Histol Histopathol (1998) 13: 657-670

http://www.ehu.es/histol-histopathol

Histology and Histopathology

Liver cell dysplasia: reactivities for c-met protein, Rb protein, E-cadherin and transforming growth factor-ß1 in comparison with hepatocellular carcinoma

M. Zhao and A. Zimmermann

Institute of Pathology of the University, Berne, Switzerland

Summary. In the present retrospective study, liver cell dysplasia (LCD) occurring in cirrhotic livers associated or not associated with hepatocellular carcinoma (HCC) was immunohistochemically analyzed for the expression of hepatocyte growth factor receptor (c-met protein), Rb (retinoblastoma gene) protein, E-cadherin, and transforming growth factor-ß-1 (TGF-ß-1). Cytoplasmic c-met protein staining was observed in about half of the HCC's, and its prevalence was about twice as high in high grade vs. low grade tumors, but it was not correlated with proliferative activity as based on PCNA labelling. In LCD, reactivity for c-met protein was restricted to the small cell type. Nuclear staining for Rb protein was found in HCC's, and was not related to type, grade or proliferative activity, whereas no immunoreactivity was observed in normal, hyperplastic or dysplastic hepatocytes. Expression of E-cadherin prevailed in HCC's of lower grade, and particularly in those with a trabecular or acinar growth pattern. E-cadherin staining was detectable in normal and large dysplastic hepatocytes, but not in small dysplastic liver cells. TGF-ß-1 reactivity was observed in more than half the HCC's, but not in normal or dysplastic hepatocytes. These findings underline the phenotypic difference between large cell and small cell liver dysplasia, and support the hypothesis that small cell dysplasia is a precursor lesion in a hepatocarcinogenic pathway.

Key words: Liver cell dysplasia, hepatocellular carcinoma, C-met protein, retinoblastoma gene product, cadherin, transforming growth factor-beta

Introduction

As hepatocellular carcinoma (HCC) is, on a worldwide scale, one of the most important solid tumors, it is of particular interest to study its precursor lesions. Whereas pathways involved in liver carcinogenesis in laboratory animals have been investigated in detail, knowledge relating to hepatic preneoplastic changes in humans is still incomplete. Probable precursors include liver cell dysplasia (LCD; Anthony, 1976; Serov and Beketova, 1990) and nodular lesions comprising atypical adenomatous hyperplasia, macroregenerative nodules with bulging activity and nodule-in-nodule lesions (for reviews, see Nakanuma et al., 1990; Okuda, 1992). LCD was originally detected in the liver of HBsAg-positive Ugandan patients and was suggested to represent a preneoplastic change (Anthony, 1976), but this hypothesis has been discussed in the following years, sometimes with a controversial outcome (Akagi et al., 1984; Roncalli et al., 1985; Chopra et al., 1987; Kovacs and Elek, 1987; Lefkowitch and Apfelbaum, 1987; Reid et al., 1987; Matturri and Bauer, 1988; Watanabe et al., 1988; Borzio et al., 1991; Hytiroglou et al., 1992). For Western countries, the etiology of liver cirrhosis has not been conclusively demonstrated to be of relevance for the presence of LCD (Theise et al., 1992), even though LCD is relatively frequently encountered in hepatitis C virus infection (Ferrel et al., 1992). Several studies support the view that dysplastic hepatocytes form a liver cell population subject to probable neoplastic evolution, based on immunohistochemical (Roncalli et al., 1985, 1986; Govindarajan et al., 1990), morphometric (Giannini et al., 1987; Roncalli et al., 1988; Zhao et al, 1994a) and DNA ploidy (Zerbini et al., 1992) findings. Recently, we could show that the prevalence of LCD was higher in cirrhotic livers carrying HCC than in those without, and that karyometric features of large cell LCD are most close to those of highly differentiated HCC's, whereas karyometric features of small cell LCD closely reflect those of poorly differentiated HCC's (Zhao et al., 1994a). Moreover, reactivity for p53 protein, the product of a tumor suppressor gene frequently mutated in HCC, has been detected in LCD, but in the small cell variant only (Zhao et al., 1994b), whereas the apoptosis protector, bcl-2 protein, expressed in a subset of HCC's, was not detectable in LCD (Zhao et al., 1994c). It

Offprint requests to: Arthur Zimmermann, M.D., Professor of Pathology, Institute of Pathology of the University, Murtenstrasse 31, CH-3010 Bern, Switzerland. FAX: 0041-31-6329938.

therefore appears that growth modulators deranged in HCC's may already undergo alterations at the level of putative precursor lesions.

Growth and differentiation of HCC's depend, among others, on a complex interplay between growth factors and factors inhibiting cell proliferation. Hepatocyte growth factor, a multifunctional cytokine (reviews: Boros and Miller, 1995; Jiang and Hiscox, 1997) and its receptor, c-met protein, have been demonstrated in human liver tumors (Selden et al., 1994; Boix et al., 1994; Suzuki et al., 1994; Grigioni et al., 1995; Noguchi et al., 1996; Annen et al., 1996; D'Errico et al., 1996), and this ligand/receptor system has also been shown to be involved in experimental hepatocarcinogenesis (Hu et al., 1996; Imai et al., 1996; Miller et al., 1996). In addition, the retinoblastoma (RB) gene, one of the best-studied tumor suppressor genes encoding a nuclear phosphoprotein found to be mutated in a wide range of human malignancies (for reviews, see Weinberg, 1995; Xu, 1995; De Luca et al., 1996), has been shown to be involved in malignant liver tumors (Murakami et al., 1991; Nakamura et al., 1991; Farshid and Tabor, 1992; Hsia et al., 1994; Kawakita et al., 1994; Zhang et al., 1994; Seki et al., 1995; Nishida et al., 1997). An additional important factor modulating growth of normal and neoplastic epithelial cells is transforming growth factor TGF-ß (TGF-ß; reviews: Massague, 1990, Wright and Huang, 1996). In most cells of epithelial origin, TGF-betas act as potent growth inhibitors, TGF-ß-1 being able to induce dephosphorylation of the RB gene product and thus block the entry of cells into the S phase of the cell division cycle (Thoresen et al., 1992; Baldwin and Korc, 1993). TGF-ß gene expression is transiently enhanced at a critical stage during liver regeneration (Armendariz-Borunda et al., 1993) and TGF-ß appears to play a role in autocrine regulation of hepatocyte proliferation (Bissell et al., 1995). HCC cells have been shown to express TGF-ß-1 either at the mRNA or the protein level (Ito et al., 1991; Bedossa et al., 1995), but TGF-ß-1 was not observed in preneoplastic nodules of the rat liver (Nakatsukasa et al., 1991). So far, there is no information present with respect to the expression of these growth stimulating or suppressing factors in human LCD.

Growth of neoplastic hepatocytes not only depends on tumor growth factors and growth-suppressor factors, but also on cell-to-cell and cell-to-matrix adhesion molecules, E-cadherin having a central position (Takeichi, 1990; Pignatelli, 1993; Cowin, 1994; Wagner, 1995). Alterations of cadherin expression are frequent in HCC's and may be of prognostic significance (Shimoyama and Hirohashi, 1991; Slagle et al., 1993; Yamaoka et al., 1995; Ihara et al., 1996; Kozyraki et al., 1996), whereas the pattern of cadherin expression in human hepatic precursor lesions is not yet known.

The aim of the present investigation was to systematically analyze the expression patterns of reactivities for c-met protein, RB protein, E-cadherin and TGF-ß in human dysplastic liver cells in comparison with HCC based on two groups of patients with liver cirrhosis associated or not associated with HCC.

Materials and methods

Material for histology

Biopsies and resection specimens from 37 Chinese patients with liver cirrhosis and HCC, and 66 Chinese patients of the same region with cirrhosis not associated with HCC were analyzed retrospectively.

The selection criterion for these sets of cases was the presence of liver dysplasia (LCD) within cirrhotic nodules, identified in 1748 liver tissue samples collected and assessed between 1957 and 1988 in a Northern Chinese center (Zhao et al., 1994a). The hepatitis B virus (HBV) status and aflatoxin exposure of these patients was not known. The material analyzed did not disclose atypical adenomatous hyperplasia (Nakanuma et al., 1990) or nodular borderline lesions (Ferrell et al., 1992). For conventional light microscopy, tissues were fixed in 10% buffered formaldehyde solution, dehydrated and embedded in paraffin. Fixation before embedding was less than 30 hours throughout. Sections were stained with hematoxylin and eosin.

Immunohistochemistry

The following antibodies were used: C-met protein, rabbit polyclonal antibody, Santa Cruz Biotechnology; Rb protein, mouse monoclonal antibody, Chemicon International; E-cadherin, mouse monoclonal antibody, Takara Shuzo Co.; and TGF-ß, chicken polyclonal antibody, R&D Systems. Immunostaining was based on a modified alkaline phosphatase-anti-alkaline phosphatase (APAAP) procedure. Tissue sections were deparaffinized and rehydrated in TRIS-sodium chloride (NaCl) buffer (0.1% TRIS and 1% NaCl, Merck, pH 7.4), followed by exposure of sections to 3% bovine serum albumin (BSA, Sigma), in TRIS-NaCl buffer with 1% normal rabbit serum (Dako) for 1 h at room temperature and incubation with primary antisera for 3 h. Rabbit anti-mouse immunoglobulin (Dako; 1:30) and APAAP mouse monoclonal antibody (Dako; 1:50) were applied for 45 min each. The AP reaction was run for 20 min in new fuchsin substrate solution, and the reaction was stopped by rinsing the sections in cold tap water.

All incubations were performed in a humidified environment at room temperature. Finally, sections were counter-stained with hematoxylin (Merck) and mounted with Aquadex (Merck). For negative control sections, primary antisera were substituted with normal mouse or rabbit serum (Dako) at a protein concentration of 0.06mg/ml, and all other steps were carried out with these preparations. As a positive control, three cases of HCC strongly expressing oncoprotein and PCNA were processed in parallel to each working step.

Classification and grading of HCC's

HCC's were classified according to published guidelines (Gibson and Sobin, 1978; Nakashima and Kojiro, 1987). Trabecular, pseudoglandular (acinar), compact, scirrhous (sclerosing) and sarcomatoid types/subtypes were distinguished. Grading of HCC's was performed employing the system proposed by Edmondson and Steiner, i.e. allocating cases to four grades (Edmondson and Steiner, 1954).

Grading of hepatic fibrosis/cirrhosis and classification of LCD

The term, hepatic fibrosis, was employed for those biopsies showing septal fibrosis with or without nodular change. The highest grades of fibrosis are associated with cirrhotic change and, therefore, biopsies with fully established cirrhosis were included in this category. For scoring of fibrosis, a system originally proposed by Knodell and coworkers (Knodell et al., 1981) was used, with some modifications (Schmid et al., 1994). In this procedure, grade 0 denotes no fibrosis, grade 1, mild fibrosis (portal tract fibrosis only), 2, portal tract fibrosis plus incomplete septa, 3, septa bridging portal-portal, 4, septa bridging portal-central and/or focal incomplete cirrhosis, 5, diffuse complete and/or focal complete cirrhosis, and 6, diffuse complete cirrhosis.

Features used to define LCD in contrast to normal or regenerating hepatocytes have previously been reported by our group (Zhao et al., 1994a). In brief, simple regenerating liver cells/hepatocytes (SRLC) are characterized as hepatocytes being smaller than normal parenchymal liver cells (NL), but whose nuclear size is apparently normal. The main difference between SRLC and NL is the typical arrangement of the former, in that SRLC have the tendency to form clusters within cirrhotic nodules, where they usually occur in peripheral parts and are then easily detectable due to nuclear crowding. Large liver cell dysplasia (LLCD) occurs in two variants, i.e. LLCD with nuclear hyperchromasia (LLCDe; Anthony, 1976; Zhao et al., 1994a) and LLCD with nuclear hypochromasia (LLCDo). In LLCDo, dysplastic hepatocytes are large cells with large nucleus and one or several prominent nucleoli. This cell type frequently exhibits an abundant, eosinophilic or clear cytoplasm. LLCDe has some features in common with LLCDo, but its cells usually exhibit a strongly eosinophilic cytoplasm and markedly polymorphous and hyperchromatic nuclei. Cells of both LLCDo and LLCDe frequently form clusters and occupy a part of a nodule, but they may form entire nodules. In small liver cell dysplasia (SLCD; Watanabe et al., 1988), both the cell size and the nuclear size are clearly smaller than in cells of LLCD. In contrast to SRLC cells, the cytoplasm is usually basophilic, and the nuclei are hyperchromatic. Cells of SLCD are, in most instances, located in peripheral parts of cirrhotic nodules, where they can form clusters, but they sometimes form entire nodules. Ductular proliferations occurring in chronic fibrosing liver disease were assessed as either present or absent.

Assessment of PCNA labelling in HCC's

The PC 10 monoclonal mouse anti-PCNA antibody (Dako) was used. With the APAAP method used, nuclei that had reacted were stained either pink or brightly red. Nucleated HCC cells were considered positive for PCNA only if a distinctly red staining of nucleus was identified. PCNA labelling was scored using a x40 objective and a x10 eyepiece, and sections were scanned to identify areas that were most evenly labelled, but within these zones areas to be analyzed were randomly chosen. The extent of PCNA positivity was evaluated by determining N-pos/100 nuclei, and this value was used as a PCNA labelling index.

Statistical analysis

For the statistical analysis of differences between groups, Pearson's Chi-Square analysis was employed. $P<0.05$ was considered as statistically significant.

Results

Hepatocellular carcinomas (HCC's)

The distribution of HCC's analyzed in the present study with respect to types, subtypes and grades is shown in Table 1. It is seen that most tumors exhibited either a trabecular or pseudoglandular (acinar) pattern, and that 11/37 were of grades 1 or 2, and 26/37 of grades 3 or 4, respectively. The proliferative activity of HCC's as based on PCNA labelling is also listed in Table 1.

C-met protein immunoreactivity was mostly detected in the cytoplasm, but staining of the surface membrane was observed as well (Table 1; Fig. 1). In one instance each, the cytoplasm of a tumor cell in mitosis and of a cell forming an apoptotic body, resepectively, was reactive. Staining either involved single cells or disclosed a more diffuse pattern. Overall, 18/37 HCC's were immunoreactive for c-met protein, and staining was moderate in 7, and marked in 2 cases. C-met positivity was twice as frequent in tumors of grade 3 or 4 as in grade 1 or 2 (38% vs. 21%; $p>0.05$). The yield of c-met protein staining was significantly higher in HCC's also expressing TGF-ß ($p<0.05$; see below).

Rb gene product (Rb protein) was expressed most frequently in tumor cell nuclei (Table 1; Fig. 2), a paranuclear and membraneous reactivity being observed only once. Overall positivity was 46%, and was more frequently of a diffuse pattern within a given tumor than focal. No significant correlation between Rb protein reactivity and tumor type or grade was found, and positive cases were not overrepresented in tumors also being positive for c-met protein, E-cadherin, or TGF-ß. A total of 20/37 (55%) of HCC's were reactive for E-cadherin, 11% exhibiting a strong, and 14% a moderate

Table 1. Hepatocellular carcinomas (HCCs): types, grades, immunoreactivity for E-cadherin, Rb protein, c-met-protein, TGF-ß1, and proliferative activity.

	HISTOPATHOLOGY						
No.	Type	Grade	E-cadherin	Rb-protein	C-met protein	TGFß1	PCNA-LI(%)
1	T+SC	1-2	+++/M/D	++/P/F	+/IN;C/S	++/M;C/F	0.0
2	P	2	+/M/F	-	+/C/S	++/M;C/F	15.0
3	P	4	-	+/N	+++/C;MI/D	+/C/F	0.0
4	T-P	2-3	-	-	-	-	57.0
5	T+SC+P	1-2	+/M/F	+++/M;P/F	+/C/S	+/C/F	68.0
6	T	3-4	-	-	-	+/C;MI/F	51.0
7	P+C+SA	3-4	-	++/N	-	-	20.0
8	C	2	+/M/F	-	+/IN;C/S	-	40.0
9	SA	4	+/M/F	+/N	++/AP/D	+/C/F;BL	37.0
10	T	2	+/M/F	-	++/IN;C/S	+/AP/F	36.0
11	T+SC	1-2	-	-	-	-	27.0
12	P	2-3	++/M/D	-	-	-	32.0
13	P	3	-	-	-	-	15.0
14	C	2-3	+/M/F	-	+/C/S	++/C/F;BL	0.0
15	T	3-4	+/M/F	-	-	+/C/S	49.0
16	T	3	+/M/F	++/N/D	-	++/C/F;BL	21.0
17	T	3	-	+++/N/D	+++/M;C/D	+/C/S;BL	38.0
18	T+SC	3-4	+++/M/F	++/N/D	++/C/S	+/C/F;BL	19.0
19	T	3	-	-	++/M;C/D	+/C/S;BL	44.0
20	C	4	-	++/N/D	-	-	62.0
21	T	3	-	-	+/C/S	++/C/F;BL	10.0
22	T	3	-	-	++/C/S	-	46.0
23	T+C	2-3	++/M/D	-	++/C/S	-	0.0
24	T	2	+/M/F	+++/N/D	-	-	58.0
25	T	2	-	-	-	+/C/S	44.0
26	T	2	-	+/N/F	-	+/C/S;BL	0.0
27	T	2	+++/M/D	-	+/C/S	++/C;AP/F;BL	49.0
28	T	2-3	-	++/N/F	-	-	20.0
29	P	2-3	++/M/F	++/N;P/F	++/M;C;AP/S	++/C/F;BL	66.0
30	C+SA	4	-	+++/N/D	-	-	33.0
31	T	4	-	+++/N/D	-	-	0.0
32	P+SA	2-3	+++/M/D	-	-	+/C/S;BL	36.0
33	T	3	+/M;AP/F	+/N/F	+/C/S	-	41.0
34	T	2-3	++/M/F	-	-	-	44.0
35	T+C	2-3	+/M/F	-	+/C/S	++/C/S;BL	0.0
36	T	3	++/M/D	+++/P/D	-	-	10.0
37	T	2	-	-	-	-	56.0

T: trabecular; P: pseudoglandular; C: compact; SC: sclerosing; SA: sarcomatoid. Grading (grades 1-4) according to Edmondson and Steiner (1954). Immunohistochemical reactivities of hepatocytes: M: membraneous; C: cytoplasmic; N: nuclear; P: paranuclear; IN: intranuclear; MI: cell in mitosis; AP: apoptotic body.. Immunohistochemical reactivity of other cells: BL, red blood cells. Staining pattern: D, diffuse; F, focal; S, single cells. Grades of reactivity: -, absent; +, weak; ++, moderate; +++, marked.

staining reaction. Staining was restricted to the surface membrane throughout (Fig. 3). With the exception of tumors showing a cellular accumulation of macrovesicular fat (fatty HCC's), all types and subtypes of HCC's were found to be reactive for E-cadherin, and the highest positivity tended to occur in trabecular, pseudoglandular and compact types ($p>0.05$). Moreover, reactivity tended to be lower in grade 4 than in grade 2 or 3 tumors ($p>0.05$). Reactivity for E-cadherin was significantly more frequent in HCC's also expressing c-met protein and TGF-ß ($p<0.05$).

Testing for TGF-ß disclosed mainly a cytoplasmic staining, 20/37 cases being reactive (Table 1). A remarkably strong staining was observed in mitotic tumor cells (Fig. 4), and few apoptotic bodies derived from tumor cells were immunoreactive. Staining for TGF-ß-1 was not related to type and grade.

There was no significant correlation between PCNA labeling and any of the four factors tested.

Liver cirrhosis associated with HCC

Data concerning this group of patients are complided in Table 2. Overall positivity for c-met protein in cells of cirrhotic nodules was 43%, and reactivity was cytoplasmic. C-met protein staining was detected in SRLC cells and in cells defined as small cell liver dysplasia (SLCD; Fig. 5), overall positivity amounting to 20%, and in epithelia of small bile ducts (30%). C-met protein staining was not correlated with TGF-ß staining, but its reactivity tended to be higher in E-cadherin-positive cases, and was significantly more frequent in Rb

protein-positive samples (bile duct cells only), all of Rb protein-labelled cirrhotic samples also being c-met protein-positive ($p<0.001$).

For Rb protein, bile duct and ductule cells were the only elements showing immunoreactivity, with a nuclear staining pattern and an overall prevalence of 30%.

The overall prevalence of E-cadherin staining was 80%. For the hepatocyte population, 77% of samples exhibited basolateral membraneous cell staining for E-cadherin, and in 57% the canalicular membrane was stained (Fig. 6A). In 67% the cell membrane of bile duct and ductule cells was reactive. Apart from normal and hyperplastic hepatocytes, large dysplastic liver cells, but not cells of SLCD (Fig. 6B), were labeled. The staining intensity was closely correlated with the distribution pattern of labeling, i.e. moderate to strong staining prevailed in a diffuse pattern, whereas weak staining was predominantly focal ($p<0.01$). E-cadherin staining was positively correlated with reactivity for TGF-ß ($p<0.05$).

Overall positivity for TGF-ß in this subgroup of cirrhosis amounted to 90%, but staining was restricted to the cytoplasm of bile duct and ductule cells, and of cells localized in the perisinusoidal space.

Liver cirrhosis not associated with HCC

Data are listed in Table 3. With 59% of cirrhotic samples stained, cytoplasmic reactivity for c-met protein was somewhat, but not significantly, higher than in the group associated with HCC. Reactivity was detected in SLCD (10%), in hyperplastic hepatocytes (23%), and in epithelia of small, in part proliferating, bile ducts (54%). A significantly higher prevalence of c-met protein staining was observed in samples positive for Rb protein ($p<0.01$), E-cadherin ($p<0.05$) and for TGF-ß-1 ($p<0.05$).

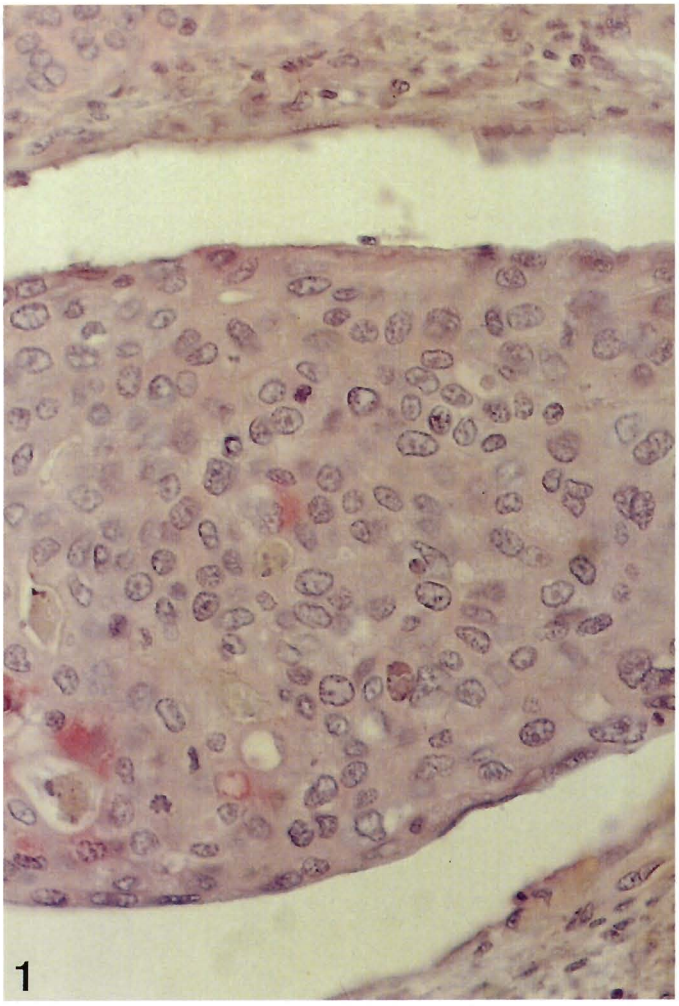

Fig. 1. C-met protein immunoreactivity in hepatocellular carcinoma. Note that reactivity is restricted to the cytoplasm of tumor cells in this area. APAAP technique, anti-c-met protein antibody, counterstained with haematoxylin. x 240

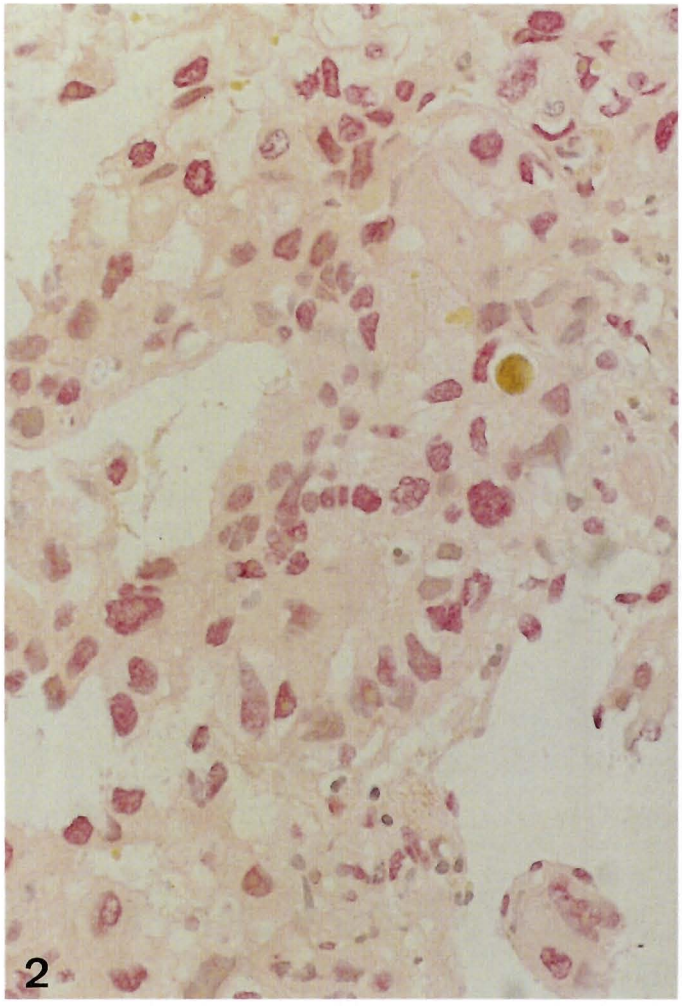

Fig. 2. Expression of retinoblastoma (Rb) protein in nuclei of a hepatocellular carcinoma, trabecular type. APAAP technique, anti-Rb protein antibody, counterstained with haematoxylin. x 240

As in cirrhosis associated with HCC, bile duct and ductule cells were the only elements staining for Rb protein, with a similar yield (34% vs. 30%).

Similar to the group with HCC, membraneous staining for E-cadherin was found in normal, hyperplastic and large dysplastic hepatocytes, and in bile duct cells, but not in SLCD, overall positivity amounting to 80%. Again, reactivity for E-cadherin was positively correlated with a higher prevalence of TGF-ß-1 reactivity.

Overall positivity for TGF-ß-1 was 92% (group with HCC: 90%), and staining was restricted to the cytoplasm of bile duct cells and of perisinusoidal cells.

Discussion

Among potential precursors of human HCC, atypical nodular hyperplasia and LCD are considered as candidate lesions. Whereas atypical nodular liver lesions are thought to represent a direct carcinogenic pathway leading to HCC, the significance of LCD as a «true» precursor for HCC is still debated. The definition of LCD is principally based on morphological (Anthony, 1976; Watanabe et al., 1988), morphometric (Giannini et al., 1987; Roncalli et al., 1988; Zhao et al., 1994a), and immunohistochemical (Roncalli et al., 1985; Govindarajan et al., 1990) criteria, and the findings obtained by these approaches only deliver very indirect evidence as to whether LCD may enter a neoplastic pathway.

However, more recent results have demonstrated that LCD cells appear to have undergone changes otherwise known for neoplastic lesions. DNA anomalies are more frequent in LCD of high grade than in low grade (Thomas et al., 1992; Zerbini et al., 1992). The tumor suppressor, p53 protein, is overexpressed in small cell

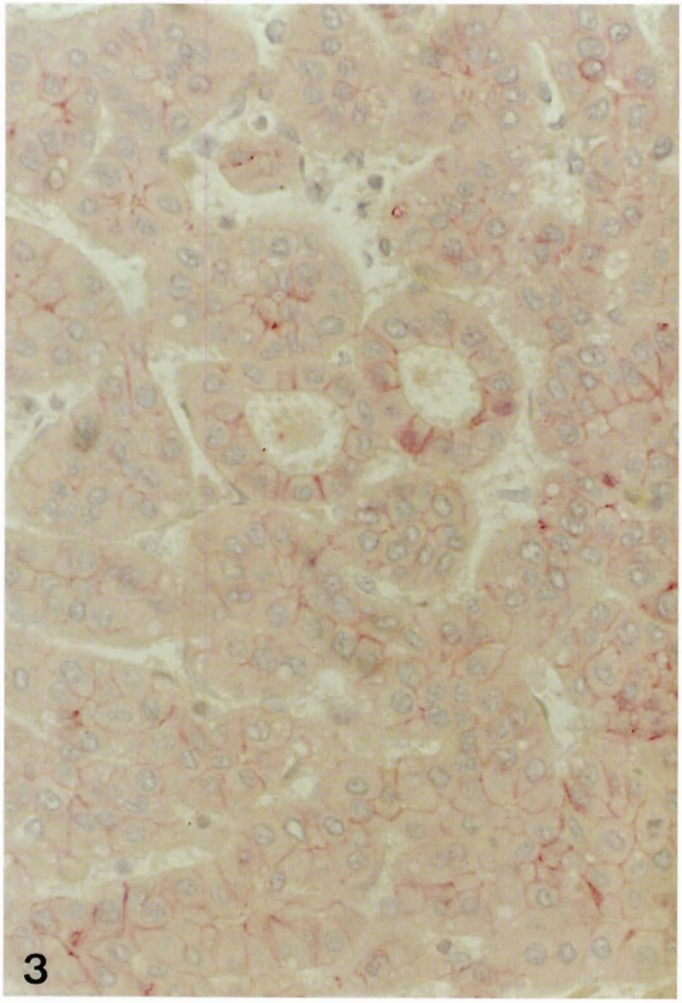

Fig. 3. In hepatocellular carcinomas, reactivity for the adhesion molecule, E-cadherin, is restricted to the surface of neoplastic cells. APAAP technique, anti-E-cadherin antibody, counterstained with haematoxylin. x 240

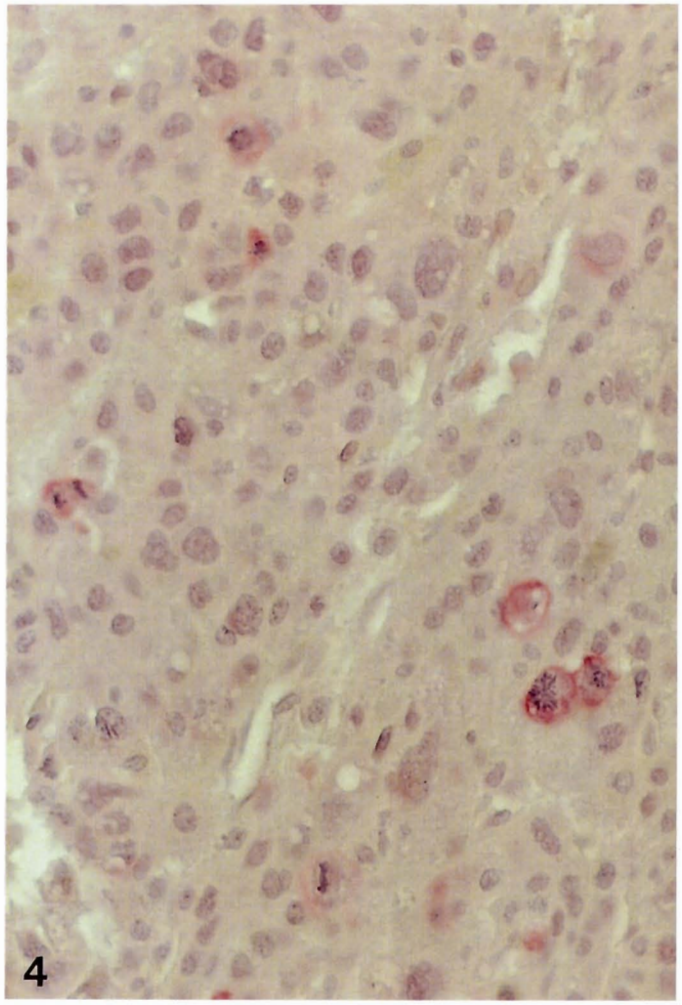

Fig. 4. Hepatocellular carcinoma, trabecular type, immunostaining for TGF-ß-1. Note that cytoplasmic reactivity is visualized in tumor cells undergoing mitosis. APAAP technique, anti-TGF-ß-1 antibody, counterstained with haematoxylin. x 240

Table 2. Liver cell dysplasia (LCD) in cirrhosis and fibrosis associated with hepatocellular carcinoma (HCC): Fibrosis/Cirrhosis scores and immunoreactivity of LCD cells for E-cadherin, c-met protein, TGF-ß1 and Rb-protein.

No.	FIBROSIS SCORES	E-CADHERIN	C-MET PROTEIN	TGF-ß1	RB-PROTEIN
1	3	++/HM,+/CM/F	+/BDC-	++/BDC,++/IC	++/BDN
2	5	+++/HM,+++/CM/D,++/BDM	-	+/BDC,+++/IC	-
3	5	+++/HM,+++/CM/D,+++/BDM	++/BDC	+/BDC,++/IC	-
4	5	++/HM,++/CM/D,++/BDM	-	++/BDC,+/IC	-
5	5	+/HM/F,SRLC,++/BDM	NO TISSUE	NO TISSUE	NO TISSUE
6	6	++/BDM	++/HC/F,SLCD	+/BDC,+/IC	+/BDN
7	5	++/HM,++/CM/F,NL,++/BDM	-	(/BDC,+++/IC	-
8	3	++/HM,++/CM/F,NL,++/BDM	+/HC/F,SLCD	++/BDC,+/IC	++/BDPN
9	4	++/HM,++/CM/F,+/BDM	+/BDC	+++/BDC,+/IC	-
10	5	+++/HM,+++/CM/D,+++/BDM	++/BDC	++/BDC,+/IC	-
11	3	NO TISSUE	-	++/IC	-
12	5	++/HM,++/CM/F,NL,SRLC,+/BDM	+/HC,LLCDe,SLCD	+/BDC,+/IC	+/BDN
13	6	-	+/BDC	+/BDC,+/IC	+/BDN
14	3	-	-	++/BDC,+/IC	-
15	5	+/HM,+/CM/F,NL,+/BDM	-	++/BDC,+/IC	-
16	5	++/HM,++/CM/F,NL,SRLC,+/BDM	+/HC/F,LLCDo,++/BDC	+/BDC,+++/IC	+/BDN
17	4	(/HM/F	NO TISSUE	NO TISSUE	NO TISSUE
18	5	-	-	++/IC	-
19	5	++/HM,+/CM/F,++/BDM	NO TISSUE	+++/BDC,+/IC	-
20	5	++/HM,+/CM/F,SRLC,+/BDM	-	+/BDC,+/IC	-
21	3	+/HM/F,SLCD,+/BDM	-	+/BDC,+/IC	-
22	3	-	-	++/IC	-
23	5	+/HM,+/CM/F,SLCD,++/BDM	++/BDC	+/BDC,+/IC	+/BDN
24	3	-	-	+/IC	-
25	3	+/HM/F,++/BDM	+/BDC	++/BDC,++/IC	+/BDN
26	3	+/HM/F	-	NO TISSUE	NO TISSUE
27	4	++/HM,+++/CM,D,++/BDM	-	++/BDC,+/IC	-
28	5	+++/HM/D,SRLC,++/DBM	++/HC/F,LLCDo,SRLC, SLCD	+/BDC,++/IC	-
29	3	++/HM,++/CM/F,+/BDM	+/HC/F,LLCDo, +/BDC	++/BDC,+/IC	+/BDN
30	4	++/HM,++/CM/F	-	+/IC	-

Immunohistochemical reactivity pattern: HM, hepatocyte membrane; HC, hepatocyte cytoplasm; CM, canalicular membrane; N, nuclear; PN, perinuclear; BDM, cell membrane of bile duct and ductule cells; BDC, cytoplasm of bile duct and ductule cells; BDN, nuclei of bile duct and ductule cells. Staining pattern: D, diffuse; F, focal. Grades of reactivity: -, absent; +, weak; ++, moderate; +++, marked. Cell types: NL, normal hepatocytes; SRLC, regenerating liver cells; SLCD, small cell liver dysplasia; LLCDo, large liver cell dysplasia with hypochromatic nuclei; LLCDe, large liver cell dysplasia with hyperchromatic nuclei; IC, perisinusoidal cells.

LCD (Zhao et al., 1994b), whereas bcl-2 protein is detectable in some HCC, but not in LCD (Zhao et al., 1994c). Moreover, small cell LCD is characterized by an abnormal tenascin and type IV collagen expression, reflecting a defective extracellular matrix (ECM) pattern previously detected in HCC (Zhao et al., 1996). Cells of LCD thus appear to not only deviate from normal hepatocytes with respect to morphological features, but also regarding growth regulation and cell-ECM relationships. In the present investigation, we therefore tested the expression patterns in LCD of additional factors having been shown to play a role in the growth of HCC.

C-met protein is the receptor of a pleiotropic epithelial growth factor, HGF, and is expressed in many cells and tissues. In the liver, the HGF/c-met ligand-receptor system may represent the most active modulator of hepatocyte proliferation (Horimoto et al., 1995). In the present group of HCC, about half of the tumors were reactive for c-met protein, and reactivity was also detected in mitotic and apoptotic cells. Expression of c-met in HCC has previously been reported, yields of positivity depending on the methods employed (Boix et al., 1994; Selden et al., 1994; Suzuki et al., 1994; Grigioni et al., 1995; Annen et al., 1996; D'Errico et al., 1996; Noguchi et al., 1996). In two analyses based on immunohistochemistry (Suzuki et al., 1994; D'Errico et al., 1996), reactivity was higher than in our study, whereas Northern blotting for c-met mRNA have yielded different prevalences up to now (Suzuki et al., 1994; 31.6%; Boix et al., 1994; 55%). Based on Western blotting, the expression of the p145 met proreceptor was significantly greater in HCC tissues than in non-tumorous areas (Annen et al., 1996). In the present study, the prevalence of c-met protein was about twice as high in high grade tumors in comparison with low grade tumors, a finding in line with a previous study showing that c-met positivity correlates with poor to moderate differentiation in HCC (Suzuki et al., 1994). No correlation was found between tumor type or the proliferation activity based on PCNA labelling, the latter being in contrast to a former report (Suzuki et al., 1994). This indicates that, in some situations, measured proliferative activity may be uncoupled from c-met expression, as has recently been shown in a rat model of hepatic carcinogenesis by use of the peroxisomal

proliferator, WY-14, 643 (Miller et al., 1996). In this study, c-met expression was upregulated, but did not correspond to the rapid proliferation of cells present in tumors. In addition to HCC, we found c-met protein reactivity in hyperplastic hepatocytes, in cells of small bile ducts and, notably, in small cell liver dysplasia, suggesting that the HGF receptor is upregulated in a subset of normal and dysplastic hepatocytes in cirrhosis. That hepatocytes in situations of increased cell turnover (e.g. hepatitis with regeneration) can express c-met has previously been shown (D'Errico et al., 1996; Noguchi et al., 1996), but we are not aware of a study having shown that this holds true for LCD in humans. However, elevation of c-met at early time points of carcinogenesis, when oval cells were proliferating, has been demonstrated in animal experimentation (Hu et al., 1996; Imai et al., 1996). The finding of an elevated expression of c-met protein in small bile ducts may either indicate proliferation of epithelial cells as such, reflect the proposed presence of precursor cells in this particular compartment, or may be related to the function of c-met protein as a morphogenetic factor, in particular for structures with lumen formation (Tsarfaty et al., 1992; Schmidt et al., 1995; Soriano et al., 1995). Taken together, c-met protein and its ligand is not only involved in the regeneration of hepatocytes, but may also control, in a paracrine growth pathway, the proliferation of oval cells and of putative cancer precursor cells.

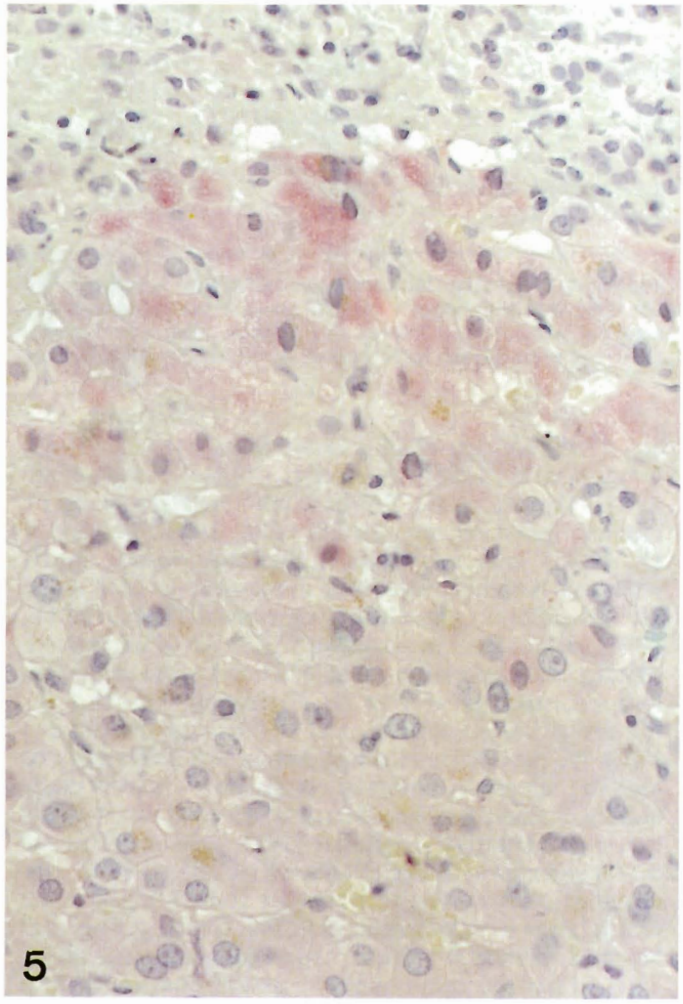

Fig. 5. Cytoplasmic reactivity for c-met protein in cells classified as small cell liver dysplasia (SLCD). APAAP technique, anti-c-met protein antibody, counterstained with haematoxylin. x 240

Rb protein, an important negative growth regulator acting in early segments of the cell division cycle (reviews: Weinberg, 1995; Xu, 1995) and playing a role in hepatocyte differentiation (Leggett and Mueller, 1994) was detected in 46% of HCC in a nuclear staining pattern, as previously reported (Hsia et al., 1994; Kawakita et al., 1994; Seki et al., 1995). There was no correlation between Rb protein reactivity and type, grade or proliferative activity. No correlation with differentiation has previously been noted (Seki et al., 1995), whereas one study demonstrated that Rb positivity increased in proportion to proliferation (Kawakita et al., 1994). Mutations of the Rb gene have been detected both in human and rat HCC (Murakami et al., 1991; Nakamura et al., 1991; Smith et al., 1993; Zhang et al., 1994). In contrast to previous studies showing Rb expression also in regenerating human or rat hepatocytes (Kawakita et al., 1994; Fan et al., 1995), Rb protein reactivity in our two groups was restricted to epithelial cells of small ducts proliferating in this situation, whereas no staining was found in dysplastic or hyperplastic hepatocytes.

E-cadherin, a calcium-dependent cell adhesion molecule controlling cell polarity, adhesion and tissue construction widely expressed in many cell systems (Takeichi, 1991) and known to be a morphogenetic regulator in several tumors, was detectable in 55% of HCC, and in 11% with a strong membraneous expression. Strongest immunoreactivity was observed in HCC forming trabecular, acinar or compact structures. Reactivity tended to be less frequent and of lower intensity in G4 than in G3 or G2 tumors, and was significantly more frequent in HCC also expressing c-met protein and TGF-ß-1. Reactivity of E-cadherin in HCC cells supports results reported in previous studies (Shimoyama and Hirohashi, 1991; Yamaoka et al., 1995; Ihara et al., 1996). Reduction or even loss of E-cadherin, as formerly demonstrated (Shimoyama and Hirohashi, 1991) and shown in our study, indicates that down-regulation of this cell adhesion molecule may be one characteristic of poorly differentiated HCC, whereas the majority of HCC of the thin-trabecular or acinar type have a preserved expression (Ihara et al., 1996). HCC with formation of cell cords and/or pseudoglandular structures appear to recapitulate normal parenchymal morphology with formation of an apical pole of the cells, which may in fact produce bile. The presence of E-cadherin in these more differentiated HCC is in line with the observation that this morphogenetic factor is

required for the formation of bile canalicular networks (Terry and Gallin, 1994). It appears, therefore, evident that loss of E-cadherin in HCC is correlated with altered growth, i.e. large tumor size and capsular and vascular invasion (Kozyraki et al., 1996).

We can confirm that variable E-cadherin staining is found in normal hepatocytes, regenerating liver cells, and epithelial cells of bile ducts and ductules (Ihara et al., 1996; Kozyraki et al., 1997). In addition, we observed reactivity in large dysplastic hepatocytes, but not in small dysplastic liver cells. A loss of E-cadherin reactivity in the latter cell system may either suggest that expression of a morphogenetic regulator is dependent on distinct phases of proliferation and differentation of a committed, but still immature cell population, or that E-cadherin is in fact down-regulated in small dysplastic liver cells representing putative neoplastic precursors. This would mimick the situation observed in dysplastic lesions of the colon, where changes of E-cadherin-associated molecules (catenins) occur early in dysplasia (Valizadeh et al., 1997), and where changes of E-cadherin reactivity and localization are correlated with adenoma size and the grade of dysplasia (Gagliardi et al., 1995).

TGF-ß-1, which has an important role in growth regulation of liver cells (Braun et al., 1988, 1990; Massague, 1990; Bissell et al., 1995; Mitaka et al., 1995) and which is upregulated during active liver disease in humans (Annoni et al., 1992), disclosed a predominantly cytoplasmic staining in more than half of the HCC's analyzed, without correlation as to type or grade. Cytoplasmic reactivity of this cytokine and elevated levels of its mRNA in HCC's has previously been reported (Ito et al., 1991), and levels of TGF-ß-1 are enhanced in patients with HCC (Shirai et al., 1994). Interestingly, a marked staining was observed in the cytoplasm of tumor cells being in mitosis and in apoptotic tumor cells, but staining prevalence was not correlated with PCNA

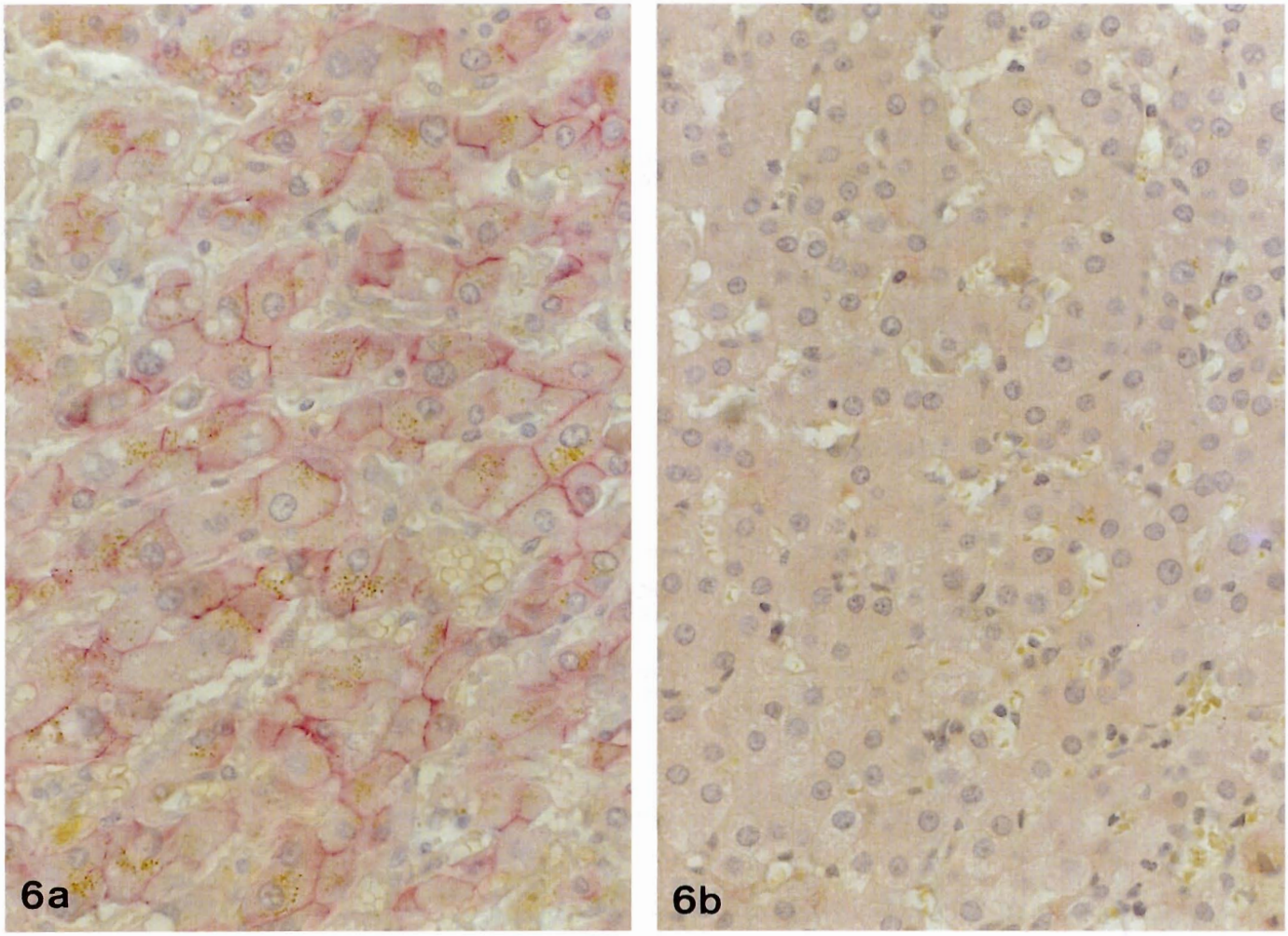

Fig. 6. A. E-cadherin membraneous staining in normal hepatocytes and liver cells classified as large liver cell dysplasia. **B.** No E-cadherin immunostaining is detectable in cells classified as small liver cell dysplasia. APAAP technique, anti-E-cadherin antibody, counterstained with haematoxylin. x 240

Table 3. Liver cell dysplasia (LCD) in cirrhosis and fibrosis not associated with hepatocellular carcinoma (HCC): Fibrosis/Cirrhosis scores and immunoreactivity of LCD cells for E-cadherin, c-met protein, TGF-ß1 and Rb-protein.

Nr.	FIBROSIS SCORE	E-CADHERIN	RB-PROTEIN	C-MET	TGF-ß1
1	4	+/HM,CM/F;+/BDM	+/BDC	+/BDC	++/BDC;++/IC
2	5	++/HM,+/CM;+/BDM	+/BDN	+/HC/SLCD;+/BDC	++/BDC;++/IC
3	4	+++/HM,CM;+++/BDM	-	+/HC/TRAN;++/BDC	+/BDC;+/IC
4	4	++/HM,CM;BDM	-	++/BDC	+/BDC;++/IC
5	4	+/BDM	+/BDN	-	+/IC
6	4	++/HM,CM;+/BDM	-	-	+/IC
7	5	+/HM,CM/NL/F;++/BDM	-	-	+/IC
8	6	++/HM,CM;++/BDM	+/BDN	+/HC/TRAN;+/BDC	++/BDM;++/IC
9	6	++/HM,CM;++/BDM	+/BDN	++/HC/LLCDo;++/BDC	++/BDC;++/IC
10	6	+++/HM,CM/D;+++/BDM	-	-	+/BDC;+/IC
11	5	+/HM,CM;+/BDM	-	++/HC/NL,TRAN;++/BDC	++/BDC;+/IC
12	4	+/HM,CM;++/BDM	+/BDN	+/HC/TRAN;+/BDC	+/BDC;++/IC
13	5	+/HM,CM;+/BDM	+/BDN	++/BDC	+/BDC;+/IC
14	5	+/HM,CM;++/BDM	+/BDC	+/BDC	+/BDC;+/IC
15	5	+/HM,CM;+/BDM	+/BDN	+/HC/NL	+/BDC;++/IC
16	6	++/HM,CM;+/BDM	+/BDN	+/BDC	+/BDC;+/IC
17	6	+/HM,CM;+/BDM	+/BDC	+/BDC	++/BDC;+/IC
18	3	-	-	-	-
19	5	+/HM,CM;+/BDM	-	+/BDC	++/IC
20	4	+/HM,CM;+/BDM	+/BDN	+/HC/NL,LLCDo	++/BDC;++/IC
21	6	+/HM	-	+/BDC	+/BDC
22	5	+++/HM,CM;++/BDM	-	-	+/BDC;+/IC
23	4	++/HM,CM;+/BDM	-	+/HC/TRAN,LLCDo;+/BDC	+/BDC;+/IC
24	6	+/HM,CM;++/BDM	+/BDC	+++/HC/TRAN,NL,LLCDo;++/BDC+/BDC;+/IC	
25	5	+/HM,CM;+/BDM	-	-	+/IC
26	5	+/HM,CM	-	-	+/IC
27	5	+/HM,CM	-	-	+/BDC;+/IC
28	6	++/HM,CM;++/BDM	-	+/HC/SLCD;+/BDC	+/BDC;+/IC
29	4	+HM,CM;+/BDM	-	-	+++/BDC;++/IC
30	4	+/BDM	+/BDN	+/BDC	+/BDC;+++/IC
31	6	++/HM,CM;+/BDM	+/BDN	+/HC/LLCDo;+/BDC	+/BDC;+++/IC
32	6	+/HM,CM	-	+/HC/NL,LLCDo;+/BDC+/BDC;+/IC	
33	6	+/HM,CM;+/BDM	-	-	+/IC
34	6	+/HM,CM;+/BDM	-	-	+/BDC;+/IC
35	6	-	-	-	+/BDC;+++/IC
36	6	++/HM,CM;+/BDM	-	-	+/BDC;+/IC
37	5	NO SECTION	-	NO SECTION	NO SECTION
38	5	+/HM,CM	-	++/BDC	+/BDC;+/IC
39	5	+/HM,CM;+/BDM	-	+/HC/NL;+/BDC	+/BDC;+/IC
40	5	+/HM,CM;+/BDM	-	+/BDC	+++/BDC;+/IC
41	5	++/HM,CM;+++/BDM	-	-	+/BDC
42	6	-	-	NO TISSUE	+/IC
43	6	-	-	-	-
44	6	+/HM,CM;+/BDM	-	-	-
45	5	-	-	+/HC/NL;++/BDC	+/BDC;+/IC
46	5	-	-	NO TISSUE	NO SECTION
47	5	+/HM,CM;+/BDM	-	-	+/BDC;+/IC
48	5	-	-	-	NO SECTION
49	4	+++/HM,CM;++/BDM	+/BDN	++/BDC	+/BDC;+/IC
50	4	+/HM,CM;+/BDM	-	+/BDC	++/BDC;+/IC
51	3	+/HM,CM;+++/BDM	+/BDN	+/BDC	+/BDC;+/IC
52	5	+/BDM	+/BDN	++/BDC	++/BDC;++/IC
53	5	+/HM,CM;+/BDM	-	-	+/BDC;+/IC
54	4	-	-	NO TISSUE	NO TISSUE
55	3	++/HM,CM;+/BDM	+/BDN	++/HC/NL,SLCD	+/BDC;+/IC
56	6	+/HM	+/BDN	++/BDC	+BDC;+/IC
57	4	+/HM;+/BDM	-	+/BDC	++/BDC;++/IC
58	5	+/BDM	-	-	+/BDC;++/IC
59	4	+/HM,CM;+/BDM	-	+/HC/TRAN;+/BDC	++/BDC;++/IC
60	5	++/HM,CM;++/BDM	-	-	+/BDC;++/IC
61	5	+/BDM	-	-	+/IC
62	4	++/HM,CM;+/BDM	+/BDN	+/BDC	+/BDC;+/IC
63	3	+/BDM	-	+/BDC	+/IC
64	4	-	-	-	+/BDC;+/IC
65	4	NO TISSUE	-	NO TISSUE	++/IC
66	5	+/HM;+/BDM	-	-	NO TISSUE

Immunohistochemical reactivity pattern: HM, hepatocyte membrane; HC, hepatocyte cytoplasm; CM, canalicular membrane; N, nuclear; PN, perinuclear; BDM, cell membrane of bile duct and ductule cells; BDC, cytoplasm of bile duct and ductule cells; BDN, nuclei of bile duct and ductule cells. Staining pattern: D, diffuse; F, focal. Grades of reactivity: -, absent; +, weak; ++, moderate; +++, marked. Cell types: NL, normal hepatocytes; SRLC, regenerating liver cells; SLCD, small cell liver dysplasia; LLCDo, large liver cell dysplasia with hypochromatic nuclei; LLCDe, large liver cell dysplasia with hyperchromatic nuclei; IC, perisinusoidal cells.

labelling. The reason why a marked reactivity occurs in mitotic HCC cells is not clear so far, but it may reflect overexpression of the cytokine counteracting progression in the cell division cycle, because TGF-ß-1 expression has been shown to act close to the G1/S border in the cell cycle (Kletsas et al.,1995). Staining of apoptotic tumor cells can be expected, because TGF-ß-1 appears to be involved in programmed cell death (Bursch et al., 1993; Takiya et al., 1995), apparently inhibited by the Rb gene product (Fan et al., 1996). Similar to a previous investigation we could not detect reactivity for TGF-ß-1 in hepatocytes or LCD cells (Nakatsukasa et al., 1991), whereas TGF-ß-1 mRNA expression has been observed in liver carcino-genesis (Braun et al., 1990) and in neoplastic liver nodules (Bedossa et al., 1995). With respect to lacking reactivity in dysplastic liver cells it is of interest to note that TGF-ß-1 partially suppresses the transformed phenotype of ras-transformed hepatocytes (Serra et al., 1992). Cytokine reactivity was detectable in epithelial cells of small bile ducts and ductular proliferations, and in perisinusoidal cells, as shown in a former study (Takiya et al., 1995).

In conclusion, the present findings show that, in liver cell dysplasia (LCD) associated or not associated with HCC, the hepatocyte growth factor receptor, c-met protein, is only detectable in the small cell variant of LCD, but not in normal hepatocytes or large cell LCD. This observation supports the hypothesis that SLCD is the most probable candidate for representing a precursor cell for carcinogenesis, also being the only cell type among LCD expressing p53 protein (Zhao et al., 1994b). Similar to less differentiated HCC, SLCD cells also lack expression of E-cadherin, further underlining the potential role of these cells in a hepatic carcinogenic pathway.

Acknowledgements. The secretarial help of Erna Müller is gratefully acknowledged.

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Accepted November 29, 1997