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Hepatic 'stem cell' malignancies in adults: four cases

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Aims: Combined hepatocellular/cholangiocarcinomas have been explained by some investigators as bidirectional differentiation of neoplastic progenitor cell populations. The presence of hepatic progenitor cells has now been confirmed in humans, though whether they can give rise to malignant tumours has not been confirmed. We report four cases of small tumours identified in livers with features of chronic hepatitis which may suggest a role for malignant transformation of hepatic stem cells in hepatic malignancies.
Methods: Tumour samples were studied from four patients by histochemistry and immunohistochemistry.
Results: Two patients had chronic hepatitis B, one had chronic hepatitis C and chronic alcoholic liver injury, and one had non-B non-C chronic hepatitis. Stages of disease ranged from portal fibrosis to cirrhosis. All tumours contained undifferentiated cells with morphological and immunohistochemical features that would be expected of hepatic progenitor cells. These cells merged with both hepatocellular carcinoma and cholangiocarcinoma components as well as with mature appearing hepatocytes within the tumours.
Conclusion: We suggest that these tumours are of hepatic progenitor cell origin, supporting the concepts that human hepatocarcinogenesis can be based on transformation of progenitor cells and that such a process may underlie development of some mixed hepatocellular/cholangiocarcinomas and dysplastic nodules.

Keywords: hepatocellular carcinoma, cholangiocarcinoma, stem cell, progenitor cell, hepatocarcinogenesis

Abbreviations: ChC, cholangiocarcinoma; HCC, hepatocellular carcinoma

Introduction

In animal models of hepatocarcinogenesis there are proliferations of small, undifferentiated, oval shaped cells, with a high nuclear-to-cytoplasmic ratio which are referred to as 'oval cells'; these cells are thought to be facultative bipotent progenitor cells (or 'stem cells') and have been linked to the subsequent development of hepatocellular malignancies in these models.[1–3] Oval cells, when they appear in such experimental situations, are often seen in continuity with nests of hepatocytes, sometimes with cells of intermediate morphology noted between them and the mature hepatocytes. It has long been debated whether such cells exist in humans and whether hepatocellular malignancies arise from their malignant transformation or from de-differentiation of neoplastically transformed mature hepatocytes. Recent studies confirm the existence of a hepatic progenitor cell population in humans, both within the liver[4] and deriving from the bone marrow,[5–7] though their role in human hepatocarcinogenesis has not been confirmed

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It has been demonstrated in vivo that a single clonally expanded tumour cell can give rise to both glandular and hepatocyte elements.[8] A well-documented, though relatively rare, subset of hepatic malignancies bears on this question: namely combined hepatocellular-cholangiocarcinoma (HCC/ChCs). These tumours contain both hepatocellular and cholangiocarcinoma features and have also been used as evidence to support both hypotheses.[9,10] Here we present four small tumours with populations of small cells suggesting that these tumours, and perhaps other hepatic malignancies, can be, in fact, 'stem cell' derived.

Patients, materials and methods

Clinical and pathological data regarding the patients and their background liver disease are summarized in Table 1.

Immunohistochemical staining was performed on all tissues according to standard techniques with antibodies to a panel of antigens commonly expressed in normal liver, HCC and ChC, including CAM5.2 [against cytokeratins (CK)8, 18, normally positive in hepatocytes and cholangiocytes; Becton Dickinson, San Jose, CA, USA], AE-1/AE-3 (a cocktail of monoclonal antibodies against high-molecular-weight cytokeratins, positive in cholangiocytes; Dako Corp., Carpenteria, CA, USA), CK19 (monoclonal antibody; Becton Dickinson), HepPar1 (monoclonal antibody specific for hepatocytes; Dako Corp.), α-fetoprotein (AFP; monoclonal antibody, positive in hepatoblasts and some hepatocellular carcinomas; Dako Corp.); carcinoembryonic antigen (polyclonal antibody, specifically cross-reacts with biliary glycoprotein 1 giving a canalicular pattern of staining in hepatocytes and HCC; Dako Corp.), CD34 (monoclonal antibody, positive in capillary endothelium, but not in normal sinusoidal endothelium, also in haematopoietic stem cells; Biogenex Corp., San Ramon, CA, USA); c-kit (also known as CD117, polyclonal antibody; Nova-Castra, Newcastle, UK); vimentin (intermediate filaments mostly expressed in mesenchymal cells, but also expressed in some primitive non-mesenchymal cell lines; Biogenex Corp.); and chromogranin-A (neuroendocrine marker, reported positive in progenitor cells; Clonatec, Paris, France).

PATHOLOGICAL FINDINGS

Case 1

The resected liver weighed 1250 g, measured 230 × 150 × 70 mm and was cirrhotic. There was a thin-walled, clear fluid cyst on the posterior surface of the right lobe. The specimen was sectioned at 5-mm intervals and the majority of the cirrhotic nodules measured <5 mm in the greatest dimension. Two distinct larger nodules were identified in the right and left lobes. The nodule in the right lobe measured 18 × 15 × 15 mm and had a rubbery consistency. The other, located in the left lobe, measured 10 × 9 × 9 mm, was a lighter tan colour and had a spongy appearance.

Histological sections of the cirrhotic liver demonstrated features of chronic hepatitis with mild interface and parenchymal necroinflammatory activity and rare lymphoid aggregates compatible with chronic hepatitis C infection. In some areas, residual terminal hepatic venules were scarred and associated with pericellular fibrosis consistent with chronic alcoholic liver injury. In addition, the fibrous septa contained many von Meyenburg complexes, usually associated with portal structures. The cystic structure identified grossly was a simple bile cyst microscopically. The left lobe lesion consisted of multiple biliary cysts lined by a predominantly cuboidal epithelium without atypia or papillary projections. The cysts were separated by a poorly cellular, loose connective tissue stroma. Bile was not identified within the cyst lumens. At one edge of this lesion, the connective tissue was invaded by a mucin-producing adenocarcinoma compatible with a ChC.

The right lobe lesion consisted of a dysplastic nodule containing mature, regenerative hepatocytes and occasional well-formed portal tracts. Within the nodule, there were several coalescent subnodules of malignant appearing cells (Figure 1). Focally, these populations invaded the fibrous septa surrounding the dysplastic nodule and spread into adjacent cirrhotic nodules. Some areas of tumour consisted of a bile-producing, well to moderately differentiated HCC with a variety of growth patterns including the formation of thickened trabeculae and pseudoacinar structures. In other areas, a distinct mucin-producing, well to moderately differentiated adenocarcinoma was identified, sometimes merging with HCC.

A third distinct population of cells was identified which had moderately pleomorphic, hyperchromatic nuclei and scant, slightly basophilic cytoplasm. These cells were organized in sheets or smaller nests and clusters, sometimes forming tubular structures, and were contained within the borders of the dysplastic nodule. Some nests of these cells contained mature appearing hepatocytes in their centre with benign appearing nuclei and a normal nuclear-to-cytoplasmic ratio. In such formations, the hepatocytes were always entirely encircled by a thin rim of the smaller

Table 1. Summary of clinical data

Case	Age	Sex	Ethnic background	Chronic liver Disease(s)	Serum AFP (nl)	Specimen type	Stem cell tumour nodule size, mm	Other tumour or tumour-like lesions	Recurrence
1	54	Male	European	1. Chronic hepatitis C, mildly active with cirrhosis 2. Chronic alcoholic liver disease	Normal range	Explant	18	1. Cholangiocarcinoma arising in association with von Meyenburg complex (10 mm) 2. Multiple von Meyenburg complexes 3. Simple biliary cyst	None at 8 years
2	71	Male	Chinese	Chronic hepatitis B, mildly active with cirrhosis	80 ng/ml (<20)	1. Left lobe hepatectomy 2. Right lobe wedge resection	25	1. Biliary papillomatosis (left lobe)	New tumour in liver at 1.5 years (not biopsied)
3	56	Male	European	Chronic hepatitis B, mildly active with portal fibrosis	53 ng/ml (<20)	Right lobe hepatectomy	40	None	None at 5 years
4	73	Female	Japanese	Chronic hepatitis, non-B non-C, with fibrous septa. (HbcAb, HbeAb positive; HCV-RNA negative)	Normal range	Right lobe hepatectomy	16	None	None at 5 years

cells; sometimes transitional features were seen between the two components. Transitions were also seen between these small cells and elements of the HCC and ChC.

Case 2

The specimens consisted of a left hepatectomy and right lobe wedge resection. Grossly, the left lobe specimen showed dilated bile ducts filled with papillary lesions. The right lobe specimen contained a 25-mm circumscribed, solid nodule. The background liver appeared cirrhotic. On microscopic examination, the left lobe specimen showed cirrhotic liver tissue with papillary tumours within dilated bile ducts in a pattern consistent with a diagnosis of biliary papillomatosis. The right lobe tumour (Figure 2) contained a mixed population: (i) pleomorphic hepatocytes with abundant cytoplasm, arranged in a predominantly trabecular pattern with focal pseudoacinar formation, were consistent with HCC; (ii) small undifferentiated cells, surrounded by extensive fibrous tissue, had basophilic cytoplasm, high nuclear-to-cytoplasmic ratio and round to oval nuclei with marked pleomorphism. In another area of the tumour, a moderately differentiated adenocarcinoma, compatible with ChC, was identified. This tumour had similar appearances in all components to the tumour presented in case 1.

Case 3

The resection specimen consisted of a right partial hepatectomy. The liver segment weighed 225 g, measured 100 × 80 × 60 mm, and included a well-circumscribed, 40-mm tumour nodule. The cut section of the tumour was yellow-white with a greenish tinge and showed a central stellate scar. Microscopic examination revealed a predominantly hepatocellular carcinoma consisting of large nests of clear cells separated by dense, fibrous septa (Figure 3). In addition, closer examination of the tumour revealed small cells at the margins of these nests, often at the stromal–epithelial interface, which had scant cytoplasm and hyperchromatic nuclei. Cells of intermediate morphology between these small cells and the hepatocytes could often be identified. Occasionally, these small cells formed glands within the fibrous stroma suggesting a cholangiocellular component (Figure 3D, arrowhead), though obvious invasion by these elements was not identified.

Case 4

The resected right lobe contained a 16-mm well-circumscribed, white-grey tumour. Histologically, the adjacent liver parenchyma showed focal fibrous septa

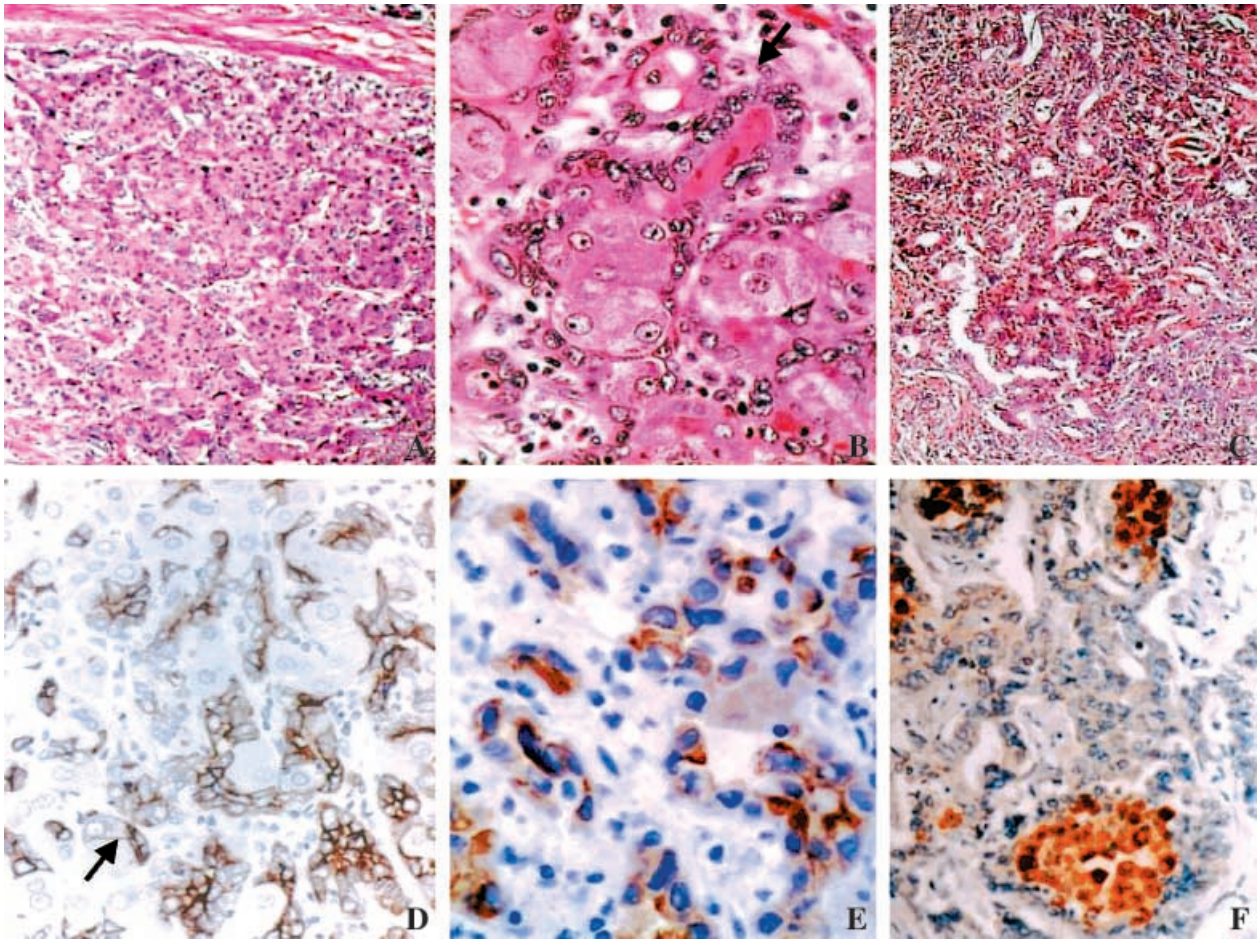

Figure 1. Case 1. **A,** Low-power view showing nests comprising mature appearing hepatocytes in the centre, peripheral small cells, and cells with intermediate morphology at their interface (H&E). **B,** Higher magnification of hepatocyte/small cell nests showing the striking similarity to oval cell proliferations with hepatocyte regeneration in animal models (H&E). **C,** Low-power view of a well to moderately differentiated cholangiocarcinoma (ChC) found within the tumour in case 1 (H&E). **D,** Immunostaining for AE1/AE3 demonstrating positive staining of small cells and negative staining of tumour hepatocytes; there are 'transitional' cells with cytoplasmic staining for high-molecular-weight cytokeratins intermediate between the small cells and fully differentiated hepatocytes (arrow) (DAB, haematoxylin counterstain). **E,** Immunostaining for AE1/AE3 demonstrating positive staining of ChC cells (DAB, haematoxylin counterstain). **F,** Immunostaining for HepPar1 demonstrating positive staining of tumour hepatocytes and negative staining of small cells (DAB, haematoxylin counterstain).

linking portal tracts, but a transition to cirrhosis was not evident and hepatitic activity was minimal. The tumour was composed of intimately mixed neoplastic hepatocytes and small undifferentiated cells, irregularly divided by thin fibrous tissue (Figure 4). The hepatocytes were small with eosinophilic or clear cytoplasm and hyperchromatic and minimally pleomorphic nuclei. The small undifferentiated cells had basophilic cytoplasm, high nuclear-to-cytoplasmic ratio and round to oval nuclei with marked pleomorphism. Focally some of these small cells seemed to form glandular units, suggesting a cholangiocellular component, but these were negative for mucin. The histological appearance of this tumour was notably similar to that of the tumour in case 3.

Immunohistochemistry results

AE1/AE3 AND CK19

Focal positivity, from faint to strong, was observed in the small cells (Figures 1D, 2C, 3C and 4C) and some HCC components of all four tumours. ChC and focal glandular elements of all tumours also stained positively (Figure 1E). Staining with AE1/AE3 was somewhat more extensive and intense than that seen with CK19.

HEPPAR1

Negative in the small cells and positive in the HCC components of all four tumours (Figures 1F, 2E, 3D

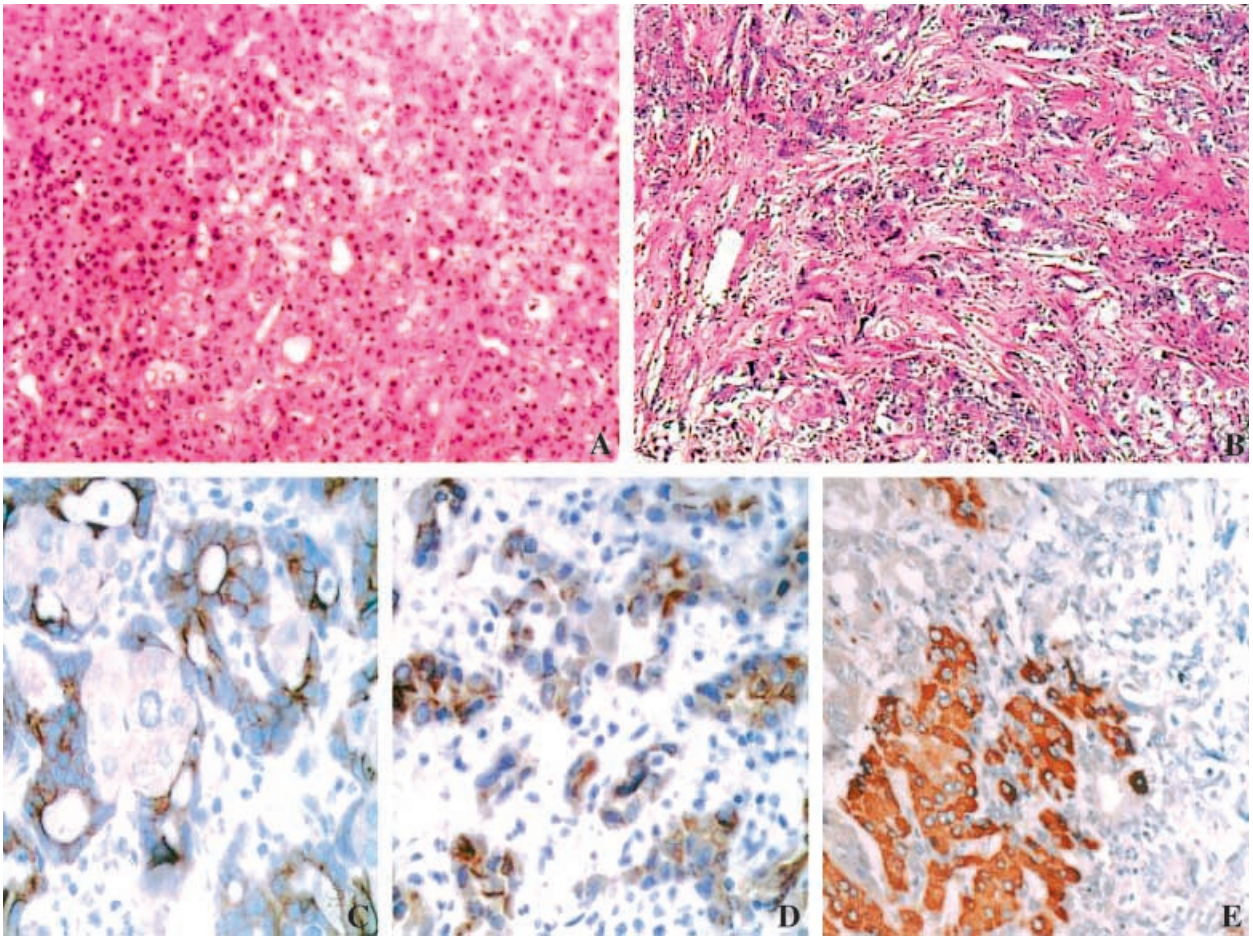

Figure 2. Case 2. **A,** Low-power view of well differentiated hepatocellular carcinoma (HCC) component, showing trabecular pattern and focal pseudoacinar structures (H&E). **B,** Low-power view of moderately differentiated cholangiocarcinoma (ChC) component identified (H&E). **C,** Immunostaining for cytokeratin 19 demonstrating positive staining of small cells at the periphery of nests, with negative staining of hepatocytes within the nests (DAB, haematoxylin counterstain). **D,** Immunostaining for cytokeratin 19 demonstrating positive staining of ChC cells (DAB, haematoxylin counterstain). **E,** Immunostaining for HepPar1 demonstrating positive staining of HCC cells and negative staining of small cells (DAB, haematoxylin counterstain).

and 4D). Cholangiocarcinoma and glandular elements in all tumours were negative.

α-FETOPROTEIN

Focally positive in the HCC components of cases 1, 2 and 3. There was also focal staining of the small cells of these cases. In the tumours from cases 1 and 2, the ChC component showed immunoreactivity, as did rare glandular elements in cases 3 and 4.

α_1-ANTITRYPSIN

Negative in the small cells and focally positive in the HCC components of all four tumours. ChC and glandular elements of all tumours were negative.

CD34, C-KIT (CD117), VIMENTIN, CHROMOGRANIN-A

Negative in all tumour cells of all types in all cases. Capillary endothelium within the tumours stained for CD34, while sinusoidal endothelium was focally positive as well, more extensively so in cirrhosis than in the non-cirrhotic livers. Vimentin was positive in stromal cells within portal tracts and fibrous scars.

Discussion

The existence of combined HCC/ChCs has been explained in three ways: (i) as 'collision', or intermixing of two distinct primary tumours growing into each other; (ii) as 'redifferentiation' of a primary HCC into an adenocarcinoma phenotype; (iii) as a tumour derived

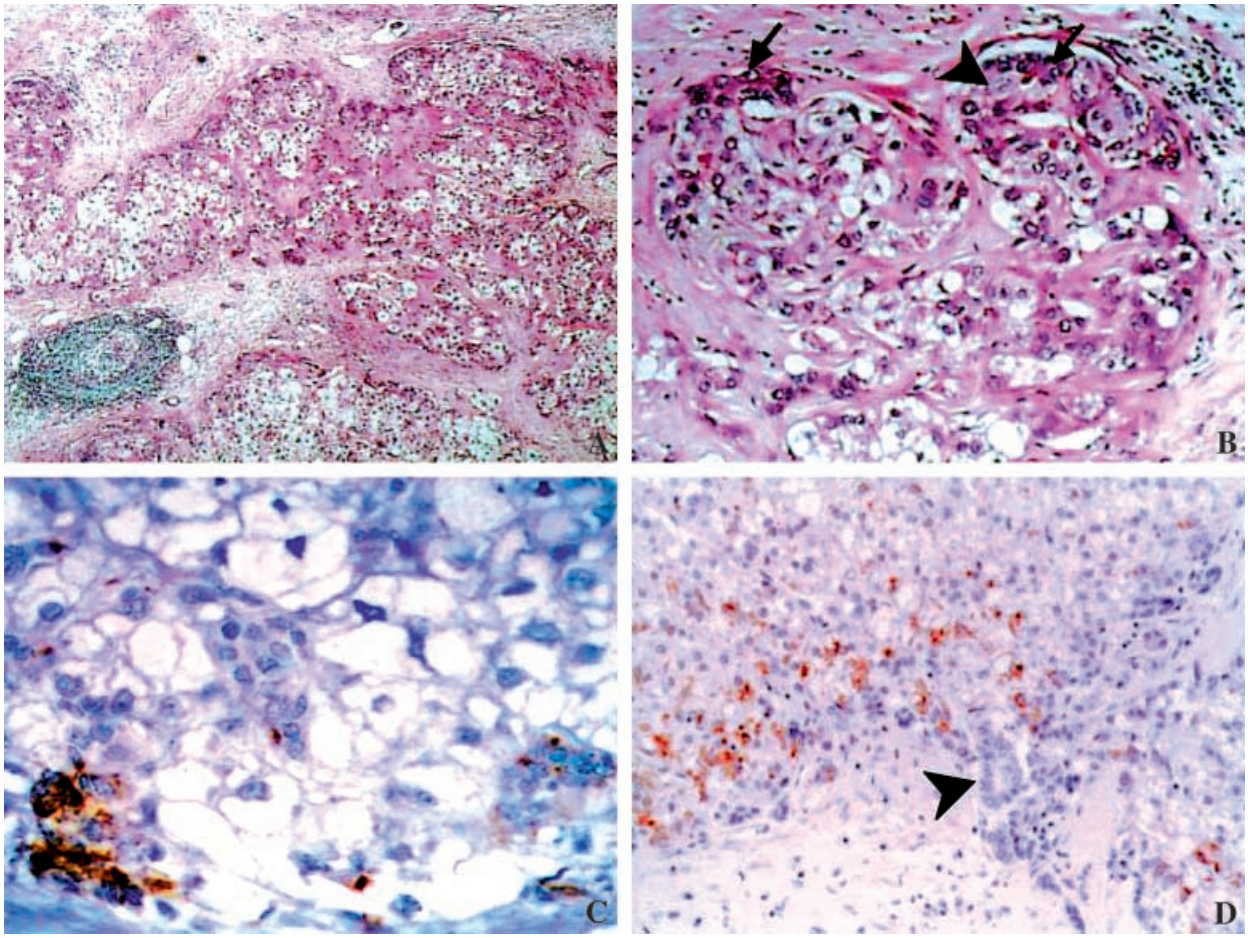

Figure 3. Case 3. **A,** Low-power view of tumour from case 3 showing nests of neoplastic hepatocytes with clear cytoplasm and pleomorphic nuclei and prominent fibrous septa consistent with a hepatocellular carcinoma (HCC), clear cell type. The second population of small cells are discernible as basophilic cells with hyperchromatic, oval nuclei (H&E). **B,** Higher magnification of HCC illustrating the intimate relationship between the small cells (arrows) and the HCC cells. A few cells with an intermediate type of appearance are seen (arrowhead) (H&E). **C,** Positive immunostaining of small cells for cytokeratin 19 and negative staining of HCC cells (DAB, haematoxylin counterstain). **D,** Positive immunostaining of HCC cells for HepPar1 and negative staining of small cells (DAB, haematoxylin counterstain).

from a bipotent hepatic progenitor cell differentiating into two morphologically distinct, though intermixed, malignant lesions. The first explanation probably explains some large tumours.[9–11] However, some mixed HCC/ChC tumours are small, well-defined nodules, an appearance making this collision hypothesis unlikely. The remaining two explanations need to be considered in such cases and as a result, these lesions have been a point of focus in the debate as to whether bipotential hepatic progenitor cells have a role in human hepatocarcinogenesis.

The highlighted features of the tumours presented here differ from previously reported mixed HCC/ChCs in that they contain a prominent third compartment of cells without features of either differentiation pathway. While one must consider the possibility that these small, hyperchromatic, 'oval-like' cells may represent a poorly differentiated population of malignant cells or 'blastoma' cells of a hepatoblastoma, this hypothesis is undermined by their growth pattern. These cells appear at first glance to be spreading along the edges of sinusoids, surrounding mature, non-neoplastic appearing hepatocytes. However, upon review of the morphology in these areas, one begins to appreciate that the mature hepatocytes in this region of the tumour are always surrounded by these undifferentiated cells, and moreover, that there are often 'transitional' cells between these undifferentiated cells and the mature appearing hepatocytes. Thus, it does appear as though these nests of cells represent a process of maturation from the undifferentiated small cells at the periphery to the mature cells at the centre. This would be a

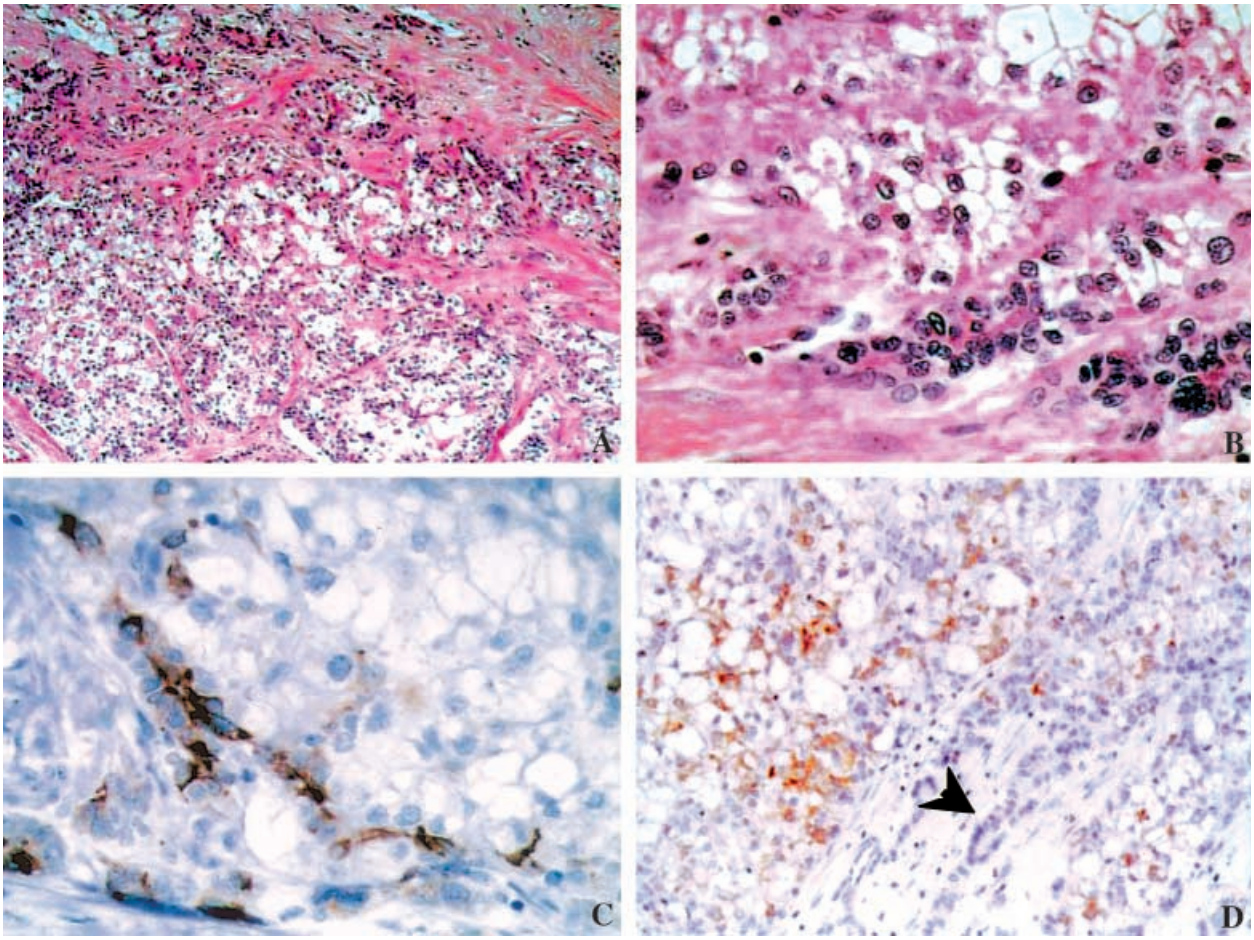

Figure 4. Case 4. **A,** Low-power view of tumour from case 4 exhibiting similar histological features as the clear-cell hepatocellular carcinoma (HCC) from case 3. The small-cell population is apparent even from this magnification (H&E). **B,** Higher magnification of the tumour shows the nests of clear-cell HCC with the small-cell component at the periphery (H&E). **C,** Immunostain for cytokeratin 19 highlights the small-cell component of the tumour. The HCC cells are negative for cytokeratin 19 (DAB, haematoxylin counterstain). **D,** Immunostain with HepPar1 is positive in the HCC cells and negative in the small cells (DAB, haematoxylin counterstain).

neoplastic version of the reactive ductular reactions seen in massive hepatic necrosis which have been shown to be stem cell derived.[4] The morphology of these nests of cells is strikingly similar to that of oval cell proliferations in animal models of hepatocarcinogenesis.

Additionally, as described in these four lesions, the undifferentiated cells also merge with both malignant hepatocellular and cholangiocellular compartments of the tumour, suggesting that the malignant components are arising from this undifferentiated population. Whether these features correspond to aspects of the 'transitional' mixed tumour category described by Goodman *et al.*[11] is unclear. In that paper, the description of some regions of the transitional tumours suggests similarities, though the photomicrographs of these lesions do not correspond precisely to what is being demonstrated in our lesions; this difference may simply reflect sampling of images.

There are no markers that are absolutely indicative of 'stem cell-ness' in the liver. While C-kit has proved to be suggestive in some work,[4] it is unclear whether it is as consistent or functional in liver lineages as it seems to be in haematopoiesis. It has been suggested that chromogranin-A may be a marker of hepatic progenitor cells.[12–14] The absence of this marker in the current cases, however, may relate to formalin fixation, rather than differentiation status, as the earlier studies of this marker were only successful on frozen tissue.[13] Previous work has demonstrated that hepatoblasts in human livers co-express CK19 and the antigen recognized by monoclonal antibody HepPar1.[15,16] Studies of ductular hepatocytes, regenerating cell populations seen in massive hepatic necrosis from viral or toxic

injury in humans, show recapitulation of the fetal phenotype in the transitional cells.[4,17,18] This fetal phenotype is often seen within these four tumours, in the islands of peripheral oval cells merging into mature-appearing hepatocytes. The presence of focal AFP staining in these cells is also similar to that seen in fetal liver tissue.

The microscopic similarity of these small cells to hepatic 'blastoma' cells may cause these tumours to be confused with hepatoblastoma. However, careful examination of their relationship to the other tumour components will reveal the true nature of the lesion. Of the 16 cases of adult hepatoblastomas reported in the literature, the most are of the mixed type, with only three presenting as epithelial type hepatoblastomas.[19–21] It is also possible that some of the cases of adult hepatoblastomas reported in the literature are actually variant forms of these tumours.

The location of one of these tumours in a dysplastic nodule also needs to be addressed. A previously reported case of a mixed HCC/ChC in a dysplastic nodule did not contain an undifferentiated population of cells as seen in this case.[22] The authors concluded that the ChC component was a 'redifferentiation' of the HCC component, basing this conclusion on the assumption that the HCC itself arose from mature hepatocytes in the dysplastic nodule. This interpretation was based on the standard hypothesis that dysplastic nodules consist entirely of mature hepatocytes, thus any tumour arising within them must be similarly derived. However, we have previously suggested that dysplastic nodules represent neoplastic clonal expansions.[23,24] This stem cell hypothesis for the origin of dysplastic nodules allows for this clonal expansion and further malignant transformation to be either the result of a dedifferentiation pathway of hepatocarcinogenesis or a progenitor cell pathway.[24,25] The tumour presented here in case 1, which contains small cells within a dysplastic nodule, supports the concept that dysplastic nodules themselves may arise on the basis of clonal progenitor cell proliferation.

Finally, it is interesting to note that these four cases actually have two distinct morphological appearances. Two cases, both arising in non-cirrhotic livers, have extensive fibrous septa coursing through the tumours and their hepatocellular components display predominantly clear cell features. Also, while glandular structures are part of these tumours, they do not have the appearance of an invasive adenocarcinoma; thus these tumours are not truly HCC/ChCs. The other two cases, both in the setting of cirrhosis, lack these features and have a more prominent cholangiocarcinoma component. It is possible that, as more such tumours are recognized, further subclassification may be warranted. It should be noted that none of these small tumours has recurred in many years of follow-up, suggesting that their behaviour is most like HCC, which, when resected early, may be considered 'cured'.

A recently described 'primary liver progenitor cell tumour' recently reported by Robrechts *et al.* is not similar to our tumours.[26] That tumour is described as having a monomorphic population of small cells with scant cytoplasm and a trabecular architecture, with an immunophenotype intermediate between hepatocytes and bile duct epithelial cells. In contrast, our tumours had a mixed population of oval cells differentiating into both hepatocytic and biliary type epithelia. In the same manner, our tumours were different from the small-cell carcinoma variant of HCC, which is described as having a monomorphic small-cell population arranged in nests and trabeculae, with positive staining for neuroendocrine markers.[27]

Summarizing, we suggest that the lesions described here are malignant tumours of hepatic progenitor cell origin with elements of the original progenitor cell population still in evidence within the tumour. We do not mean to say that these represent distinctive lesions that warrant a separate diagnostic category from those already identified. Rather, we propose that these are lesions where the persistent stem cell component is particularly prominent and easy to identify. As has been shown elsewhere,[4,12,13,15] immunohistochemical staining for biliary-type cytokeratins may highlight the small, progenitor-like cell components, but these would be difficult to see by routine H&E staining. In conclusion, we therefore support the concept not only that human hepatocarcinogenesis is at least sometimes based on a transformation of progenitor cells, but that such a process may underlie development of at least some mixed HCC/ChC tumours and dysplastic nodules.

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