Early hepatocellular carcinoma with high-grade atypia in small vaguely nodular lesions

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Multistep hepatocarcinogenesis is characterized by the following three phases: premalignant dysplastic nodules (DN) (low grade or high grade), early hepatocellular carcinoma (eHCC) and finally advanced HCC. Histopathological diagnosis of these three types of lesions was established by an international consensus in 2009 and was also described in the 4th edition of the World Health Organization (WHO) classification in 2010. Imaging diagnosis of these lesions also supports the presence of premalignant or early malignant lesions without definite features of advanced HCC that can transition to advanced HCC during follow up. In particular, hemodynamic changes detected by contrast-enhanced computed tomography or ultrasonography and hepatocellular changes detected by gadolinium ethoxybenzyl diethylene triamine pentaacetic acid-enhanced magnetic resonance imaging are useful for evaluating the malignant potential of these lesions. Clinically, equivocal nodules smaller than 2 cm without definite features of HCC are encountered in high-risk patients, such as those with chronic hepatitis and liver cirrhosis, and are generally followed up without treatment. Follow up of these nodules has revealed various possible changes, from disappearance or stability without change of nodule size or hemodynamics to increased nodule size and blood supply; that is, hypervascularization of the nodule. It is hypothesized that equivocal nodules on imaging diagnosis include regenerative nodules, dysplastic nodules (DN) and eHCC; however, the clinical behavior of these nodules is quite heterogeneous, and, in particular, clinical diagnosis of eHCC is difficult.

Early hepatocellular carcinoma (eHCC) is characterized by an increased cell density, cellular and structural atypia, decreased intratumoral portal tracts, and the presence of variable numbers of unpaired arteries. In addition, stromal invasion is frequently observed; indeed, the presence of stromal invasion is one of the pathological features used to make a diagnosis of HCC. Together with these histological features, a panel of immunohistochemical markers is reportedly useful in making a diagnosis of eHCC. These molecular markers include heat shock protein 70 (HSP70), glypican-3, glutamine synthetase, adenylate cyclase-associated protein 2 (CAP2) and the polycomb gene product Bmi-1. Most published studies have focused on the differential diagnosis of eHCC from DN; however, a detailed histopathological study of eHCC itself has not yet been conducted.

The aim of the current study was to investigate the detailed histopathological features of eHCC. Consequently, we conducted immunohistochemical analyses of vascular changes...
Materials and Methods

Patients. From 40 patients who underwent hepatectomy at Keio University Hospital from 1990 to 2013, 66 nodules smaller than 25 mm in diameter that showed as small vaguely nodular lesions macroscopically and were pathologically diagnosed as DN or eHCC were selected for this study from pathological records. The nodule sizes ranged from 5 to 25 mm, with a mean value of 11.1 mm and a median value of 10 mm. The patients were 34 men and 6 women aged between 46 and 79 years (mean age: 62.6 years; median age: 60 years.) Operative indications were tumor resection in 21 patients and living donor liver transplantation in 19 patients. The patient backgrounds are summarized in Table S1. This study was approved by the institutional review boards of Keio University School of Medicine.

Pathological analysis. All histological diagnoses and evaluations of immunohistochemical stainings were conducted by two pathologists (H.O. and Y.M.) without reference to clinicopathological data. If the initial evaluation provided different results, a consensus interpretation was reached after re-examination.

Histological diagnosis. Nodular lesions were re-diagnosed as low-grade dysplastic nodules (LGDN), high-grade dysplastic nodules (HGDN), or eHCC based on the pathological diagnostic criteria of the WHO classification(6) and the international consensus classification.(5)

Using H&E-stained specimens, we observed and evaluated cellular atypia (size of the nucleus), structural atypia and tumor cell density throughout the whole tumor. Nuclear size (equal to/enlarged compared with non-tumoral liver tissue) and tumor cellularity (more than twofold/less than twofold compared with non-tumoral liver cellularity) were determined. Structural atypia was evaluated based on the presence of a scirrhous hepatocellular carcinoma-like pattern composed of fibrous stroma (denoting a “scirrhous component”) in the lesion (Fig. 1a). Stromal (portal area) invasion was evaluated in elastic Van Gieson-stained sections. The extent of tumor invasion was evaluated according to the following four grades with reference to the elastic fiber seen in the outer circumference around the portal area: None, invasion was not found or was unclear; Suspected invasion, tumor cells were observed on the border part of the elastic fiber; Distinct invasion/mild, despite distinct invasion of the stroma in the portal area, further severe invasion was not found; Distinct invasion/severe, massive invasion (Fig. 1b,c) or invasion enclosing the portal vein and periphery of bile duct, invasion in an “Indian file pattern,” or hepatic vein wall invasion (Fig. 1d–f).

Immunohistochemical analysis. Formalin-fixed, paraffin-embedded serial tissue sections 4-μm thick were placed on silane-coated slides for immunohistochemical analysis. The sections were deparaffinized, rehydrated in xylene and graded-diluted ethanol (50%–100%), and then immersed for 20 min in...
0.3% hydrogen peroxide in absolute methanol to block endogenous peroxidase activity. Immunohistochemical staining using mouse anti-human HSP70 monoclonal antibody (1:500 dilution; SC-24; SantaCruz Biotechnology, Santa Cruz, CA, USA), mouse anti-human CD34 monoclonal antibody (1:50 dilution; QBEnd-10; Dako, Glostrup, Denmark) and mouse anti-human h-caldesmon monoclonal antibody (1:100 dilution; hCD; Dako) was performed using a Leica Bond-Max automated immunostainer (Leica Microsystems, Bannockburn, IL, USA). For CAP2 and Bmi-1, the tissue sections were heated at 120°C in 0.01 mol/L sodium citrate buffer, pH 7.0, for 10 min before incubation with rabbit anti-human CAP2 polyclonal antibody (1:4000 dilution) and mouse anti-human Bmi-1 polyclonal antibody (1:200 dilution; Upstate Biotechnology, Lake Placid, NY, USA), and immunohistochemical staining was performed using the ImmPRESS system (Vector Laboratories, Burlingame, CA, USA).

For CAP2 and HSP70, tumor cells showing strong expression compared with non-tumoral liver were considered to be positive (Fig. 2a,b), and for Bmi-1, tumor cells showing clear dot-pattern staining were considered to be positive (Fig. 2c). Subsequently, for each molecular marker, the number of positive tumor cells was counted by random selection of three areas of the tumor, and the average positive rate was calculated for evaluation. For CD34, positive sinusoidal vascular architectural areas in the scirrhus component of the tumor and in the rest of the tumor (Fig. 2d) were counted separately. We determined each areal rate of CD34 in 10% increments.

All values less than 10% were counted as 5%, because there are no completely negative cases in the liver parenchyma in general, and a detailed measurement of positive areas evaluated at 10% or less may be difficult. For h-caldesmon, the number of intratumoral positive vessels (arterial tumor vessels) in the non-scirrhus component was counted in 10 visual fields at ×200 magnification (i.e. ×20 objective lens and ×10 ocular lens). In the scirrhus component; the number of h-caldesmon-positive vessels was counted in all countable visual ×200 fields. Then, the mean number per field was calculated and this was taken as the arterial vessel density (AVD) in the two regions (Fig. 2e).

Statistical analysis. The Mann–Whitney U-test was used to analyze the immunohistochemical staining results. A $P$-value of <0.05 between two groups was judged to be statistically significant. Hierarchical cluster analysis of all analyzed factors was performed using TM4 MeV (http://www.tm4.org/index.html). Heat maps were generated using Microsoft Excel 2010.

Results

Histological features of small vaguely nodular lesions and subclassification of high-grade and low-grade of early hepatocellular carcinoma. Of the 66 nodules, 10 were diagnosed as LGDN (15.1%), 10 as HGDN (15.1%) and 46 as eHCC (69.8%). In the eHCC nodules, no or unclear stromal invasion, suspected stromal invasion, mild distinct stromal invasion and severe

![Fig. 2. Positive immunohistochemical expression pattern for each antibody. (a) CAP2 and (b) HSP70 showed a strong expression in the tumoral compared with the non-tumoral liver ((b) inset) and (c) Bmi-1-positive regions showed clear "dot-pattern" staining (inset). (d) Areas of CD34-positive sinusoidal vascular architectural and (e) h-caldesmon-positive unpaired vessels were seen in high-grade early hepatocellular carcinoma (HGeHCC). N, non-tumoral liver; T, tumor. Scale bars = (a),(d),(e) 100 μm; (b),(c) 50 μm.](http://www.wileyonlinelibrary.com/journal/cas)
distinct stromal invasion were observed in 5, 12, 15 and 14 nodules, respectively. In addition, distinct structural atypia with the scirrhous component was found in 10 nodules. Some scirrhous components showed one regional feature per nodule, whereas other patterns showed multiple small scirrhous foci in a single nodule. Six nodules exhibited both the scirrhous component and severe distinct stromal invasion (13.0% of eHCC). Accordingly, we divided eHCC into two groups: 18 nodules (39.1% of eHCC) in which marked stromal invasion and/or the scirrhous component foci were found, and 28 nodules (60.9% of eHCC) that did not possess these features.

These two types of lesions were provisionally subclassified as high-grade early hepatocellular carcinoma (HGeHCC) and low-grade early hepatocellular carcinoma (LGeHCC) (Fig. 3a, b) according to our proposed diagnostic criteria (Fig. 4), and a further study was conducted based on this classification.

The number of nodules with large nuclei showed a tendency to increase from LGDN, to HGDN, to LGeHCC, and eventually to HGeHCC. Tumor cellularity was less than twofold compared with non-tumoral liver in all LGDN cases and was more than twofold in approximately 80% of each other nodule type. LGeHCC and HGeHCC nodules tended to be larger than DN nodules (Fig. 5a).

Immunohistochemical features of small vaguely nodular lesions. We further evaluated these lesions immunohistochemically. CAP2 expression-positive rates tended to increase step-wise from LGDN to HGeHCC. Although the immunohistochemical expression of CAP2 did not show a statistically significant difference between LGDN and HGDN or between HGDN and LGeHCC, there was a statistically significant difference between LGeHCC and HGeHCC ($P = 0.0145$) and between LGDN and LGeHCC ($P = 0.0034$) (Fig. 5b). In the present study, it was noted that a strong expression of CAP2 was frequently observed in tumors showing stromal invasion, hepatic vein wall invasion or a scirrhous component (Fig. 6a–c).

Both HSP70 and Bmi-1 showed statistically significant (or tendencies to have) low positive rates in DN but higher rates in eHCC. The immunohistochemical expression of HSP70 showed statistically significant differences between LGDN and LGeHCC ($P = 0.0008$) and between LGDN and HGeHCC ($P = 0.0018$) (Fig. 5c). The immunohistochemical expression of Bmi-1 showed a statistically significant difference between LGDN and LGeHCC ($P = 0.0193$) but no significant differences between other combinations (Fig. 5d).

Vascularization of high-grade early hepatocellular carcinoma and their scirrhous component. Vascular changes were examined in 65 nodules (excluding 1 nodule that became too small to examine during the preparation of serial sections). The area of CD34-positive sinusoid-like vascular architecture (sinusoidal capillarization) in lesions tended to gradually increase from LGDN to HGeHCC, but there were no significant differences. The scirrhous component of HGeHCC showed a very high positive rate compared with the non-scirrhous component of HGeHCC ($P = 0.0014$) (Fig. 5e). AVD was low in LGDN, HGDN and LGeHCC, with no significant differences between them. However, AVD was high in the non-scirrhous component of HGeHCC, being significantly different from that of LGeHCC ($P = 0.0054$). Moreover, the scirrhous component of HGeHCC was highlighted by a very high AVD compared with...
the non-scirrhous component of HGeHCC \( (P = 0.0047) \) (Figs 5f,6d).

Comparison between pathological diagnoses and clustering of pathological factors in small vaguely nodular lesions. A heat map was created for the four types of lesions (LGDN, HGDN, LGeHCC and HGeHCC) with raw data for each parameter analyzed (lesion size; tumor cellularity; size of nucleus; structure of tumor cells; extent of stromal invasion; immunohistochemical reactivity of CAP2, HSP70 and Bmi-1; the areal rate of CD34; and AVD) (Fig. S1). In addition to the original criteria defining high grade (i.e. a scirrhous component and severe stromal invasion), HGeHCC showed a tendency toward nuclear swelling and high positive expression of CAP2, CD34 and AVD compared with LGeHCC and DN.

In addition, we performed cluster analysis on each pathological factor (Fig. 7a), and this resulted in lesions being divided into three groups (Fig. 7b). Group I was composed of HGeHCC only. Group II mainly comprised HGDN and LGeHCC. Group III was mainly composed of LGDN; in fact, all LGDN appeared in group III.

Discussion
In the present study, we divided eHCC into two subclasses, HGeHCC and LGeHCC, and conducted a detailed histological study comparing them with LGDN and HGDN. We found that HGeHCC and LGeHCC accounted for approximately 40% and 60% of eHCC, respectively. Compared with LGeHCC and DN, HGeHCC tended to have large nuclear size, high cellularity, structural atypia (including scirrhous pattern), and large tumor size together with marked stromal invasion. Immunohistochemically, both the expression of CAP2 and the areal ratio of sinusoidal capillarization were upregulated in a stepwise manner from LGDN, to HGDN, to LGeHCC, and eventually to HGeHCC. In contrast, AVD was almost unchanged in LGeHCC compared with DN, whereas AVD in HGeHCC was...
Fig. 6. Immunohistochemical findings of CAP2 and h-caldesmon in high-grade early hepatocellular carcinoma (HGeHCC). The strong expression of CAP2 was frequently observed in tumors showing (a) portal area invasion (arrowheads, the same area of Fig. 2b), (b) hepatic vein wall invasion (arrowheads, the same area of Fig. 2d and e) and (c) scirrhous component. (d) Areas of scirrhous component in HGeHCC were highlighted by very high levels of arterial vessel density (AVD), as indicated by the h-caldesmon-positive vessels. Scale bars = 100 μm.

Fig. 7. Cluster analysis of pathological factors. (a) The lesions were clearly divided into three groups based on pathological factors. (b) Group I was composed of high-grade early hepatocellular carcinoma (HGeHCC) only. Group II mainly comprised high-grade dysplastic nodules (HGDN) and low-grade early hepatocellular carcinoma (LGeHCC). Group III was mainly composed of low-grade dysplastic nodules (LGDN); all LGDN were included in group III.
higher. Statistically significant differences between LGeHCC and HGeHCC were found for CAP2 expression and AVD. Moreover, vascularization in the scirrhous component of HGeHCC was different from that in the non-scirrhous component of HGeHCC. Based on the above findings, we considered that HGeHCC is histopathologically distinct from LGeHCC.

In a previous study using an oligonucleotide array, we found that CAP2 was upregulated in early HCC, and high immunohistochemical expression of CAP2 was shown in HCC with increased malignant potential, such as those with poor tumor differentiation, vascular invasion and intrahepatic metastasis. Therefore, a significant overexpression of CAP2 in HGeHCC likely indicated increased malignant potential of the lesion. Moreover, hypervascularization in small lesions in chronic liver disease is thought as an indicator of increased malignant potential. AVD and the area with sinusoidal capillarization in HGeHCC were high in comparison with LGeHCC and DN. Indeed, during follow up, small vaguely nodular lesions generally show poor arterial blood flow on hemodynamics imaging, however, approximately 10–40% of such lesions may become hypervascular tumors within a year. It has also been reported that tumor cells of nodular lesions larger than 15 mm, which is similar to the 13 mm mean size of HGeHCC, actively proliferate with the emergence of unpaired arteries. We consider that HGeHCC thus distinct histological malignant features that may represent a transitional stage to advanced HCC.

While HSP70 and Bmi-1 are not correlated with the malignant potential of HCC, they are valuable for differentiation between DN and eHCC, as reported previously. We found no significant differences in the expressions of HSP70 and Bmi-1 between LGeHCC and HGeHCC, although Bmi-1 expression tended to decrease slightly. This is in agreement with our previous observation of a higher expression of Bmi-1 between DN and eHCC, as reported previously.

In conclusion, we investigated detailed histological and immunohistochemical features of small vaguely nodular lesions. Our results indicated that from the perspective of histopathology, immunohistology and changes in vascularization, approximately 40% of eHCC cases belonged to a distinct, highly malignant tumor group that we subclassified as HGeHCC. In addition, we believe that HGeHCC may already be a transitional stage to advanced HCC. We consider that our grading classification system may be valuable for evaluating treatment strategies for eHCC with a size of approximately 2 cm.

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Disclosure Statement
The other authors have no conflict of interest to declare.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Heat map of the raw analyzed parameters in each lesion.
Table S1. Patient background summary.