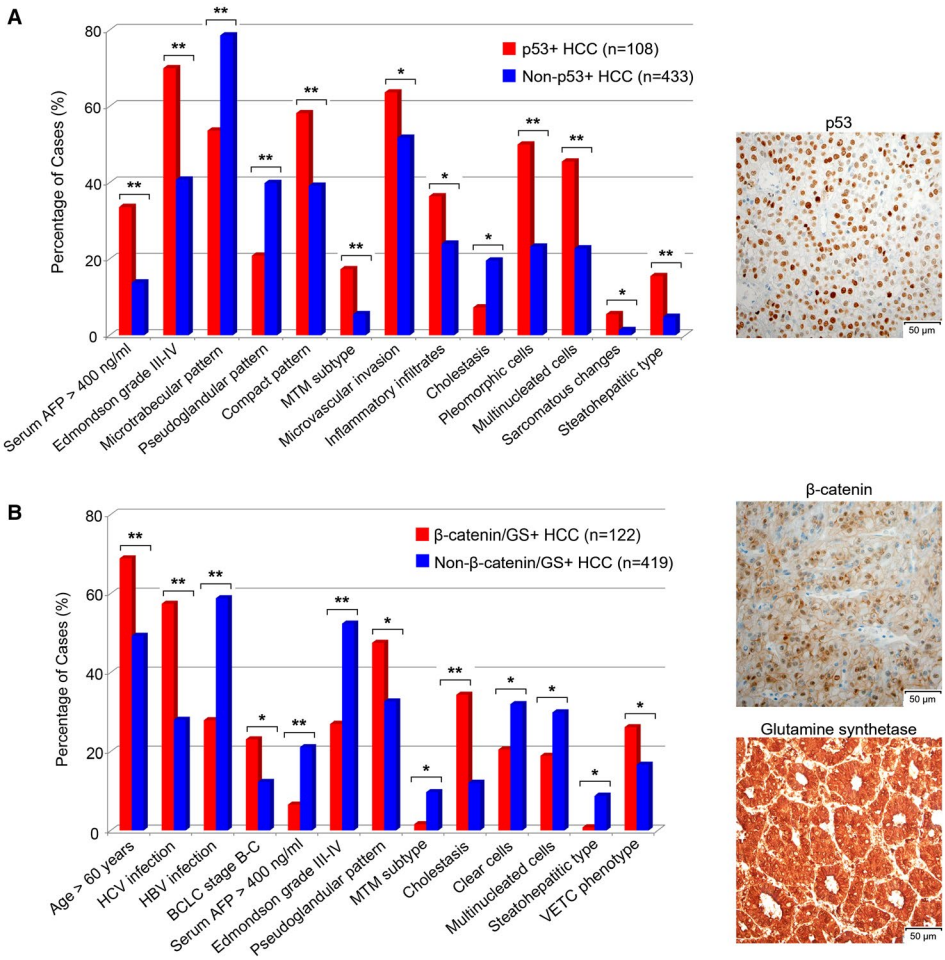

FIG. 3. Clinicopathologic features associated with p53+ HCC (A) and β-catenin/GS+ HCC (B). (*$P < 0.05$, **$P < 0.001$; χ^2 test).

the VETC phenotype, a greater proportion compared with the Wnt/β-catenin-unrelated group (16.7%, 70 of 419; $P = 0.025$). Interestingly, VETC was also significantly enriched ($P = 0.006$) in MTM HCC (35.7%, 15 of 42) as compared with non-MTM HCC (17.4%, 87 of 499). VETC enrichment in a subpopulation of Wnt/β-catenin deregulated HCCs, which usually have a better outcome, as well as in MTM HCC, which usually have a poorer prognosis, could seem contradictory. However, in the clinically robust molecular HCC classification,[15] the S1 subclass—with an aberrant activation of the canonical Wnt/β-catenin pathway (by transforming growth factor β rather than by β-catenin mutations)—was characterized by a greater risk of earlier recurrence and more vascular invasion, two features in keeping with the profile of our VETC HCC. It has been also shown that the Wnt/β-catenin pathway contributes to angiogenesis, infiltration, and metastasis by regulating the expression of angiogenic factors.[16] We are therefore tempted to speculate that VETC enrichment might have occurred in an HCC subpopulation with Wnt/β-catenin deregulation not necessarily related to gene mutations.

Notably, in the present study we also noticed consistent pathologic/molecular correlations with the data shown by Calderaro et al. (Supporting Table S5), emphasizing the role of gene drivers (or lack of them) in modulating HCC morphology and growth.[13] Despite being associated with the p53 phenotype, high AFP levels, poor differentiation and VETC phenotype, the recently reported MTM subtype of HCC did not show an independent prognostic value at multivariable analysis. However, as also suggested by Ziol et al., MTM HCC was associated with an unfavorable

outcome (univariable analysis; early recurrence and OS) in tumors greater than 5 cm.[14] Overall, this study suggests that MTM HCC likely identifies a subset of aggressive HCCs of larger size, which was a small proportion in our series. Additional useful histopathological features provided here with negative impact on survival were poor tumor differentiation (Edmonson III-IV), the exceptionally rare sarcomatoid variant, microvascular invasion, and hyaline bodies.[17]

Reliable prognostic parameters of HCC behavior are still an unmet need in the clinical practice. In this study, we have proven that the VETC phenotype, a distinct pattern involving most of the tumor area, is found in about one-fifth of resectable HCCs, and is significantly associated with OS, DFS, and early recurrence. This vascular phenotype can be detected with increasing frequency, from the earliest stages of HCC, and its assessment is easy and reliably reproducible even in small-sized tissues such as liver biopsy. In advanced HCC, VETC has been recently suggested to act as a predictor of sorafenib benefit[12]; however, our data support the assessment of the VETC phenotype also in nonadvanced and resectable HCC for the identification of HCC patients at high risk of a more rapid tumor progression. VETC is a noncapillary network of HCC vessels that is prone to open into larger ones, as to deliver metastatic tumor cell clusters.[11]

The results presented here emphasize the role of the microenvironment, particularly of endothelial cells, in the aggressive behavior of HCC and highlight the heterogenous features of HCC angiogenesis. We also believe that the characterization of the vascular phenotype and the recognition of the vascular heterogeneity can be helpful, in the future, for an individual anti-angiogenetic treatment of HCC.

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