Nestin as a diagnostic and prognostic marker for combined hepatocellular-cholangiocarcinoma

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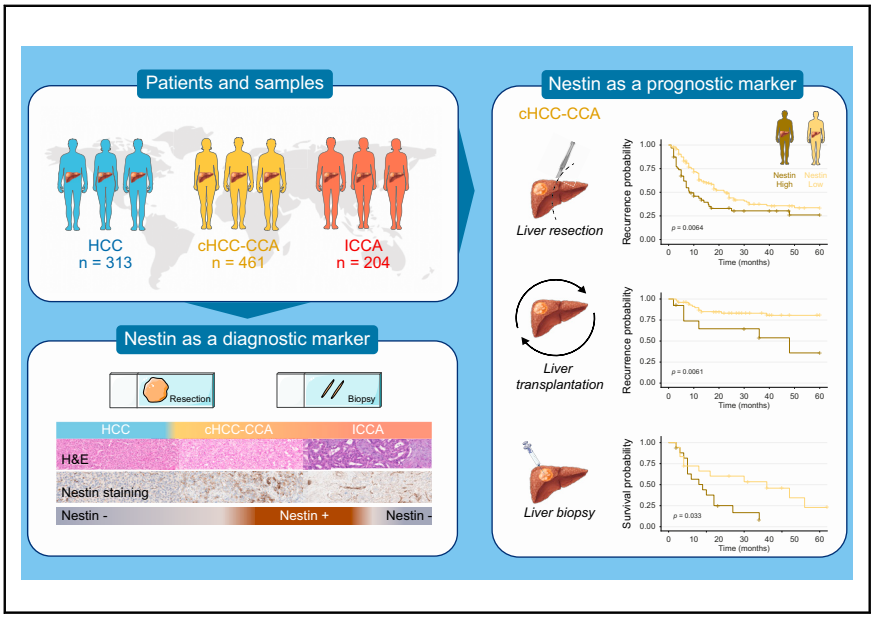
Graphical abstract



Highlights

- Biomarkers for combined hepatocellular-cholangiocarcinoma (cHCC-CCA) are critically needed.
- Nestin immunohistochemical expression is able to identify the subset of cHCC-CCA associated with the worst clinical outcome.
- cHCC-CCA with >30% of neoplastic cells expressing Nestin are classified “Nestin High”.
- Nestin High cHCC-CCA are associated with an adverse outcome after surgical resection and liver transplantation.

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Lay summary

There are different types of primary liver cancers (*i.e.* cancers that originate in the liver). Accurately identifying a specific subtype of primary liver cancer (and determining its associated prognosis) is important as it can have a major impact on treatment allocation. Herein, we show that a protein called Nestin could be used to refine risk stratification and improve treatment allocation for patients with combined hepatocellular carcinoma, a rare but highly aggressive subtype of primary liver cancer.

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Nestin as a diagnostic and prognostic marker for combined hepatocellular-cholangiocarcinoma

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Background & Aims: Combined hepatocellular-cholangiocarcinoma (cHCC-CCA) is a rare primary liver cancer (PLC) associated with a poor prognosis. Given the challenges in its identification and its clinical implications, biomarkers are critically needed. We aimed to investigate the diagnostic and prognostic value of the immunohistochemical expression of Nestin, a progenitor cell marker, in a large multicentric series of PLCs.

Methods: We collected 461 cHCC-CCA samples from 32 different clinical centers. Control cases included 368 hepatocellular carcinomas (HCCs) and 221 intrahepatic cholangiocarcinomas (iCCAs). Nestin immunohistochemistry was performed on whole tumor sections. Diagnostic and prognostic performances of Nestin expression were determined using receiver-operating characteristic curves and Cox regression modeling.

Results: Nestin was able to distinguish cHCC-CCA from HCC with AUCs of 0.85 and 0.86 on surgical and biopsy samples, respectively. Performance was lower for the distinction of cHCC-CCA from iCCA (AUCs of 0.59 and 0.60). Nestin, however, showed a high prognostic value, allowing identification of the subset of cHCC-CCA ("Nestin High", >30% neoplastic cells with positive staining) associated with the worst clinical outcome (shorter disease-free and overall survival) after surgical resection and liver transplantation, as well as when assessment was performed on biopsies.

Conclusion: We show in different clinical settings that Nestin has diagnostic value and that it is a useful biomarker to identify the subset of cHCC-CCA associated with the worst clinical outcome. Nestin immunohistochemistry may be used to refine risk stratification and improve treatment allocation for patients with this highly aggressive malignancy.

Lay summary: There are different types of primary liver cancers (*i.e.* cancers that originate in the liver). Accurately identifying a specific subtype of primary liver cancer (and determining its associated prognosis) is important as it can have a major impact on treatment allocation. Herein, we show that a protein called Nestin could be used to refine risk stratification and improve treatment allocation for patients with combined hepatocellular carcinoma, a rare but highly aggressive subtype of primary liver cancer.

Introduction

Primary liver cancers (PLCs) encompass different entities with varying degrees of hepatocytic and/or biliary differentiation, the ends of the spectrum being hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (iCCA).[1–4] The former is thought to derive from the malignant transformation of hepatocytes and therefore most often mimics the trabecular architecture of the non-neoplastic liver, while the latter represents an intrahepatic adenocarcinoma with biliary epithelial differentiation.[3] These malignancies are characterized by distinct risk factors, clinical outcomes, molecular alterations and therapeutic modalities. iCCA is noticeably associated with a poorer prognosis, with 5-year survival rates lower than 10-15%.[3]

Combined hepatocellular-cholangiocarcinoma (cHCC-CCA) is a rare variant of PLC that exhibits a dual hepatocellular and biliary phenotype.[1,3] It is linked to an adverse clinical outcome similar to that of iCCA and is thus currently considered a contraindication to liver transplantation.[5] Although the

biological mechanisms leading to its development remain unclear, one hypothesis is that this subset of PLC may arise from progenitor cells, therefore conferring a high degree of cellular plasticity.[5] These tumors are consequently often difficult to diagnose, with low inter-observer agreement even among expert liver pathologists.[5]

Accurate classification of PLCs has a major impact on clinical outcome and treatment. HCC unfortunately presents few therapeutic options that include the atezolizumab-bevacizumab or durvalumab-tremelimumab combinations.[6,7] Molecular profiling of iCCA has shown that a significant subset of tumors harbor actionable alterations, such as *FGFR2* rearrangements and *IDH1*, *BRAF* or *BRCA* mutations.[8–11] Recent clinical trials have demonstrated that targeting these genetic defects may result in significant improvement in disease-free or overall survival.[12–15] Interestingly, although no conventional anti-cancer therapies have shown efficacy against cHCC-CCA, several studies have reported that a significant subset of these tumors show an overlapping molecular profile with that of iCCA.[16,17] Molecular screening may thus be warranted.

Given the challenges in cHCC-CCA diagnosis and its associated clinical implications, the development of biomarkers relevant to this type of PLC is a critical unmet need.[5] Several immunohistochemical stainings, such as cytokeratin 19 (CK19) or CD56, are commonly used but they show low sensitivity and/or specificity.[18] Recent studies, performed on limited numbers of cases, have suggested that the expression of Nestin could serve as a marker of cHCC-CCA.[17–19] Nestin is a class IV intermediate filament expressed by bi-potential liver progenitor cells.[20–22] It has been shown to be a key regulator of cellular plasticity through the maintenance of an undifferentiated state, thereby enabling the transdifferentiation of neoplastic cells.[21,23] We thus aimed, in the present study, to investigate the potential diagnostic and prognostic value of Nestin immunohistochemistry in an overall series of 1,050 PLC samples collected from multiple European, American and Asian centers.

Materials and methods

Patients and samples

We retrospectively collected 461 cHCC-CCA samples from the archives of 32 European (n = 20), Asian (n = 6) and American (n = 6) Pathology Departments. The inclusion criteria were as follows: 1) patients in whom a surgical resection, liver transplant or tumor biopsy was performed between the years 2000 to 2019, 2) available paraffin-embedded material, histological slides and baseline clinical data, and 3) pathological diagnosis of cHCC-CCA confirmed by at least 2 expert liver pathologists, according to the most recent guidelines.[24]

The following features were systematically recorded: age, sex, risk factors for liver disease, preoperative alpha-fetoprotein serum level (for HCC and cHCC-CCA), preoperative anti-tumor treatment, tumor multinodularity, largest nodule diameter (size of the tumor or size of the largest tumor in the case of multinodular disease), macrovascular and microvascular invasion, tumor differentiation (for HCC and iCCA) and resection margin status. We were not able to include CA19-9 serum levels in our analysis, as the vast majority of tumors were classified as HCC before surgery meaning these data were not available.

Control cases consisted of a series of 589 patients with non-cHCC-CCA PLC treated by surgical resection or with biopsy samples available (resection: HCC n = 313 and CCA n = 204 and biopsy: HCC n = 55 and CCA n = 17). They were randomly selected and provided by 9 clinical centers. Inclusion criteria were 1) available histological slides and paraffin-embedded material, 2) available baseline clinical data and 3) unequivocal diagnosis of HCC or CCA (confirmed by at least 2 expert liver pathologists). This study was approved by a review board (CPP Ile de France V), conducted in accordance with the Declaration of Helsinki and the legislations of each participating center. All necessary informed consents were obtained from patients.

The study flowchart is presented in Fig. 1.

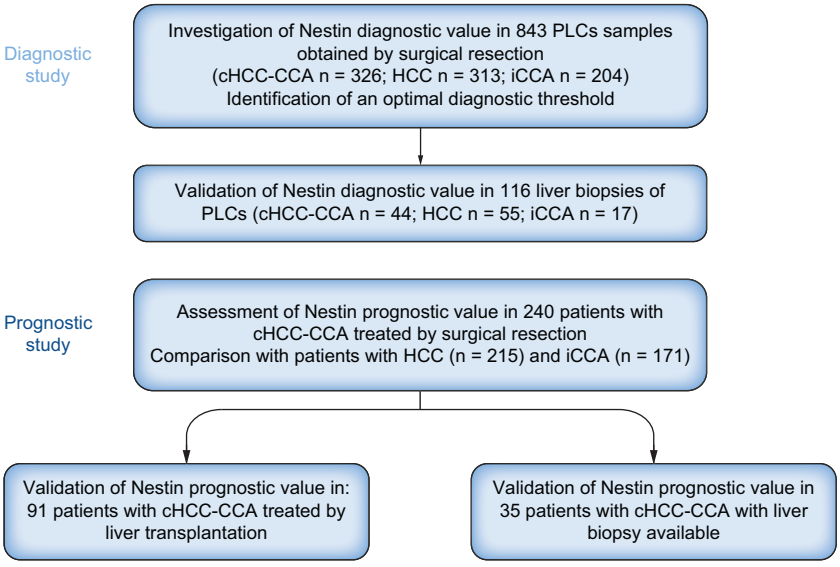

Fig. 1. Flowchart of the study. cHCC-CCA, combined hepatocellular-cholangiocarcinoma; HCC, hepatocellular carcinoma; iCCA, intrahepatic cholangiocarcinoma; PLC, primary liver cancer.

Immunohistochemistry

Five micrometer thick sections were cut from the tissue blocks and all stainings were performed on whole tissue sections to avoid heterogeneity inherent to tissue microarray-based approaches. All slides were further processed on an automated autostainer (Leica Bondmax). After deparaffinization and rehydration, endogenous peroxidase was blocked with the Peroxyde Block reagent (Leica Biosystems). Antigen retrieval was performed using the E1 (pH6) (Nestin, Glypican 3, EpCAM) or E2 (pH9) (CK19) solution (Bond Polymer Refine Detection Kit, Leica Biosystems). We further applied the anti-Nestin (Millipore, MAB5326, Clone 10C2, Dilution 1/100), anti-CK19 (Leica, clone b170, ready-to-use no dilution), anti-Glypican 3 (Diagomics, clone IG12, Dilution 1/100), and anti-EpCAM (Dako, M0804, Clone Ber-EP4, Dilution 1/100) primary antibody. Detection was performed using the Polymer reagent (Bond Polymer Refine Detection Kit, Leica Biosystems) and 3,30 di-aminobenzidine.

All immunohistochemical slides were reviewed by a pathologist specialized in liver disease (JC). The assessment of the percentage of tumor staining was performed using the following semi-quantitative scale: 0-1% (negative class), >1-10%, >10-30%, >30-50%, >50-70% and >70%. Endothelial cells and nerves served as positive internal controls. We also investigated Nestin expression in the different morphological contingents of cHCC-CCA: HCC, equivocal (intermediate features between HCC and iCCA) and iCCA.

The inter-observer agreement was determined through kappa statistics (weighted kappa) using the vcd package in R on a set of 250 randomly selected slides that were reviewed by a second pathologist (CTN).

Statistical analysis

Statistical analysis and data visualization were performed using R statistical software version 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org) and Bioconductor packages (version 3.4).

Comparison of qualitative variables in 2 or more than 2 groups was performed using either parametric test (*t* test or ANOVA) if the variable was normally distributed or non-parametric test (Mann-Whitney or Kruskal-Wallis test). Qualitative data were compared using Chi-square test. Standardized mean difference (SMD) was also calculated for each comparison using the *tableone* package in R.

The diagnostic performance of Nestin was estimated using the area under the receiver-operating characteristic curves (AUCs) together with their 95% CI. Nestin cut-offs were identified using values that maximized the Youden index. For each cut-off value, we reported sensitivity and specificity with 95% CIs. The estimated coefficients of a logistic regression were used to calculate a diagnostic score with multiple markers.

Disease-free survival was defined by the interval between surgery or biopsy and disease recurrence whereas overall survival was defined by the interval between surgery or biopsy and death or last follow-up. To evaluate prognostic performance, Nestin expression was discretized by selecting a cut-off with the greatest discriminative power for disease-free survival. The optimal cut-off point was calculated using maximally selected rank statistics as implemented in the *maxstat* R package. Survival curves were represented using the Kaplan-Meier method compared with log-rank statistics. Univariate analysis was performed using the Cox proportional-hazards regression model;

Table 1. Clinical, biological and pathological features of patients with PLC treated by surgical resection.

Variables	cHCC-CCA (n = 326)		HCC (n = 313)		iCCA (n = 204)		*p* value	SMD
	Available data	n (%)	Available data	n (%)	Available data	n (%)		
Sex (male)	317	244 (77.0)	313	255 (81.5)	204	118 (57.8)	<0.001	0.353
Age at surgery (mean (SD))	304	62.60 (11.85)	313	63.66 (12.30)	204	65.21 (11.05)	0.052	0.149
Etiology								
Alcohol	280	59 (21.1)	296	91 (30.7)	187	15 (8.0)	0.011*	0.222*
HBV	280	60 (21.4)	296	71 (24.0)	187	20 (10.7)	0.527*	0.061*
HCV	280	58 (20.7)	296	77 (26.0)	187	13 (7.0)	0.161*	0.125*
NASH	280	55 (19.6)	296	60 (20.3)	187	30 (16.0)	0.933*	0.016*
Undetermined	279	67 (24.0)	296	37 (12.5)	187	72 (38.5)	0.001*	0.301*
Treatment before intervention	266	29 (10.9)	313	7 (2.2)	204	11 (5.4)	<0.001	0.241
Largest nodule diameter (≥50 mm)	316	129 (40.8)	313	155 (49.5)	204	126 (61.8)	<0.001	0.284
Multinodularity	314	58 (18.5)	312	44 (14.1)	201	47 (23.4)	0.027	0.16
Preoperative AFP (>20 ng/ml)	167	98 (58.7)	255	150 (58.8)	-	-	1*	0.003*
Surgical margins (R1)	274	53 (19.3)	312	41 (13.1)	174	29 (16.7)	0.124	0.113
Macrovascular invasion	292	30 (10.3)	313	55 (17.6)	204	12 (5.9)	0.014*	0.212*
Microvascular invasion	300	169 (56.3)	313	140 (44.7)	201	87 (43.3)	0.003	0.175
WHO differentiation	-						<0.001**	0.782**
Well differentiated		-	313	62 (19.8)	204	32 (15.7)		
Moderately differentiated		-	313	205 (65.5)	204	89 (43.6)		
Poorly differentiated		-	313	46 (14.7)	204	83 (40.7)		
Cirrhosis	263	117 (44.5)	295	106 (35.9)	200	21 (10.5)	0.049*	0.175*

cHCC-CCA, combined hepatocellular-cholangiocarcinoma; HCC, hepatocellular carcinoma; iCCA, intrahepatic cholangiocarcinoma; SMD, standardized mean difference.
As clinical and pathological features may radically differ and are also not equally recorded among patients with PLCs, *indicate comparison between cHCC-CCA and HCC only and **between HCC and iCCA only. Statistical tests: Student's *t* test for continuous outcomes or Chi-square test for categorical outcomes.

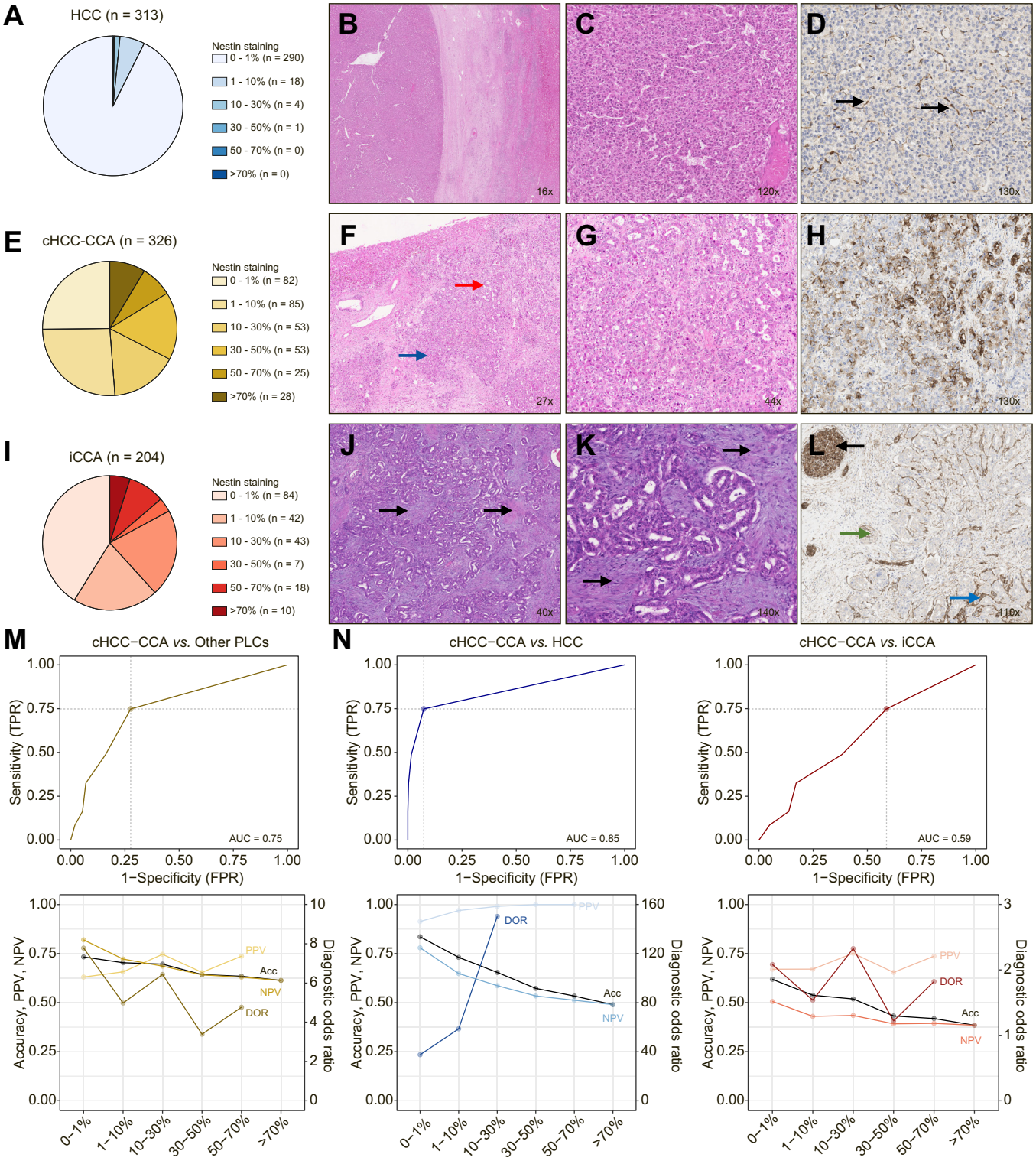

Fig. 2. Nestin diagnostic value in resected samples of PLCs. Nestin staining is very rarely observed in HCC (A). This classical HCC consists in a well-circumscribed nodule surrounded by a fibrous capsule (B), with a typical microtrabecular architectural pattern (HES) (C). There is no Nestin expression by neoplastic cells. A positive staining is observed on endothelial cells (black arrows, internal controls) (D). Most cHCC-CCAs (approximately 75%) show Nestin expression, although the percentage of positive neoplastic cells is variable (E). Microscopic examination of this cHCC-CCA reveals 2 different areas: one has a solid/compact architecture (blue arrow) while the other displays glandular formations (red arrow) (HES) (F, G). Cytoplasmic Nestin expression is observed in a significant fraction of tumor cells (H). Nestin is expressed in more than half of iCCA (I). This iCCA features clusters of neoplastic cells arranged in glands and an abundant fibrous stroma (black arrows) (HES) (J, K). No Nestin expression is identified in this iCCA (L). Positive controls include nerves (black arrow), endothelial cells (green arrow) and fibroblasts (blue arrow) (L). Nestin diagnostic performances, assessed by AUCs are 0.75 (cHCC-CCA *vs.* other PLCs) (M), 0.85 (cHCC-CCA *vs.* HCC) and 0.59 (cHCC-CCA *vs.* iCCA) (N). Dotted lines on the receiver-operating characteristic curves indicate the cut-offs maximizing Youden's index. Lower panels show other diagnostic performance indicators (accuracy, PPV, NPV on the left y-axis and DOR on the right y-axis, respectively). AUC, area under the receiver-

variables with a p value <0.05 were selected for multivariate analysis. All tests were 2-tailed and a p value <0.05 was considered significant.

Results

Assessment of Nestin expression and diagnostic performance in surgical and biopsy PLC samples: Nestin distinguishes cHCC-CCA from HCC

To assess the diagnostic performance of Nestin immunohistochemical expression, we first investigated a series of 843 PLC samples obtained by surgical resection (cHCC-CCA n = 326, HCC n = 313, iCCA n = 204). The main clinical and pathological features of the patients and tumors are described in Table 1. For patients with cHCC-CCA, we observed a strong male predominance (sex ratio=3.34) and mean age at diagnosis was 63 years. As expected in patients treated by surgical resection, a relatively low rate of cirrhotic livers was observed (45%). Disease was multinodular in 19% of the patients, and positive surgical margins, microvascular invasion and macrovascular invasion were identified in 19%, 56% and 10% of the cases, respectively. The clinico-pathological features of patients with HCC or iCCA were relatively common for these malignancies (Table 1).

Nestin expression on tumor cells was cytoplasmic or more rarely membranous and detected (>1%) in 75% (244/326) of cHCC-CCA, 7% (23/313) of HCCs and 59% (120/204) of iCCA (Fig. 2A-L). Proportions according to each category are presented in Table S1. Representative slides are provided at the following website: https://chcc-cca-study.crosscope.com. In order to assess whether Nestin expression was associated with a distinct morphological component of cHCC-CCA, we reviewed the slides of cHCC-CCA cases showing positive Nestin staining (n = 244). Interestingly, Nestin expression was more often observed in equivocal/intermediate or iCCA contingents (Fig. S1).

To assess the reproducibility of Nestin expression assessment, a second pathologist reviewed a total of 250 randomly selected Nestin immunostained samples. The inter-observer agreement was considered substantial with an estimated weighted kappa of 0.73 (95% CI 0.68-0.78).

Nestin exhibited a good diagnostic performance in distinguishing cHCC-CCA from other PLCs with an AUC of 0.75 (95% CI 0.72-0.79), a sensitivity of 0.75 (95% CI 0.70-0.79) and specificity of 0.72 (95% CI 0.68-0.76) using the threshold of >1% tumor cells with positive staining (selected by maximizing Youden's index) (Fig. 2M). In particular, the diagnostic performance of Nestin improved for the distinction of cHCC-CCA from HCC (the most critical challenge from a clinical standpoint) with an AUC of 0.85 (95% CI 0.82-0.88), a sensitivity of 0.75 (95% CI 0.70-0.79) and a specificity of 0.93 (95% CI 0.89-0.95). Its performance in distinguishing cHCC-CCA from iCCA dropped to an AUC of 0.59 (95% CI 0.55-0.64), a sensitivity of 0.75 (95% CI 0.70-0.79) and a specificity of 0.41 (95% CI 0.34-0.48) (Fig. 2N).

To compare the diagnostic performance of Nestin with that of other conventional biomarkers, we analyzed a subset of 240 randomly selected resected PLCs (cHCC-CCA n = 104, HCC n = 73, iCCA n = 63) using Glypican 3, EPCAM (epithelial cell adhesion molecule) and CK19 immunohistochemistry. Compared to other markers, Nestin showed a higher overall performance to distinguish cHCC-CCA from other PLCs (Fig. S2A-D). Nestin AUC was lower than that of the other markers to differentiate cHCC-CCA from iCCA (Fig. S3A-D). Both Nestin and CK19 exhibited good performance in distinguishing cHCC-CCA from HCC (a score using both markers reached an AUC of 0.92, Fig. S4E).

Biomarkers are particularly important for the evaluation of biopsies, and we therefore validated our findings in our series of 116 PLC biopsy samples (cHCC-CCA n = 44, and non-cHCC-CCA PLC n = 72, including 55 HCCs and 17 iCCAs). The clinical, biological and pathological features of the patients are described in Table S2. Our results were consistent with those observed in the resected PLCs (Fig. 3A-L). Nestin expression was mainly observed in cHCC-CCA and iCCA, and AUCs were 0.80 (95% CI 0.71-0.88, for cHCC-CCA *vs.* other PLCs), 0.86 (95% CI 0.78-0.93, for cHCC-CCA *vs.* HCC) and 0.60 (95% CI 0.45-0.76, for cHCC-CCA *vs.* iCCA) (Fig. 3M-N).

Nestin expression has a prognostic impact in patients with cHCC-CCA treated by surgical resection

Improved prognostication of clinical outcomes in patients with cHCC-CCA is of paramount importance in order to guide treatment strategies. To this end, we further examined whether Nestin could serve as a prognostic biomarker. We first excluded patients with metastatic disease at the time of surgery, R2 resection or preoperative anti-tumor treatment, and then investigated a series of patients with cHCC-CCA treated by surgical resection for whom disease-free (n = 212) and/or overall survival (n = 240) data were available (Fig. 4A-D). During a median follow-up period of 19 months, we recorded 119 tumor relapses and 96 deaths. We then assessed the impact of Nestin expression (based on the subgroups from our semi-quantitative scale) on disease-free survival, our main clinical endpoint. Using maximally selected rank statistics, 30% was identified as the optimal prognostic cut-off. Cases with >30% and ≤30% of tumor cells with positive staining were designated as "Nestin High" (106/326, 32%) and "Nestin Low" (220/326, 68%), respectively. No differences in clinical, biological and pathological features were observed based on Nestin status (Table S3).

Nestin High tumors were significantly associated with worse disease-free survival (log-rank p = 0.006) (Fig. 4B). We next sought to evaluate the independent predictive value of Nestin High expression using Cox regression modeling. Baseline features associated with disease-free survival in univariate analysis were largest nodule diameter (≥50 mm, hazard ratio [HR] 1.63, 95% CI 1.13-2.34, p = 0.009), multinodularity (HR 2.33, 95% CI 1.53-3.54, p <0.001), microvascular invasion (HR 2.44, 95% CI 1.64-3.64, p <0.001) and Nestin expression (HR 1.69, 95% CI 1.15-2.47, p = 0.007) (Table S4) (one limitation is the lack of data regarding CA19-9 serum levels). Multivariate analysis confirmed the independent prognostic value of Nestin expression (p = 0.031) (Fig. 4C and Table S4).

We then investigated overall survival, our second descriptive endpoint (Fig. 4D). Patients with Nestin High tumors (>30%) showed a significantly shorter overall survival (log-rank p =

operating characteristic curve; cHCC-CCA, combined hepatocellular-cholangiocarcinoma; DOR, diagnostic odds ratio; FPR, false positive rate; HCC, hepatocellular carcinoma; HES, hematein-eosin-saffron; iCCA, intrahepatic cholangiocarcinoma; PLCs, primary liver cancers; NPV, negative predictive value; PPV, positive predictive value; TPR, true positive rate. (This figure appears in color on the web.)

0.012) (Fig. 4E). Using Cox regression modeling, features associated with overall survival were largest nodule diameter (≥50 mm, HR 1.52, 95% CI 1.01-2.28, p = 0.044), microvascular invasion (HR 2.31, 95% CI 1.48-3.61, p <0.001) and Nestin High expression (HR 1.70, 95% CI 1.12-2.57, p = 0.01) (Table S5). The independent predictive value of Nestin was confirmed by multivariate analysis (p = 0.039) (Fig. 4F) (Table S5).

Importantly, none of the other conventional biomarkers, including Glypican 3, EPCAM and CK19, showed any prognostic value for disease-free or overall survival (Table S8). We also analyzed the prognostic impact of Nestin among the overall series of patients with PLC treated by surgical resection for whom disease-free (n = 577, including 212 cHCC-CCAs, 214 HCCs, and 151 iCCAs) and/or overall survival (n = 626, including 240 cHCC-CCAs, 215 HCCs, and 171 iCCAs) data were available (Fig. S5A-C). Consistent with our findings for cHCC-CCA, Nestin expression was significantly associated with 5-year disease-free (log-rank p <0.0001, Fig. S5B) and overall survival (log-rank p <0.0001, Fig. S5D) in the overall series of patients with PLC. Multivariate analysis using Cox regression also confirmed that Nestin was an independent prognostic factor in terms for both disease-free (HR 1.89, 95% CI 1.36-2.64, p <0.001, Table S6) and overall survival (HR 1.92, 95% CI 1.32-2.8, p <0.001, Table S7).

We could not determine the prognostic impact of Nestin among patients with HCC as all but one were classified as Nestin Low. Interestingly, Nestin High expression was also significantly associated with worse disease-free survival in patients with iCCA (Fig. S6, no difference in overall survival). There was no association between Nestin status and any other clinical or pathological features (Table S9).

We finally investigate the potential impact of intratumor heterogeneity on the concordance of classification as Nestin High or Nestin Low. We thus assessed, for 60 randomly selected PLCs, Nestin expression in another tumor area (another tissue block was selected). Nestin staining was quite consistent in different regions of the same tumor, regardless of diagnosis (Fig. S7). The classification of cases as Nestin positive or negative was consistent across the different tumor areas investigated (Cohen's kappa = 0.84, 95% CI 0.62-1.00).

The prognostic role of Nestin is validated in patients with cHCC-CCA treated by liver transplantation

We then aimed to validate the prognostic value of the Nestin High 30% threshold on other clinical settings. We were able to collect samples from 91 patients with cHCC-CCA who underwent liver transplantation (diagnosis was performed during the pathological examination of liver explants). Clinical, biological and pathological features of the patients and tumors are presented in Table S10. Mean age at transplantation was 61, and the most frequent risk factors for liver disease were excessive alcohol consumption (55%, 47/86) and HCV (31%, 27/86). The diameter of the largest nodule was higher than 50 mm in 17% (15/90) of the samples and multinodularity was observed in 72% (63/88) of patients. Microvascular invasion was detected in 34% (30/89) of tumors. Almost all patients had established cirrhosis (96%, 77/80). Eighteen relapses and 25 deaths were recorded during a median follow-up of 33 months.

Nestin immunohistochemistry was performed in all cases, and the frequency of tumors classified as Nestin High was lower than observed in patients treated by surgical resection (14% *vs.* 32%). No difference in any clinical, biological or pathological features was observed according to Nestin status (Table S11).

Patients with Nestin High tumors had a significantly shorter disease-free survival after transplantation (Fig. S8A-B, log-rank p = 0.006). As performed for resection samples, we then assessed the prognostic value of Nestin using Cox-regression modeling. In univariate analysis, features associated with disease-free survival were HBV infection (HR 6.66, 95% CI 1.89-23.52, p = 0.003), largest nodule diameter (≥50 mm, HR 3.12, 95% CI 1.17-8.35, p = 0.024), microvascular invasion (HR 3.59, 95% CI 1.41-9.15, p = 0.007) and Nestin expression (HR 3.64, 95% CI 1.36-9.73, p = 0.01) (Table S12). In multivariate analysis, a trend was observed for Nestin High expression (HR 2.30, 95% CI 0.73-7.3, p = 0.15), however the statistical power may be limited by the low number of events (Table S12).

Nestin expression was also significantly associated with overall survival, our second descriptive endpoint (Fig. 5A-B, log-rank p = 0.016). Factors influencing overall survival were largest nodule diameter (HR 2.86, 95% CI 1.23-6.65, p = 0.015), microvascular invasion (HR 2.41, 95% CI 1.1-5.29, p = 0.029) and Nestin High tumors (HR 2.81, 95% CI 1.18-6.74, p = 0.02 (Table S13). Multivariate analysis confirmed that Nestin expression was independently associated with overall survival in patients with cHCC-CCA treated by liver transplantation (HR 2.8, 95% CI 1.1-7.1, p = 0.03) (Table S13).

Patients with Nestin High cHCC-CCA diagnosed on liver biopsies have shorter overall survival

Among the patients with cHCC-CCA and liver biopsy samples, 20 were classified as Nestin High and 24 as Nestin Low. No differences in clinical, biological or pathological features were observed between the 2 groups (Table S14). We then validated the prognostic impact of Nestin in patients for whom liver biopsy and follow-up data were available (n = 35). Most patients had advanced disease not amenable to curative treatment and disease-free survival could therefore not be analyzed. Median follow-up was 10 months, and a total of 25 deaths were recorded. Interestingly, Nestin expression was significantly associated with worse overall survival (Fig. 5C-D, log-rank p = 0.033). Nestin status was also the only factor significantly associated with shorter overall survival using Cox regression analysis (HR 2.54, 95% CI 1.04-6.16, p = 0.04) (Table S15).

Discussion

PLCs are a heterogenous group of cancers with different clinical, pathological and molecular features.[1–4] Among them, cHCC-CCA represents an important challenge as this entity is difficult to diagnose and is considered to be more aggressive than conventional HCC.[5] Its distinction from HCC is particularly important as 1) it is currently considered a contraindication to liver transplantation and 2) it may harbor the targetable molecular alterations usually observed in CCA.[5,24] Misdiagnosis of cHCC-CCA as HCC may therefore lead to inappropriate therapeutic strategies.

To the best of our knowledge, our series is the largest collection of cHCC-CCA samples ever reported. We first validated that Nestin immunohistochemistry may be helpful for cHCC-CCA diagnosis.[18,19] Indeed, its expression was observed in approximately 75% of cHCC-CCAs, 60% of iCCAs while it was very rarely detected in HCCs. Nestin is thus not useful to distinguish cHCC-CCA from iCCA, but it may however help to differentiate cHCC-CCA from HCC, which is the most critical issue from a clinical

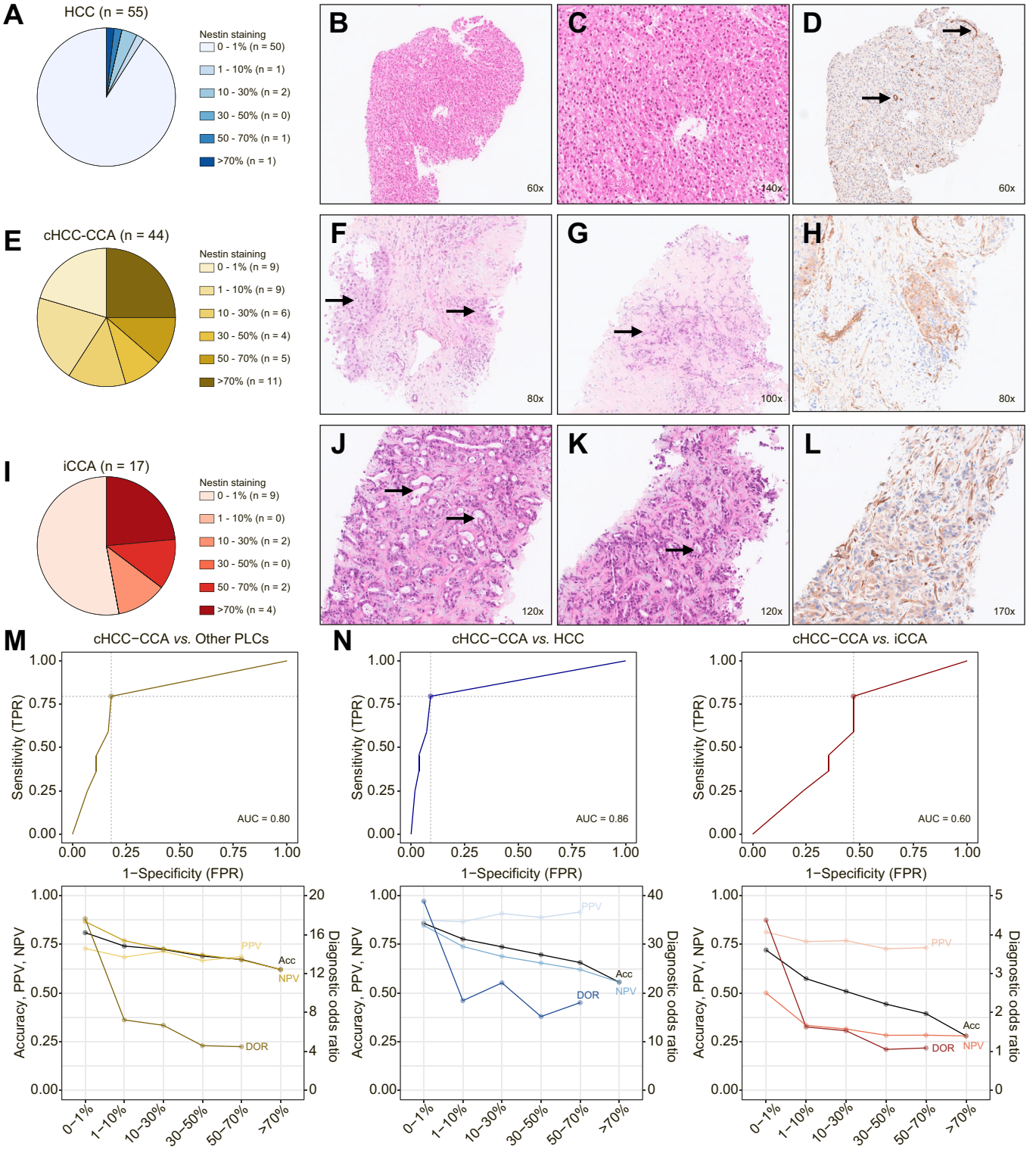

Fig. 3. Validation of Nestin diagnostic value on biopsy samples of PLCs. As observed in resected samples, Nestin staining is negative in the vast majority of HCC biopsies (A). Microscopic examination of this HCC biopsy shows a compact architectural pattern and large neoplastic cells with an eosinophilic cytoplasm (HES) (B, C). None of the HCC tumor cells show Nestin expression, endothelial cells serve as an internal positive control (black arrows) (D). The majority of cHCC-CCA biopsies stain positive for Nestin (E). This cHCC-CCA shows distinct areas with compact clusters of eosiniphilic cells (F) and elongated structures resembling ductules (black arrow) (HES) (G). Cytoplasmic Nestin expression is identified in most tumor cells (H). The rate of Nestin positive iCCA is similar to that observed in resected samples (I). This iCCA features clusters of neoplastic cells arranged in glands (black arrows) and interspersed in a dense fibrous stroma (HES) (J, K). A small subset of tumors cells show a weak cytoplasmic staining. A coarse, probably unspecific staining is also observed in some neoplastic cells (L). Nestin diagnostic performances, assessed by AUCs, are 0.80 (cHCC-CCA *vs.* other PLCs) (M), 0.86 (cHCC-CCA *vs.* HCC) and 0.60 (cHCC-CCA *vs.* iCCA) (N). Dotted lines on the receiver-operating characteristic curves indicate the cut-offs maximizing Youden's index. Lower panels show other diagnostic performance indicators (accuracy, PPV, NPV on the left y-axis and DOR on the right y-axis, respectively). AUC, area under the receiver-operating characteristic curve; cHCC-CCA, combined hepatocellular-cholangiocarcinoma; DOR, diagnostic odds ratio; FPR, false positive rate; HCC, hepatocellular carcinoma; HES, hematein-eosin-saffron; iCCA, intrahepatic cholangiocarcinoma; PLCs, primary liver cancers; NPV, negative predictive value; PPV, positive predictive value; TPR, true positive rate. (This figure appears in color on the web.)

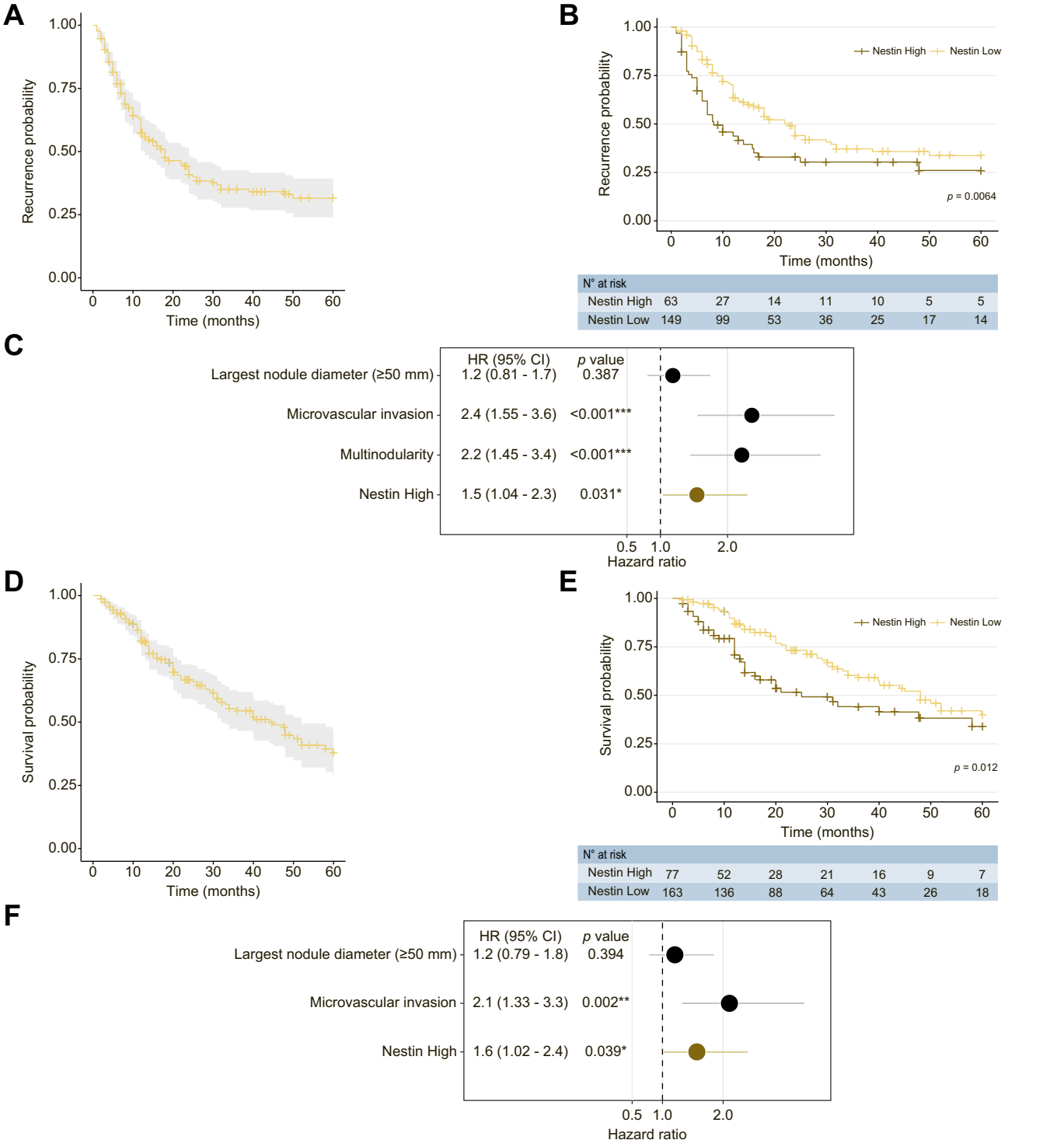

Fig. 4. Nestin is predictive of shorter disease-free and overall survival in patients with cHCC-CCA treated by surgical resection. Kaplan-Meier curve for disease-free survival in patients with cHCC-CCA treated by surgical resection (A). Patients with Nestin High tumors show shorter disease-free survival ($p = 0.0064$, Log-rank test) (B). Multivariate analysis confirms the independent predictive value of Nestin (n = 200). Levels of significance: $^{*}p$ <0.05; $^{**}p$ <0.01; $^{***}p$ <0.001 (Cox proportional-hazards model) (C). Kaplan-Meier curve for overall survival in patients with cHCC-CCA treated by surgical resection (D). Nestin High tumors are associated with shorter overall survival ($p = 0.012$, Log-rank test) (E). Factors that have a significant impact on overall survival in multivariate analysis are Nestin High tumors and microvascular invasion (n = 229) (F). cHCC-CCA, combined hepatocellular-cholangiocarcinoma; HR, hazard ratio. (This figure appears in color on the web.)

standpoint (addition of CK19 immunostaining may also be helpful in this setting). The assessment of Nestin needs to follow several guidelines that are presented in Box 1.

Although the suboptimal sensitivity of Nestin staining for cHCC-CCA may be seen as a potential weakness, we further showed that it allows, using the 30% threshold, the identification

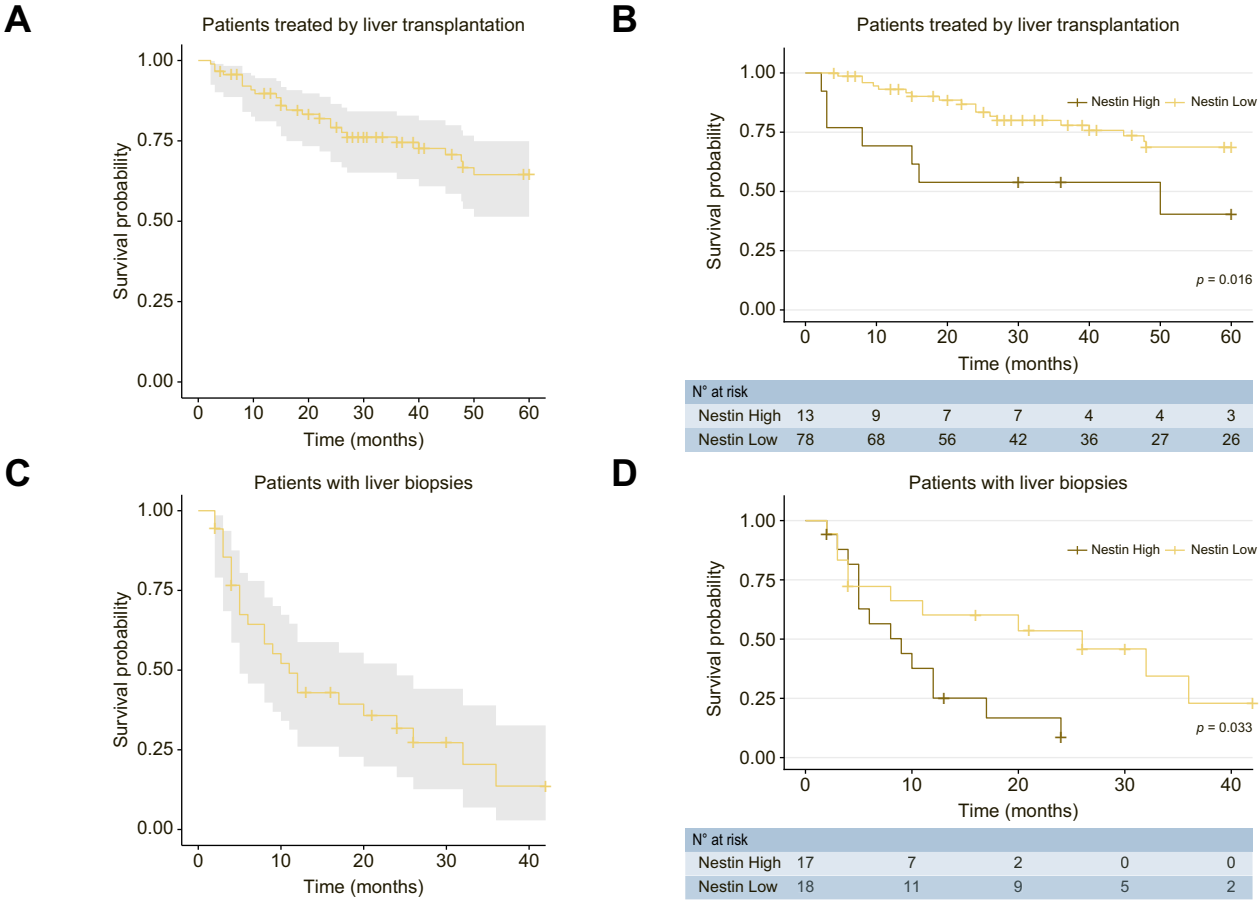

Fig. 5. Nestin is predictive of overall survival in patients with cHCC-CCA in different clinical settings. Kaplan-Meier curve for overall survival after liver transplantation (A). Nestin High cHCC-CCA are also significantly associated with shorter overall survival after transplantation ($p = 0.016$, Log-rank test) (B). Kaplan-Meier curve for disease-free survival in patients for whom cHCC-CCA diagnosis was performed on liver biopsies (C). Patients with cHCC-CCA classified as Nestin High on liver biopsies show shorter overall survival ($p = 0.033$) (D). cHCC-CCA, combined hepatocellular-cholangiocarcinoma. (This figure appears in color on the web.)

of the subset of cHCC-CCA that bear the most adverse clinical outcome. We were first able to confirm, on a larger scale, the prognostic impact of Nestin in patients treated by surgical resection.[17,21] Importantly, none of the other biomarkers (CK19, EpCAM and GPC3) investigated showed any value to predict disease-free or overall survival.

Interestingly, we then demonstrated its value to predict recurrence after liver transplantation and prospective studies will thus have to confirm that transplantation may be considered for patients with Nestin Low cHCC-CCA. A limitation of our results in transplanted patients is that, for all cases, the diagnosis of cHCC-CCA was made incidentally during the microscopic examination of the explant, thus these tumors may not reflect the full spectrum of this entity. We may in particular hypothesize that these patients who were able to undergo transplantation had less aggressive tumors.

We were finally able to investigate a series of patients with cHCC-CCA diagnosed by liver biopsies. As a significant subset of cHCC-CCA are misdiagnosed as HCC by non-invasive criteria, such samples are scarce. Using the same 30% threshold, patients with biopsies showing Nestin High cHCC-CCA had a significantly shorter overall survival (Nestin was also the only feature significantly associated with survival). Our series was however heterogeneous and these results will need to be further validated. There is a renewed interest in biopsy for PLC, in particular within the context of clinical trials, and it will be important to determine if Nestin staining may be used for patient stratification and treatment allocation. Overall, Nestin-positive PLCs (including cHCC-CCA and iCCA), using the 30% threshold, appear to be associated with similarly poor outcomes, and further studies will have to determine if they should be considered as a distinct entity.

Our findings are consistent with data from former studies that aimed to determine the biological function of Nestin. Initially identified as a marker of stemness in neural progenitor

Box 1. Guidelines for Nestin expression assessment.

- Sections should include positive controls (*e.g.* nerves, endothelial cells).
- Staining is cytoplasmic and/or membranous.
- Staining is most often observed in poorly differentiated, equivocal or iCCA contingents.
- Cases with >30% of neoplastic cells showing Nestin expression are classified "Nestin High".

iCCA, intrahepatic cholangiocarcinoma.

cells and malignant gliomas, it was further reported to play a broader role in normal stem cell biology and cancer.[25]

The study by Tschaharganeh and collaborators noticeably demonstrated that the transcriptional repression of Nestin by TP53 was able to restrict cellular plasticity in liver cancer, in line with the morphological spectrum of Nestin-positive tumors that we observed.[21] They further showed that Nestin was not merely a stem cell marker but that it also directly supported tumor growth by promoting progression through the G2M phase of the cell cycle.[21] This role of Nestin in carcinogenesis was also reported in other malignancies including gliomas and lung cancer, and may contribute to the overall poor prognosis observed in patients with Nestin-positive PLC.[25–27]

One important perspective will be to determine if Nestin-positive PLCs, which mainly include tumors with morphological features of cHCC-CCA or CCA, should be considered as a distinct biological entity from other PLCs. Integrative molecular studies will be needed to elucidate this question.

In conclusion, we have shown in different clinical settings that Nestin is a useful biomarker to identify the subset of cHCC-CCAs that bear the worst clinical outcome. Subject to further validation, Nestin immunohistochemistry may be used to refine patient stratification and improve treatment allocation.

Abbreviations

AUC, area under the receiver-operating characteristic curve; cHCC-CCA, combined hepatocellular-cholangiocarcinoma; CK19, cytokeratin 19; EpCAM, epithelial cell adhesion molecule; HCC, hepatocellular carcinoma; HR, hazard ratio; iCCA, intrahepatic cholangiocarcinoma; PLC, primary liver cancer.

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Conflict of interest

JC consults for Crosscope, KB is Crosscope Chief Technology Officer, JS is Crosscope Chief Executive Officer.

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

Authors' contributions

Study design: JC, SC. Obtained funding: JC. Statistical analysis: SC. Data acquisition: All authors. Data analysis: All authors. Drafting the manuscript: JC, SC. Approval of the manuscript: All authors.

Data availability statement

All data relevant to the study are included in the article or uploaded as supplementary information.

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2022.07.019.

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Author names in bold designate shared co-first authorship

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