Research Article

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Morphophenotypic changes in human multistep hepatocarcinogenesis with translational implications

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Background & Aims: Human hepatocarcinogenesis in cirrhosis is thought to be multistep and characterized by a spectrum of nodular lesions, ranging from low to high grade dysplastic nodules (LGDN and HGDN) to early and progressed hepatocellular carcinoma (eHCC and pHCC). The aim of this study was to investigate the morphophenotypical changes of this sequence and their potential translational significance.

Methods: We scored the vascular profile, ductular reaction/stromal invasion and overexpression of five biomarkers (GPC3, HSP70, GS, CHC, and EZH2), in a series of 100 resected nodules (13 LGDN, 16 HGDN, 42 eHCC and 29 small pHCC).

Results: The score separated the four groups of nodules as individual entities (p <0.01). In the sequence, biomarker's overexpression progressively increased with parallel decrease of ductular reaction; the vascular remodeling started very early (LGDN) but did not further develop in a proportion of HCC. eHCC was the most heterogeneous entity, with marginal overlap with HGDN and pHCC. Liver environment (fibrosis, etiology) did not impact on the phenotype of the different nodules. A subclass of eHCC (16/42) without evidence of stromal invasion was identified, suggesting a "preinvasive stage" (p <0.05). For diagnosis, the application of four and five biomarkers (rather than the usual three) improved the sensitivity of the assay for the detection of eHCC (76% and 93% *vs.* 52%); biomarkers in alternative combinations, and also increased the sensitivity of the assay (GS + CHC + EZH2: 76%; GS + CHC + EZH2 + HSP70: 90%).

Conclusions: This study supports the multistep nature of human hepatocarcinogenesis, and suggests that eHCC is more heterogeneous than previously thought. This provides further information of the potential translational significance into clinical practice.

Introduction

The sequence cirrhosis-hepatocellular carcinoma (HCC) is characterized by nodular lesions such as low and high grade dysplastic nodules (LGDN and HGDN, respectively), early HCC (eHCC) and progressed HCC (pHCC). Their morphological features have been detailed in a consensus paper between Eastern and Western pathologists [1]. Whether human hepatic carcinogenesis is in fact multistep is still controversial and there are no animal models strictly mirroring the human changes. This may be due to the lack of models generating a true cirrhotic liver background, which is a cardinal step for HCC development. A further confounding issue is represented by the great molecular and etiopathological heterogeneity of HCC, which contemplates a potential variability in precursor lesions. Indeed, several molecular classifications of HCC and driver genes have been proposed based on different molecular pathways and clinicopathological features [2]. The recent study showing a progressive increase in the rate of mutations of the telomerase reverse transcriptase (TERT) promoter from cirrhosis (no mutation) to LGDN (6%), HGDN (19%), eHCC (61%), small pHCC (42%) and then to advanced HCC (64%), is the first molecular proof of human hepatocarcinogenesis being multistep [3]. A further understanding can be provided by a deep analysis of the early most critical phases of the sequence, namely

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Abbreviations: HCC, hepatocellular carcinoma; LGDN, low grade dysplastic nodule; HGDN, high grade dysplastic nodule; eHCC, early hepatocellular carcinoma; pHCC, progressed hepatocellular carcinoma; CK7, cytokeratin 7; GPC3, Glypican 3; HSP70, Heat shock protein 70; GS, Glutamine synthetase; CHC, Clathrin heavy chain; EZH2, Enhancer of zeste homologue 2; DR, ductular reaction; CD34, cluster of differentiation 34.

dysplastic nodules and eHCC. A few studies have investigated the natural history of these lesions showing that HGDN are the most advanced HCC precursors [4,5] and that eHCC can transform at a variable rate into small, pHCC [6]. Of importance is a recent report demonstrating that eHCC rarely shows microvascular invasion and intrahepatic metastasis, with marginal survival benefit after the surgical treatment [7]. A detailed clinicopathological study of the sequence dysplasia-HCC is expected to provide important information with potential impact on nodule management and treatment. In the clinical practice, small nodules detected in cirrhotic patients under surveillance are now subjected to screening with intense radiological investigation and, in controversial cases, histopathological characterization. Because HCC at an early stage can be treated by either surgery or ablation procedures with very good outcome, the elucidation of the morphophenotypic steps of the human liver transformation from a clinical, radiological and pathological profile is of paramount importance.

The morphophenotypical changes taking place in dysplastic nodules, eHCC and pHCC include, among others, progressive cytoarchitectural alterations, neo-arterial vascularization, decrease to loss of reticulin support, stromal invasion by malignant hepatocytes and biomarker overexpression. All these features have to be carefully examined for a correct diagnosis, particularly when the lesions are very small and the material is fragmented, as it occurs in the liver biopsy [8]. CK7 immunostaining has also been reported as a useful surrogate marker to identify stromal invasion [9]. Ductular reaction (DR), highlighted by CK7 staining, is frequently found in non-cancerous hepatocellular nodular lesions while it tends to disappear at the invasive edges of HCC. During carcinogenesis, a change in the vascular support is a hallmark of the evolution to pHCC, which can be demonstrated by the use of endothelial markers [10,11]. Difficult cases however are in need of a more dedicated approach, contemplating the use of a robust panel of biomarkers to those that have already been validated (GPC3, Glypican 3; HSP70, Heat shock protein 70; GS, Glutamine synthetase) [12–14], which has absolute specificity but limited sensitivity and of markers recently suggested as diagnostically useful, such as CHC (clathrin heavy chain) [15] and EZH2 (enhancer of zeste homologue 2) [16].

In the present study, we have extensively characterized a series of small (up to 2 cm) and well differentiated hepatocellular nodules in cirrhosis with the aim to further illustrate the morphological, vascular, stromal and phenotypical changes occurring from HGDN to eHCC to pHCC.

Materials and methods

Cases under study

The series under study was composed of 100 small well differentiated hepatocellular nodules obtained from 83 patients. Surgery (resection/transplantation) was the elective treatment of all the nodules of the series under study. All the nodules were up to 2 cm in size, radiologically detected or incidentally discovered after pathological examination. Nodules were located in a cirrhotic/hepatitic background. Presence of a synchronous overt (>2 cm) HCC and/or previous treatment (resection or ablation) were also noted. Cases were collected from Humanitas Research Hospital, San Paolo Hospital, Policlinico Maggiore Hospital (Milan, Italy) and Ofunachuo Hospital, (Kamakura, Japan) from 2006 to 2012. All the original H&E slides were blindly reviewed at Humanitas Research Hospital (M.R., L.D.T., A.S.) and cases showing LGDN (n° 13) and small pHCC (n° 29) morphology were preliminary distinguished from the group of well differentiated hepatocellular nodules requiring further study for the differential diagnosis between LGDN and eHCC (n° 58). Clinicopathological baseline data of patients and nodules are reported in Table 1.

Table 1. Clinical and pathological baseline parameters of the cases under study.

Variable	Category	Number
Gender	Male	64 (77%)
	Female	19 (23%)
Age	Mean (years)	68
	SD	10.4
Etiology	Viral (HCV, HBV)	69 (83%)
	Non viral	14 (17%)
Synchronous HCC	y	72 (87%)
	n	11 (13%)
Previous treatment for other HCC (resection, LRT)	y	14 (17%)
	n	69 (83%)
Type of treatment	Resection	62 (75%)
	OLT	21 (25%)
Background liver disease	Fibrosis (METAVIR 1-3)	23 (28%)
	Cirrhosis (METAVIR 4)	60 (72%)
Diagnosis	LGDN	13/100 (13%)
	HGDN/eHCC	58/100 (58%)
	Small pHCC	29/100 (29%)
Size (largest nodule)	Mean (cm)	1.1
	SD	0.6

LRT, loco-regional treatment; OLT, orthotopic liver transplantation; LGDN, low grade dysplastic nodule; HGDN, high grade dysplastic nodule; eHCC, early HCC; pHCC, progressed HCC.

Immunohistochemistry

Seven consecutive recuts from the original paraffin blocks were obtained in all cases and were stained with antibodies against CK7, CD34, GPC3, HSP70, GS, CHC and EZH2. Supplementary Table 1 details the reagents used in this study, the working dilutions, and the detection system. Immunocytochemical analysis was performed according to standard procedures. The staining assessment was made by three observers (M.R., L.D.T., A.S.) at multihead microscope and a semiquantitative evaluation was always performed through the comparison between lesional *vs.* extralesional immunoreactivity. The specimens were evaluated with the criteria illustrated below.

CK7 staining was used to evaluate the DR. CK7 + DR was analyzed at the epithelial-stromal boundaries within and at the outer edge of each nodule. DR was semiquantitively scored as follows: 0 = maintained (as in the extralesional liver); 1 = focal decrease (50–99% DR as compared to extralesional liver); 2 = diffuse decrease (1–49% DR as compared to extralesional liver) and 3 = extinguished (0% DR). Fig. 1 illustrates the four categories of CK 7 staining.

CD34 was used to assess the extent of the intratumoral vascular network, which was semiquantitively scored as follows: 0 = focal staining of low density capillarized vessels; 1 = focal staining of high density capillarized vessels; 2 = diffuse staining of low density capillarized vessels; 3 = diffuse staining of high density capillarized vessels. Fig. 2 illustrates the four categories of CD34 staining.

Biomarkers staining: GPC3 (cytoplasmic/membrane), HSP70 (nucleo/cytoplasmic), GS (cytoplasmic), CHC (cytoplasmic) and EZH2 (nuclear) were scored as positive when unequivocally overexpressed, regardless the staining intensity, in at least a consistent hepatocellular tumor subpopulation (groups of hepatocytes accounting for not less than 5–10% cellularity of the lesion) as compared to the extranodular liver. Isolated tumor cells were not considered sufficient criteria for positivity. For GS, in particular, protein overexpression was ascertained

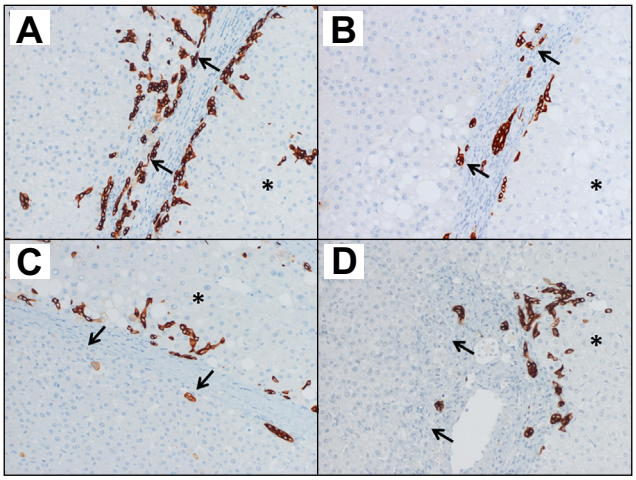

Fig. 1. Illustration of criteria used to score CK7+DR. Categories of CK7+DR: (A) Maintained: preserved and continuous immunoreactivity at the nodule periphery (arrows) as compared to the extralesional liver (asterisk); score = 0. (B) Focal loss: reduced and discontinuous immunoreactivity at the nodule periphery (arrows) as compared to the extralesional liver (asterisk); score = 1. (C) Diffuse loss: a few residual ductules at the nodule periphery (arrows) as compared to the extralesional liver (asterisk); score = 2. (D) CK7+DR extinguished: no immunoreactivity at the nodule periphery (arrows) as compared to the extralesional liver (asterisk); score = 3. (This figure appears in colour on the web.)

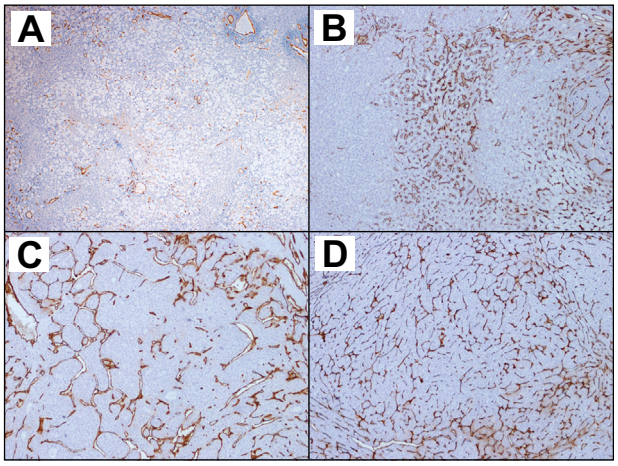

Fig. 2. Illustration of criteria used to score CD34 staining. Categories of CD34 + vascular network: (A) focal staining of low density capillarized vessels; score = 0. (B) Focal staining of high density capillarized vessels; score = 1. (C) Diffuse staining of low density capillarized vessels; score = 2. (D) Diffuse staining of high density capillarized vessels; score = 3. (This figure appears in colour on the web.)

whenever a non-canonical (i.e.: pericentral/periseptal) pattern of staining was seen. We refer this abnormal and non-canonical GS expression as "patternless pattern of staining". Fig. 3 illustrates positive immunoreactions for these five biomarkers.

Positive staining for each biomarker was scored 1 so that each individual nodule had a final score ranging from 0 (all negative) to 5 (all positive).

Cumulative nodule score: CK7, CD34 and biomarkers staining were then cumulated in a score for each individual nodule ranging from 0 to 11.

Diagnostic criteria for LGDN, HGDN, eHCC and pHCC

Following criteria detailed by the International Consensus Group for Hepatocellular Neoplasia [1] and EORT guidelines [17] we classified as LGDN those showing portal tracts, mild increase in cell density with a monotonous pattern of growth,

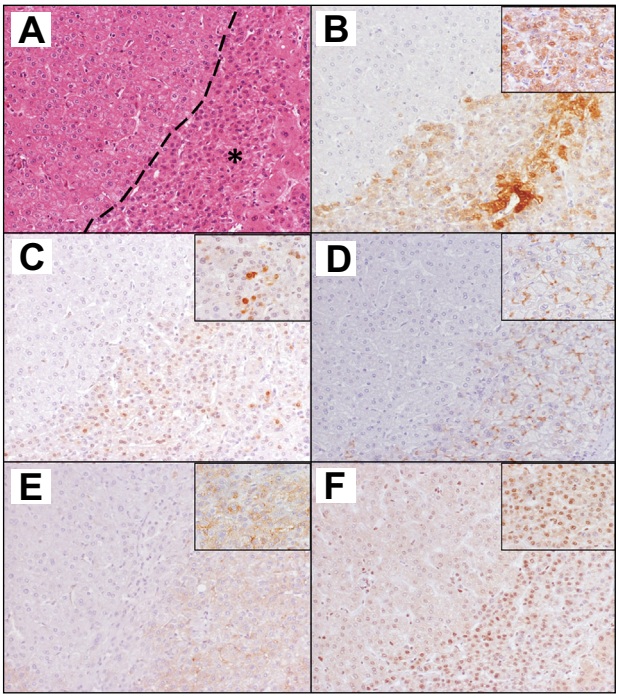

Fig. 3. Positive immunoreaction of GPC3, HSP70, GS, CHC and EZH2. (A) A case of eHCC (asterisk) characterized by increased cell density and irregular unencapsulated borders (dashed line), showing immunoreactivity for the five biomarkers in the same tumoral area. (B) Diffuse faint to moderate tumoral cytoplasmic GS staining, highlighted in the inset. Notice the completely unstained adjacent non-tumoral parenchyma. (C) Faint to moderate tumoral nucleocytoplasmic HSP70 staining, highlighted in the inset. Notice the completely unstained adjacent non-tumoral parenchyma. (D) Faint tumoral membrane GPC3 staining, highlighted in the inset. Notice the completely unstained adjacent non-tumoral parenchyma. (E) Faint cytoplasmic CHC, highlighted in the inset. Notice the completely unstained adjacent non-tumoral parenchyma. (F) Moderate nuclear EZH2 staining, highlighted in the inset. Notice that a few hepatocyte and non-hepatocyte nuclei of the adjacent non-tumoral parenchyma are also immunostained.

without cytological atypia or architectural changes (pseudoglands) beyond those clearly regenerative.

We classified as HGDN those showing residual portal tracts, cytoarchitectural abnormalities including rare pseudoglands, retained reticulin framework, no evidence of stromal invasion and focal but facultative immunoreactivity for up to one biomarker.

We classified as eHCC those showing a few residual portal tracts, replacing and not substitutive pattern of growth, cytoarchitectural abnormalities including pseudoglands, well differentiated histology (G1), focal but facultative steatosis, retained or focally decreased reticulin framework, evidence of stromal invasion and/or immunoreactivity for at least two biomarkers.

We classified pHCC as those showing lack of portal tracts, expansile/substitutive pattern of growth, important cytoarchitectural abnormalities, well to moderate histology (G1–2), and discrete loss of reticulin framework. In these tumors, malignancy was clearly evident on H&E so that there was no need to demonstrate stromal invasion and/or biomarker immunoreactivity for the original diagnosis. Supplementary Fig. 1 illustrate the main H&E features of HGDN, eHCC and pHCC.

Statistical analysis

All variables were reported as numbers and percentages. Continuous variables were summarized as mean (± SD) or median with range, and categorical variables as frequency and percentage. Comparisons between groups of quantitative variables were performed using the Mann-Whitney *U* test or Kruskal-Wallis test. Comparisons among groups of qualitative variables were performed using χ^2 and Fisher exact tests, as appropriate. All tests were two-sided and used a signifi-

cance level of 0.05. All analyses were performed with SPSS 22.0 (®2013 SPSS Inc., Chicago, IL, USA).

Results

According to criteria detailed above, LGDN was diagnosed in 13 cases, HGDN in 16, eHCC in 42 and progressed HCC in 29.

CK7 + DR in LGDN/HGDN/eHCC/pHCC

Supplementary Table 2 details the distribution of CK7 + DR in the four types of nodules.

The main feature of LGDN and HGDN was a retained pattern of DR or a focal decrease (100% and 81% respectively). This feature was never seen in association with evidence of stromal invasion. On the opposite end of the spectrum of nodules, DR was largely decreased but mostly extinguished in the pHCC. A gradual loss from focal (50%) to diffuse (31%) decrease to extinction (17%) was seen in eHCC suggesting a greater heterogeneity in terms of DR modulation at this step of carcinogenesis.

CD34 + vascular network in LGDN/HGDN/eHCC/pHCC

Supplementary Table 3 details the distribution of CD34 immunoreactivity in the four types of nodules. The main pattern of CD34 staining seen in dysplastic nodules and eHCC was a focal low density vascular pattern ranging from 77% in LGDN to 59% in eHCC to 45% in progressed HCC. High density CD34+ vessels were only seen in pHCC (35%).

Biomarkers overexpression in LGDN/HGDN/eHCC/pHCC

Supplementary Table 4 details overexpression of GPC3, GS, HSP70, CHC and EZH2 using the panel of five biomarkers. A clear-cut progression in the overexpression of biomarkers was seen from LGDN to pHCC. In particular, one biomarker was seen 2/13 LGDN (15%), in 10/16 HGDN (62%), while eHCC showed 2–3 markers in 69% of cases and pHCC showed 4–5 markers in 66% of the cases. None of eHCC/pHCC was unstained after panel application while one marker staining was seen in 7% eHCC (CHC, HSP, EZH2) and in 3% pHCC (EZH2).

Supplementary Table 5 details the distribution of individual biomarkers in the lesions, which progressively increased from dysplasia-eHCC-pHCC. The most overexpressed markers were HSP70 (HGDN), GS (eHCC) and HSP70/CHC in pHCC. GPC3 was mostly expressed in pHCC.

Cumulative score (CK7/CD34/5 biomarkers) in LGDN/HGDN/eHCC/pHCC

The cumulative score of the nodules is detailed in Supplementary Table 6 and the mean score is illustrated in Fig. 4. LGDN and HGDN showed values in the 0–3 range, eHCC in the 3–8 range and pHCC in the 5–11 range. eHCC had a 3–4 score in 50% of cases. Score 3 was also seen in LGDN (7.7%) and HGDN (37.5%) while score 4 was only seen in eHCC. A score ⩾9 was only seen in a fraction of pHCC (38%). As shown in Fig. 4 the mean score was significantly different among the four diagnostic categories (p <0.01). The mean values were 0.9 (SD 1) for LGDN, 1.9 (SD 1) for HGDN, 4.9 for eHCC (SD 1.5) and 7.9 for pHCC (SD 2).

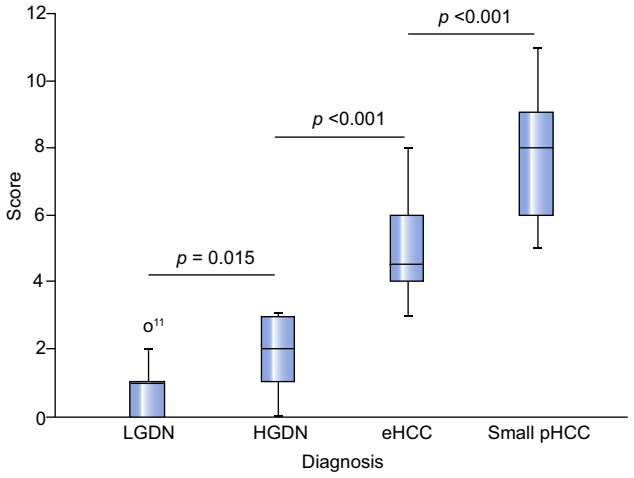

Fig. 4. Cumulative semiquantitative score (CK7/CD34/5 biomarkers) of LGDN, HGDN, eHCC and pHCC, expressed as mean (±SD).

Accuracy of biomarker panels for the diagnosis of eHCC

We also explored the accuracy of the possible combinations of biomarkers in different panels for the diagnosis of eHCC. We first used a panel of three biomarkers (HSP70, GPC3 and GS, 3M), then supplemented by the addition of CHC (4M) and finally of EZH2 (5M) in a sequential procedure. We then evaluated the accuracy of panels of 3 and 4 biomarkers by assessing all the possible combinations in a non-sequential procedure. Results are shown in Supplementary Tables 7 and 8.

Early HCC was documented by a 3M panel (the one recommended) in 52% of the cases, by a 4M panel in 76% (addition of CHC) and by a 5M in 93%. When all the possible biomarker combinations were tested, a 3M panel composed by GS + CHC + EZH2 identified 76% of eHCCs and a 4M panel composed by GS + CHC + HSP70 + EZH2 identified 91% of eHCCs. Notably GPC3 was the less performant marker in both the sequential and non-sequential procedures. As shown in Fig. 5, the area under the curve values, analyzed with Receiver Operating Characteristic (ROC) curve analysis for the diagnosis of eHCC, showed an improvement in the accuracy from the traditional diagnostic tools to those under study. Results ranged from 0.810 (95% confidence interval [CI], 0.702–0.917) for stromal invasion and 0.762 for the classical 3M panel (95% CI, 0.643–0.881), to 0.978 with the use of 5M panel (95% CI, 0.946–1.000) and to 0.964 (95% CI, 0.924–1.000) when the cumulative comprehensive score was evaluated. The accuracy of alternative biomarker panels such as GS + CHC + EZH2 (3M) and GS + CHC + HSP70 + EZH2 (4M) is also illustrated in Supplementary Fig. 2.

Subclass analysis: eHCC

We sought to further evaluate the heterogeneity of eHCC. This diagnosis was made in 42 cases and in 26 of these (62%) we had evidence of stromal invasion by H&E corroborated by diffuse decrease or extinction of CK7 + DR in the majority of cases (61%). However, in 16 cases (38%) no clear-cut evidence of stromal invasion by malignant hepatocytes could be demonstrated after H&E staining, although a diffuse decrease or even extinction of DR was seen in 25% of them. In these cases, the diagnosis of eHCC was

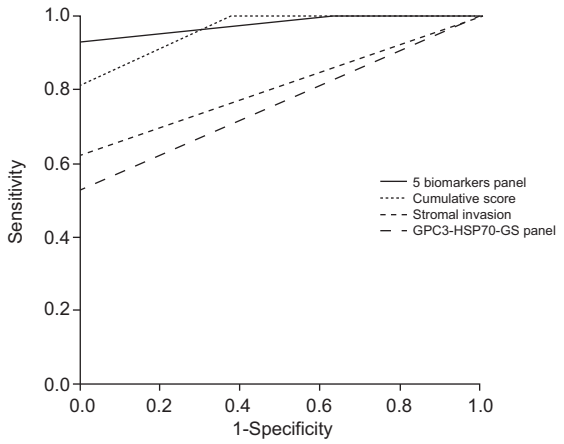

Fig. 5. Receiver operating characteristic curves for the diagnosis of eHCC considering as parameters stromal invasion, 3M panel (at least two positive among GPC3-HSP70-GS), 5M panel (at least two positive among GPC3-HSP70-GS-CHC-EZH2) and the cumulative score.

based on the general features of the tumors and on the overexpression of at least two biomarkers. Not having been able to demonstrate stromal invasion we provisionally named this subclass of eHCC as "preinvasive" eHCC. Table 2 and Supplementary Fig. 3 illustrate CK7, CD34 and biomarkers staining in these "preinvasive" as opposite to "invasive" eHCC, where we have been able to document invasion of liver parenchyma by malignant hepatocytes. "Preinvasive" differed from "invasive" eHCC also in term of biomarker overexpression. All the biomarkers under study were seen more often overexpressed in "invasive" rather than in "preinvasive" eHCC. The "preinvasive category" showed a cumulative mean score of 4.2. When compared to the score of "invasive" eHCC (mean score = 4.9) the test showed to be significantly different ($p = 0.03$). The majority of these cases (62.5%) were resected and associated with an overt HCC (75%). Fig. 6 shows representative features of "preinvasive" eHCC.

Impact of extratumoral environment on morphology and phenotype of lesion

Finally, we investigated if the underlying liver disease, namely etiology and stage of fibrosis, and type of treatment were able to impact on the vascular remodeling, stromal invasion and biomarker overexpression of the nodules. However, the nodule phenotype was scarcely affected by these variables as shown in

Table 2. CK7, CD34 and biomarkers overexpression in "preinvasive" and "invasive" eHCC.

Biomarker	Score	"Preinvasive" eHCC (n = 16)	"Invasive" eHCC (n = 26)
CK7	0-1	12 (75%)	10 (38.5%)
	2-3	4 (25%)	16 (61.5%)
CD34	0	11 (68.8%)	14 (53.8%)
	1-3	5 (31.2%)	12 (46.2%)
At least 3/5 biomarker	3	7 (43.7%)	17 (65.4%)
At least 4/5 biomarker	4	2 (12.5%)	8 (30.8%)

eHCC, early HCC.

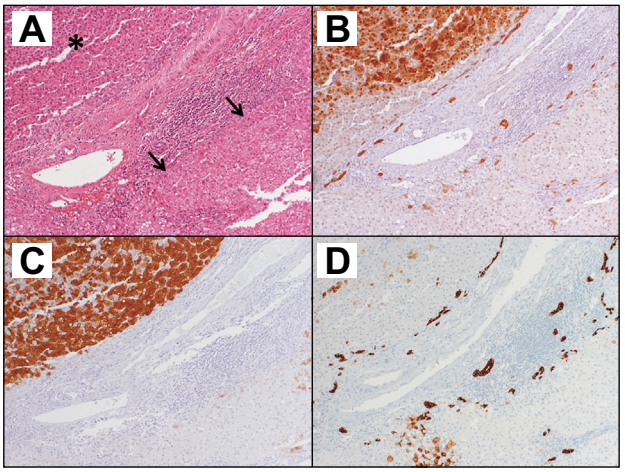

Fig. 6. Features of "preinvasive" eHCC. This particular lesion epitomizes a nodular growth resembling eHCC from a morphological analysis: as shown by H&E (A), the nodule cellularity (asterisk) doubles that seen in the adjacent parenchyma (arrows) and it is characterized by a thicker trabecular arrangement; in addition the lesion clearly overexpresses HSP70 (B) and GS (C). However the search of stromal invasion is negative as also highlighted by the preserved CK7+DR (D). The latter feature supports the concept of "preinvasive" eHCC. (This figure appears in colour on the web.)

Supplementary Figs. 4–6. Notably no LGDN/HGDN were observed in the context of fibrosis stage F1-F2, while only two HGDN developed in fibrosis stage F3. HCC onset in fibrosis stage F1 was also an uncommon event in this series, occurring in 4/71 cases (5.6%).

Discussion

The concept of eHCC is a relatively recent morphological acquirement having been formally introduced in the diagnostic workout of pathologists after a consensus agreement between Eastern and Western pathologists in 2009 [1]. Whether this entity originates from high grade dysplasia is supported by the recent demonstration of the stepwise increase in the mutation rate of the TERT promoter from LGDN to eHCC, the first molecular proof supporting human hepatocarcinogenesis being multistep [18]. In this study, we aimed to illustrate in greater detail the morpho-phenotypical changes of the sequence LGDN-HGDN-eHCC-pHCC in cirrhosis. In fact, a solid understanding of these lesions is essential for their earliest detection, better treatment and even tumor prevention. To this aim, we decided to select a series of Italian and Japanese cases, taking profit of the larger experience of Eastern countries (Japan in particular) in the identification and treatment of eHCC. We believe this as the first joint effort between Eastern and Western pathologists in the full characterization of lesions encompassing the spectrum of sizable lesions during the human carcinogenesis, in cirrhosis. We focused our attention on three cardinal features of the lesions, namely the vascular support, the stromal invasion and the expression of biomarkers. It is known indeed that only a careful analysis of a number of morphophenotypical features can provide diagnostic information, which might go undetected using the routine stains, as suggested by EASL-EORTC practical guidelines [17].

We have seen in the sequence that the earliest of the studied alterations was the vascular remodeling of nodules, already

detectable in low grade dysplasia. By using a well-accepted marker such as CD34 we have seen in HGDN an increased vascularization, which is, however and mostly, of low density and focal. To our surprise, the vascular remodeling did not linearly develop in the sequence because this pattern of vascularization was also seen in 59.5% of eHCC and even in 44.8% of pHCC. Notably 37.5% of HGDN showed a pattern of focal high density CD34+ vascular network, suggesting a vascular heterogeneity of these lesions. Also only 1/3 of pHCC showed the expected pattern of diffuse, high density vascularization. These features suggest that most of the early lesions still have a very immature/incomplete neovascularization and that even small and progressed HCC are largely incompletely vascularized, in keeping with the radiological concept of hypovascular small HCC [19]. We thus emphasize that a diffuse CD34 staining, which is considered a diagnostic hallmark of HCC, is uncommon in small HCC. As such pathologists have to be careful and to consider that a focal CD34 pattern of staining "*per se*" cannot be taken as a diagnostic proof against malignancy. An accurate pathological and radiological study of nodule vascularization is therefore expected to provide important information on nodule biology, progression and sensitivity to therapy [20].

The DR is a hyperplastic regenerative response of the biliary tract, taking place in chronic hepatitis/cirrhosis. When a neoplastic hepatocellular process is ongoing (dysplastic nodule and eHCC) the nodule growth, the vascular remodeling and the stromal fibrosis all likely concur to interfere with this regenerative process. When malignant cells start to invade the fibrous stroma the ensuing desmoplastic changes take the place of the biliary proliferation to the point that the disappearance of DR has been considered as a surrogate marker to look for in the search of stromal invasion by malignant hepatocytes [9]. These changes in the CK7 + DR are nicely illustrated in our series showing intact or focally reduced DR in the LG/HGDN but diffuse decrease or extinction in 48% of eHCC and in the vast majority of pHCC (72%). Interestingly around half of eHCC showed a pattern of DR relatively preserved (focal decrease) as seen in HGDN. Thus, in terms of DR, eHCC was heterogeneous, with a profile partly overlapping that is seen in precancerous lesions and partly seen in pHCC. Of importance, in 11/22 cases of eHCC with preserved/-focally lost DR, evidence of stromal invasion by malignant hepatocytes was missing. This feature prompted us to further analyze eHCC distinguishing them into two categories, those showing clear-cut evidence of stromal invasion and those without, as it will be discussed later.

The further step of this study was to analyze the overexpression of the five biomarkers in the four lesional categories (LGDN, HGDN, eHCC and small pHCC). These biomarkers (GPC3, GS, HSP70, CHC and EZH2) have been proposed in the literature as useful in the differential diagnosis between precancerous lesions and eHCC [12,15,16]. To date, three of them (GPC3, GS and HSP70) have been endorsed by international guidelines [17]. The analysis of the five biomarkers nicely separated the three diagnostic categories, with an increased number of biomarkers expressed in more advanced lesions. The majority of small pHCC showed at least four biomarker staining (66%) while the majority of eHCC showed at least two (93%). This progressive increase in the overexpression of biomarkers along the spectrum of human hepatocarcinogenesis is again in support of a multistep process. This concept was also enforced when biomarkers data were integrated with those of vascular remodeling and stromal invasion. The cumulative score we obtained was able to separate the three diagnostic categories with statistical significance.

From a diagnostic point we have seen that the most challenging lesions (i.e. eHCC) were immunoreactive in 52% of the cases for at least two markers when using the recommended 3M panel, as also previously reported [12–14]. The panel resulted even more effective (76% sensitivity) when integrated by the addition of CHC (4M) and EZH2 (5M, 93% sensitivity). Interestingly, when we evaluated all the possible combination of the five biomarkers, we were surprised to find that a 3M panel composed by GS, CHC and EZH2 detected eHCC in 76% of cases and that the same panel integrated with the addition of HSP70 (4M) had a sensitivity of 90%. Thus, this study suggests the opportunity to validate alternative panels of biomarkers for the diagnosis of eHCC, which may have an increased accuracy over those already validated, thus resulting more cost-effective.

Interestingly, GPC3, one of the first biomarkers to be suggested as helpful for HCC diagnosis, was shown to be the least sensitive of all of the investigated markers, particularly in early HCC. This is in keeping with the biology of the molecule, which has been associated to tumor dedifferentiation, unfavorable prognosis and recurrence [21,22].

When we further analyzed the expression of the five biomarkers in the two subclasses of eHCC, namely those showing clear-cut evidence of stromal invasion and those without, we saw that, individually taken, each biomarker was more overexpressed in the former group as opposed to the latter. This confirmed to us that eHCC is a heterogeneous category of nodules, with possibly different malignant behavior. In order to distinguish between these two categories we propose to name eHCC without clear proof of stromal invasion as "preinvasive" eHCC as opposed to eHCC with clear-cut evidence of stromal invasion.

We acknowledge that the classification of these lesions and the parameters we have used to analyze them should be supported and validated in the clinical practice also by follow-up data. The retrospective series we have studied, their different source, the alternative surgical treatment (resection and transplantation) and the presence, in most of the cases, of a synchronous HCC in the proximity of the lesions under study, were all factors preventing us to evaluate the impact of the lesions on the clinical history of the patients. Prospective studies of these lesions documented in the liver biopsy during surveillance programs constitute the best strategy to evaluate the clinical impact of this spectrum of early hepatocellular nodules.

In this study we have demonstrated that in the sequence LGDN-HGDN-eHCC-pHCC, biomarkers overexpression progressively increased and DR in parallel decreased, contrasting with the very early but mostly incomplete vascular remodeling. Early HCC resulted very heterogeneous with its own phenotype but also with features overlapping those seen in HGDN and in pHCC. More specifically a subclass of eHCC (16/42) characterized by the overexpression of least two biomarkers but lacking evidence of stromal invasion was identified and suggested to be a very early "preinvasive stage" of human hepatocarcinogenesis.

For diagnostic purposes the application of four and five biomarkers significantly improved the sensitivity of the panel for the detection of eHCC (76% and 93% *vs.* 50%). Of importance, alternative combinations of the biomarkers contributed to increase the sensitivity of the immunocytochemical assay (GS + CHC + EZH2: 76%; GS + CHC + EZH2 + HSP70: 90%).

Finally the evaluation of the tumor environment indicated that the phenotypic profile of the different nodules was relatively independent from the liver background (fibrosis/cirrhosis and etiology), suggesting to us that the carcinogenetic process under study relies upon lesions with precise and consolidated structural features.

In conclusions, our data support the multistep nature of human hepatocarcinogenesis and suggests that eHCC is a heterogeneous entity likely featuring also a "preinvasive" stage of disease; finally, the diagnostic use of biomarkers in an immunocytochemical assay can benefit from the addition of validated molecules or by their use in alternative combinations.

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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.

Authors' contribution

Amedeo Sciarra, Luca Di Tommaso, Masayuki Nakano: data collection and analysis, drafting.
Annarita Destro: technical work.
Guido Torzilli, Matteo Donadon, Marco Maggioni, Gaetano Bulfamante, Silvano Bosari, Masanori Matsuda, Hideki Fujii, Tomoaki Ichikawa, Hiroyuki Morisaka, Katsuhiro Sano, Shintaro Ichikawa: cases selection, clinical and radiological information.
Massimo Roncalli: study design, data analysis, drafting.

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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.2015.08.031.

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Author names in bold designate shared co-first authorship

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