Histol Histopath (1993) 8: 617-626

Histology and Histopathology

«Neuroendocrine» differentiation in hepatocellular carcinomas (HCCs): Immunohistochemical reactivity is related to distinct tumor cell types, but not to tumor grade

M. Zhao, J.A. Laissue and A. Zimmermann
Institute of Pathology of the University, Bern, Switzerland

Summary. We have analyzed neuroendocrine differentiation (ND) in hepatocellular carcinomas (HCCs) of fifty patients. It turned out that ND is frequent in HCCs, and that it is not restricted to fibrolamellar hepatocellular carcinoma (FL-HCC). Multiexpression is seen in a quarter of the cases, and marker coexpression may occur within the same tumor cell. ND predominates in trabecular and mixed HCCs, but does not appear to be related to grade. Most positive cases showed a hepatocyte-like cell morphology, frequently associated with bile formation. It thus appears that the HCC cell type most likely to show ND is a hepatocyte-like one, i.e. differentiated cell, frequently polarized and producing bile, rather than a small and poorly-differentiated cell. Possible pathogenic mechanisms leading to ND in HCCs are briefly discussed.

Key words: Neuroendocrine cells, Hepatocellular carcinoma

Introduction

Far more widespread than anticipated, neuroendocrine differentiation (ND) has now been identified not only in neoplasms originating in neuroendocrine tissues, but also in tumors without an evident neuroendocrine phenotype in conventional histological sections (Bosman, 1989; Wright, 1990). Several studies on neuroendocrine expression patterns both in normal liver tissue components and in hepatic tumors have been reported. Bile duct epithelial cells may, in some instances, express markers regarded as typical for neuroendocrine differentiation (Roskams et al., 1990), whereas the expression of these markers by hepatocytes and their neoplastic offspring is the subject of controversy. Hepatocellular carcinomas (HCCs) and hepatoblastomas (Ruck et al., 1990) may contain a carcinoid component or show carcinoid features (Barsky et al., 1984). More intriguing are primary hepatic tumors that do not show the morphology of carcinoids or apudomas, but nevertheless display neuroendocrine features revealed by immunohistochemical or ultrastructural studies. Thus, ultrastructural examination of fibrolamellar hepatocellular carcinoma (FL-HCC) revealed the presence of neurosecretory granules, sparse in some cells, abundant in others (Payne et al., 1986). Studies based on the use of antibodies directed against general neuroendocrine markers, and on the Grimelius method, have suggested that ND may occur in several neoplasms of the liver, including HCCs (Wang et al., 1991). These observations deserve further investigation, because tumors with ND may behave more aggressively than their counterpart without neuroendocrine features (Arends et al., 1986; Sarsfield and Anthony, 1990; Hsu et al., 1991). It is not known whether neuroendocrine reactivity in hepatocellular tumors is related to type or grade, and it has not been studied whether HCC cells with a morphology close to that of normal hepatocytes also express neuroendocrine markers or not. To address these questions, we have done a systematic retrospective study on HCCs of fifty patients. The neoplasms cover a broad range of gross growth patterns, histological subtypes and grades. We have used immunohistochemistry for the detection of markers known to be expressed in neuroendocrine cells.

Offprint requests to: Prof. Dr. A. Zimmermann, Pathologisches Institut der Universität, Murtenstrasse 31, CH-3010 Bern, Switzerland

Materials and methods

Based on archive material, HCCs from fifty patients were used for the present study. Age and sex distribution of patients, size (where known), type, grade and cellular compositions of the tumors are listed in Table 1. Typing and grading was performed according to published criteria (Edmondson and Steiner, 1954; Gibson and Sobin, 1978; Nakashima and Kojiro, 1987).

For light microscopy, formalin-fixed material was embedded in paraffin, and sections were stained with haematoxylin-eosin, PAS, Van Gieson's, and Gomori's reticulin stains. Antisera used for immunohistochemistry

Table 1. Histopathology of 50 cases of HCC.

CASE	AGE	SEX	TUMOR	SIZE (cm)	TYPE	GRADE	CELLS	LC	BILE	MB
1	69	M	HCC	1.6 x 1.6	T+P	2	Cl+Gr	MI	+	+
2	74	M	HCC	N.B.	T+P	2	Gr+Cl		+	
3	43	M	HCC	N.B.	T	2	Cl			
4	85	M	HCC	N.B.	T	2+3	Gr			
5	69	F	HCC	14 x 13 x 8	T+P	2	Cl+Gr+Gi			
6	39	M	HCC	N.B.	T	3	Gr+Sp			
7	79	M	HCC	N.B.	T+P	3	Gr+Eo		+	
8	55	M	HCC	8 x 8	T	2	Gr			
9	72	M	HCC	mn	T+C	3	Gr	FI		
10	67	M	HCC	N.B.	T	2	Cl			
11	83	M	HCC	N.B.	T	2+3		FI		
12	67	M	HCC	N.B.	T	1		MI		
13	64	M	HCC	N.B.	T	3	Gr+Cl		+	
14	55	M	HCC	16 x 14 x 11	T	2+3	Gr			
15	70	M	HCC	15 x 12.3 x 11	T+P	3+4	Po			+
16	80	M	HCC	N.B.	T+P	2+3	Gr			
17	65	M	HCC	N.B.	T	2	Gr			+
18	55	M	HCC	N.B.	T	2+3	Gr			
19	51	M	HCC	0.4 x 0.3	T	2	Gr+Eo	FI		
20	55	F	HCC	5 x 4.5 x 3	A: T	2	Gr+Cl			
					B: T+P	1	Eo			+
21	69	M	HCC	N.B.	T	2	Gr+Eo			
22	33	M	HCC	N.B.	T+P	1+2	Eo+Gr		+	
23	65	M	HCC	N.B.	T+P	2	Gr	FI		
24	75	M	HCC	6 x 5.5 x 4.5	T	2	Gr+Cl	FI		
25	69	M	HCC	N.B.	T	1+2	Cl+Gr	MI	+	
26	47	M	HCC	N.B.	T	2+3	Eo+Gr		+	+
27	47	M	HCC	N.B.	T	3	Eo+Gr	FI		
28	1	M	HCC	0.8 x 0.8	T+P	3	Gr+Cl	MI	+	
29	41	M	HCC	N.B.	T	3	Gr+Gi			
30	47	M	HCC	7 x 6 x 5	T+P	2	Gr			
31	61	F	HCC	N.B.	T	2	Gr			
32	43	F	HCC	N.B.	T	3	Gr+Gi			
33	55	M	HCC	N.B.	T	2	Eo			
34	47	M	HCC	N.B.	T+P	2	Gr+Fa	MI	+	
35	55	M	HCC	1.5 x 1 x 1	T	1+2	Gr			
36	56	M	HCC	5 x 2	T+P	2+3	Eo		+	+
37	63	M	HCC	12 x 8	T+P	2+3	Eo+Po+Gi		+	+
38	71	M	HCC	N.B.	T	2				
39	62	M	HCC	5 x 3 x 4	T+P	1+2	Gr+Eo	MI	+	+
40	67	F	HCC	4 x 3 x 2	T	2+3	Eo		+	
41	69	M	HCC	15 x 13.3 x 9.3	T	1	Eo+Fa			
42	11	M	HCC	6.5 x 5.5 x 5	T	1+2	Gr		+	
43	6	M	HCC	8 x 3 x 4mn	T+C	3	Eo+Gr		+	
44	26	M	HCC	1 x 0.4	T	2	Eo+Gr		+	
45	45	F	Fa-HCC	10 x 10	A: C	2+3	Fa+Gr+Cl		+	
					B: T	3+4	Ox+Po			
46	42	F	Fa-HCC	5 x 7.5	T+P	3	Fa+Gr+Cl+Gi			
47	50	M	Fa-HCC	4.5 x 3.5 x 3.5	T	2+3	Fa+Gr+Cl+Gi			+
48	45	F	Fa-HCC	15 x 13	T	3	Fa+Cl+Eo+Gr+Gi			
49	26	M	FL-HCC	11 x 10 x 9						
50	34	M	FL-HCC	3 x 2 x 1 mn						

Tumors: HCC, hepatocellular carcinoma; FL-HCC, fibrolamellar HCC; Fa-HCC, fatty HCC. N.B., needle biopsy. mn, multinodules. HCC type: T, trabecular (sinusoidal); P, pseudoglandular (acinar); C, compact (solid). HCC grade (Edmondson and Steiner, 1954): grades 1 to 4. Cell type: Eo, eosinophilic cell; Cl, clear cell; Fa fatty cell; Gi, giant cell; Gr, granular cell; Sp, spindle cell; Po, polymorphous cell; Ox, oxyphilic cell. LC, liver cirrhosis. MI, micronodular. FI, fibrosis. MB, Mallory body.

are listed in Table 2. Polyclonal antibodies were diluted in TPA buffer (10% physiogel and 0.1% NaN_3 in TBS buffer, pH 7.5; TBS: 0.13 µM NaCl, 7 µMNa_2HPO_4 and 3 µM NaH_2PO_4, pH 7.5) for the ABC method. Monoclonal antibodies were diluted in Tris-NaCl buffer (0.1% Tris and 1% NaCl with 0.1% NaN_3, pH 7.4) for the APAAP method.

Optimal dilution screening was performed using both negative and positive controls, and blocking procedures. A series of biopositive control sections with respect to each neuroendocrine antibody were processed in consistence with theoretical anticipations. The optimal

Table 2. Antisera used in this study.

ANTIBODY	A/C	DILUTION	SOURCE	POSITIVE CONTROL
CYT* (Pancytokeratin)	M,M	1:50	DAKO	GIT mucosa
CAM (CAM 5.2)	M,M	1:2	Becton-Dickinson	Liver (hepatocytes)
CEA (Carcinoembryonic antigen)	M,M	1:20	DAKO	Colonic carcinoma
AFP (Alpha fetoprotein)	M,M	1:500	DAKO	Colonic carcinoma
AT (Alpha-l-antitrypsin)	R,P	1:100	DAKO	Liver (hepatocytes)
FER (Ferritin)	R,P	1:1000	DAKO	Liver (hepatocytes)
MEL (HMB 45 anti-human melanoma)	M,M	1:100	DAKO	Junctional nevus
NSE (Neuron-specific enolase)	M,M	1:1000	DAKO	Brain, spinal cord
5HT (Serotonin)	M,M	1:50	DAKO	Duodenal mucosa
S100 (S 100 protein)	R,P	1:200	DAKO	Skin
SOM (Somatostatin)	R,P	1:3000	DAKO	Pancreas, hypothalamus
SYN (Synaptophysin)	M,M	1:100	DAKO	Adrenal gland
CG(Chromogranin)	M,M	1:200	DAKO	Adrenal gland
NEU (Neurotensin)	R,P	Kit:10	Biogenex	Brain
VIP (Vasointestinal peptide)	R,P	Kit:10	Biogenex	Gastric mucosa
BOM (Bombesin)	R,P	Kit:5	Biogenex	Lung, gastric mucosa
LEU (LEU-7)	M,M	1:5	Becton Dickinson	Prostate, pancreas, adrenal gland
PAP (Pancreatic polypeptide)	R,P	1:3000	DAKO	Pancreas
PGP (PGP 9.5, Protein gene product)	R,P	1:3000	Ultraclone	Brain

*: abbreviations used in the other tables and in the text. A, animal; R, rabbit; M, mouse; C, clone; M, monoclonal; P, polyclonal; GIT, gastrointestinal tract.

working dilution was worked out based on the staining conditions of positive control sections, under which positive cells exhibited the strongest staining, in the absence of background staining. Primary antisera were substituted with normal mouse and rabbit sera (DAKO), at the dilutions used for specific antisera, to verify that DAB and APAAP reactions were not caused by second antisera, chromogen-containing complex, or by endogenous enzyme activity (Table 2).

As background staining is a well-known problem in immunohistochemistry of the liver, the following procedures were used to confirm the specificity of the reactions: 1) 3% H_2O_2 and 5 μM levamisole (Sigma) to block the effects of endogenous peroxidase and alkaline phosphatase, respectively; 2) preincubation of the sections with 0.1% avidin (DAKO) and 0.01% avidin and 0.01% biotin (DAKO) or streptavidin kit mixture (DAKO) to block endogenous biotin and lecitin-like substances; 3) absorption of primary and linking antibodies with normal human, horse and rabbit sera (DAKO) to diminish nonspecific binding; 4) incubation of the sections with 3% bovine serum albumin (Serva) plus 0.1% NaCl in buffer to decrease hydrophobic reactivity between antisera and formalin-fixed liver tissue; and 5) by use of hypertonic buffer (equal to 1% NaCl) to wash the sections between incubations, in order to counteract ionic reactions at charged residues of protein molecules. Cloudy background staining patterns were clearly removed by these procedures.

Immunohistochemistry itself was performed using a modified ABC and APAAP procedure, in humidified environment at room temperature, unless stated otherwise. Sections were deparaffinized, rehydrated in TBS for ABC, in Tris buffer for APAAP, then preincubated at 37 °C in trypsin or protease solution (0.02% trypsin Difco 1:250 and 12.4 μM $CaCl_2.2H_2O$ in TBS buffer, pH 8.0; 0.1% protease Sigma in Tris-NaCl buffer, pH 7.4) for 10 to 20 minutes. Afterwards, 3% bovine serum albumin (Serva) in TBS, or Tris buffers were applied on the sections for 30 minutes at room temperature. The sections were washed three times in the buffers after each preincubation. Primary antisera were applied for one to three hours. Biotinylated swine anti-rabbit (DAKO) for polyclonal antisera (1:200 in TPA with 2% normal human serum), and rabbit anti-mouse (DAKO) for monoclonal antisera (1:30 in Tris buffer with 0.1% NaN_3 and 25% normal human serum) were applied for 45 minutes. Sections were then incubated for 45 minutes with avidin-biotin horseradish peroxidase complex (DAKO) and APAAP complex (DAKO), respectively. DAB (Sigma) and alkaline phosphatase (New fuchsin substrate solution, DAKO) reactions products were counterstained with haematoxylin and mounted with aquadex (Merck).

In two cases showing strong reaction to more than one marker, double immunohistochemical staining was performed (Table 5). First, a brown reaction product was elicited by use of polyclonal antisera and ABC staining, then a bright red reaction product, by use of monoclonal antisera and APAAP staining. Positive control sections containing both immunodeterminants were processed simultaneously.

Results

Types, grades and cellular features of HCC

Of the 50 cases of HCC, 2 were FL-HCC. A purely trabecular growth pattern was observed in 31/48 HCC (64.6%), whereas a mixture of trabecular and pseudo-

Table 3. Neuroendocrine markers in control sections.

Abs	NORMAL TISSUE	CELL TYPE	POSITIVE STAINING	
			PATTERN/INTENSITY	BACKGROUND
LEU	Prostate	Epithelium	G,D/+++	(-)
	Adrenal gland	Medullary zone	G,D/+++	(-)
	Pancreas	Islet	G,D/+++	(-)
S100	Skin	Nevus	G,D/+++	
		Sweat gland	D/++	(-)
		Langerhans cell	D/+++	(-)
SYN	Adrenal gland	Medullary zone	G,D/+++	(-)
NSE	Brain	Neuron	D/+++	(-, in white part)
	Spinal cord	Motor neuron	D/+++	(-, in tracts)
CG	Adrenal gland	Medullary zone	G/+++	(-)
	Pancreas	Islet	G,D/+++	(-)
5HT	Duodenum	Epithelium	G,D/+++	(±/cloudy)
SOM	Pancreas	Islet	D/+++	(-)
PGP	Brain	Neuron	D/++	(±/cloudy)
PAP	Pancreas	Islet	D/+++	(-)
VIP	Gastric mucosa	Epithelium	G,D/++	(-)
NEU	Brain	Neuron	D/++	(-)
BOM	Duodenum			
MEL	Junctional nevus	Nevus	G,D/+++	(-)

Abs, antibodies. G, granular reaction product. D, diffuse reaction product. Intensity: +++, marked; ++, moderate; ±, weak; -, none.

glandular patterns was found in 16/48 (33.3%) (Table 1). Only two HCCs displayed a compact (solid) component (4.1%). There was no purely pseudoglandular HCC. Within the trabecular group, 6.25% were grade 1, 27.08%, grade 2, 27.08%, grade 3, and 2.08%, grade 4. In the mixed trabecular and pseudoglandular group, the grade distribution was 2.08%, 16.67%, 12.50%, and 2.08%, respectively (Table 1). If the histological grade varied within one sample, the highest grade observed was assigned to this neoplasm. The overall distribution of grades was 6.25%, 45.83%, 43.75% and 4.17% for grades 1 to 4, respectively. Next, we analyzed the distribution of neoplastic cells with a distinct morphology. Most tumors contained a cell type with a clearly granular cytoplasm; i.e. closely resembling that of normal hepatocytes (77.1%). One third of the tumors contained cells with strongly eosinophilic cytoplasm, even though fibrous bands as seen in FL-HCC were not visible. Clear cells were found in many cases (29.2%), whereas only 2 HCCs were entirely composed of this cell type (clear cell HCC; 4.2%). 6.2% were predominantly clear cell lesions. Giant cells, which appear to occur most frequently in grade 3 lesions (Nakashima and Kojiro, 1987), were found in 14.6% (overall frequency of grade 3 in this material: 43.75%). Fatty change (macrovesicular) in neoplastic hepatocytes was present in 6/48 HCC (12.5%), and 4 cases were identified as being so-called «fatty HCC». One HCC was characterized by large, polygonal cells showing a strongly oxyphilic cytoplasm, but without fibrous bands. Tumours of this type have been termed oxyphilic or oncocytic carcinomas. Bile formation, as visualized in conventional sections, was detected in 34.00% (17/50). 18% (9/50) of the HCCs contained typical Mallory bodies.

In 44/48 cases, there was enough adjacent liver tissue for structural analysis. Micronodular cirrhosis was present in 13.6% of 44 livers, and 6/44 (13.6%) showed fibrosis only. Neither cirrhosis nor fibrosis were noted in the vicinity of «fatty HCC».

Neuroendocrine markers in non-neoplastic control tissues (Table 3)

With the exception of bombesin, all antisera directed against «neuroendocrine» determinants used in the present study led to a visible reaction in the tissues in which expression of the corresponding marker was anticipated. Cloudy staining was observed at high antibody dilutions only and could easily be blocked by preincubating the sections with 3% BSA.

Neuroendocrine markers in HCCs

Thirty HCCs (60%) displayed one or more neuroendocrine markers, but out of the 13 antisera tested, only 8 gave positive results in HCC sections (Table 4). Positivity for Leu-7 (LEU) and S-100 protein (S100) was encountered most frequently (13/50, 26%), followed by NSE and synaptophysin (SYN) (10% each), somatostatin (SOM, 8%), chromogranin (CG) and neurotensin (NEU) (6% each), and by VIP in one case (2%). Reactivity for several neuroendocrine markers was observed in 12/50 HCCs (24%; Table 4), involving all of

the 8 kinds of antisera showing positive results. Leu-7 reacted together with another marker in 8 instances, S100 in 7, NSE in 5, SYN in 3, and NEU and VIP in 1, respectively.

Reaction products for Leu-7, S100 and CG were intensively stained in at least 60% of the cases (10/13, 11/13 and 2/3, respectively).

In contrast, SYN, NSE, SOM and VIP elicited a weak staining intensity in about 80% (Table 4). The staining pattern depended on the antiserum used. Thus, staining for Leu-7 was cytoplasmic and granular in 10/13 cases (Fig. 1) and, in 5/13 tumors, staining occupied a distinct paranuclear area. A diffuse, finely granular cytoplasmic staining was also noted for S100 protein, NSE and SOM (10/13, 4/5 and 4/4 cases, respectively), but testing for these markers also resulted in nuclear staining (7/13, 4/5 and 2/4, respectively; Fig. 2). Staining for NEU, CG and VIP was also diffuse and cytoplasmic, whereas in 4/5 SYN-positive cases a linear staining pattern along the cell membrane was found (Fig. 3).

The technique of double immunostaining was employed in two HCCs. One tumor reacted with both Leu-7 and S100, the other with NSE and SOM in a subset of neoplastic cells, and a mixture of red and brown reaction products was discerned in the cytoplasm of some involved cells (Table 5, Fig. 4).

Neuroendocrine markers in HCCs in relation to HCC type, grade, cytological type and presence of cirrhosis

The majority of HCCs showing reactivity for neuroendocrine markers were either of the trabecular or mixed trabecular-pseudoglandular types (WHO classification; 92.2%; Table 1). Most of the positive cases were of grades 2 and 3 (Edmondson-Steiner grading system; 88.6%), and 50% of the HCCs showing

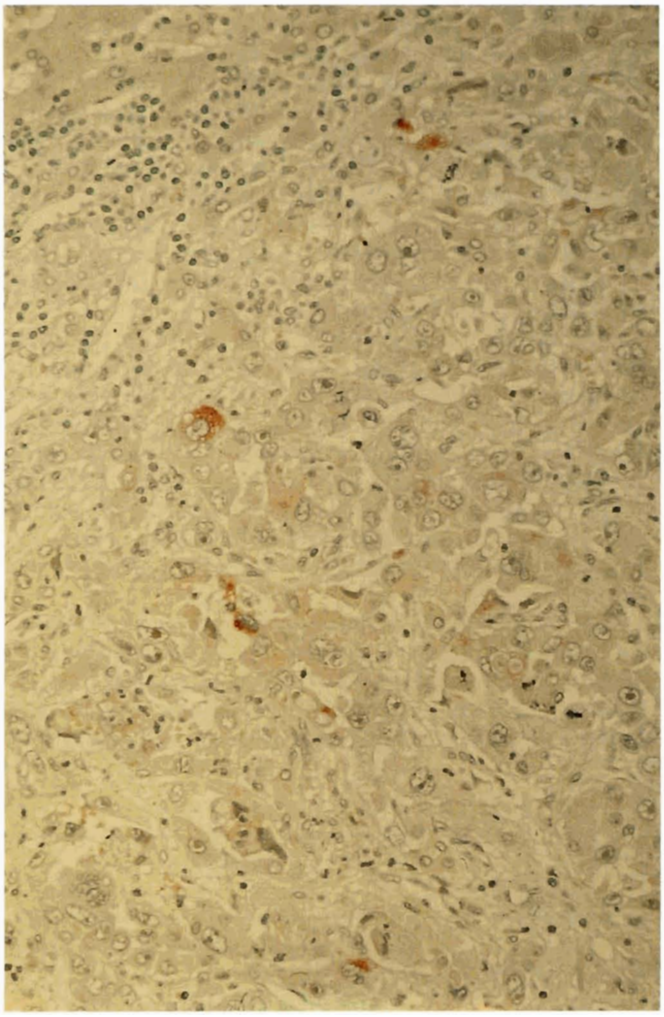

Fig. 1. Trabecular HCC, predominantly grade 3 tested with the anti-Leu 7 antibody (case 15). Granular red reaction product is located in the cytoplasm of few tumor cells. APAAP immunostain. x 250

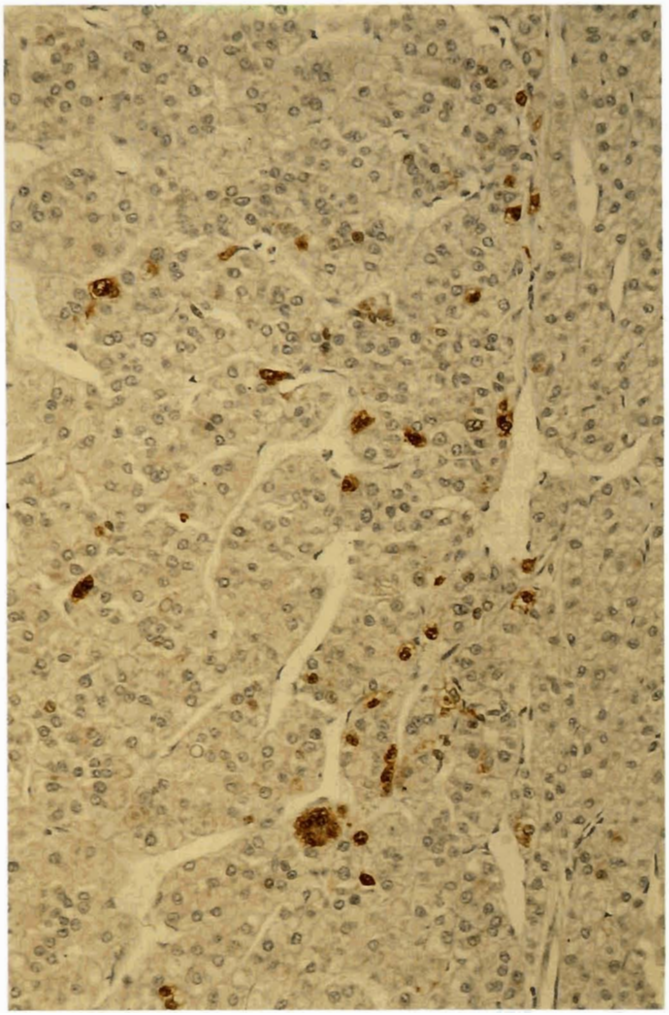

Fig. 2. Positive reaction for S-100 protein in a trabecular HCC, grade 2 (case 1). This HCC is bile-producing and contains clusters of Mallory bodies. S-100 protein is visualized both in the cytoplasm and in the nuclei. Peroxidase immunostain. x 250

clear-cut expression of one or more neuroendocrine markers obviously produced bile, even though most of these tumors were predominantly trabecular. 15.9% of the positive HCCs exhibited cells containing typical Mallory bodies.

In HCCs of a given type and grade, the largest cell population reactive for neuroendocrine markers showed a clearly granular, but not strongly eosinophilic cytoplasm; i.e. a neoplastic cell type morphologically most close to normal hepatocytes (85.1%; Table 5). Conversely, tumor components characterized by a predominance of strongly eosinophilic and large cells, large and granular cells with cytoplasmic eosinophilia (oxyphilic cells), giant cells and clear cells amounted to 10.6% of positivity only (Table 6).

Four HCCs (one Leu-7$^+$, two S-100$^+$, and one NSE$^+$) were associated with micronodular cirrhosis, and fibrosis of adjacent liver tissue was observed in one case (S-100$^+$; Table 6). Two HCCs were of the purely clear cell type; in one of these, cells reacted with NEU, while in the second there was no reactivity for neuroendocrine markers. In two cases with a large fraction of clear cells, this cell type was S-100$^+$ in one case and negative for any marker in the other. Of the two FL-HCCs analyzed, one contained S-100$^+$ cells and the other Leu-7$^+$ and VIP$^+$ cells.

Table 4. Neuroendocrine expression in 30/50 cases of HCCs.

CASE	LEU	S100	SYN	NSE	SOM	NEU	CG	VIP	M
1	±	++	-	-	-	-	-	-	2
2	-	-	-	-	-	+	-	-	
3	-	-	-	-	-	+	-	-	
4	-	-	-	-	+	-	-	-	
5	+	-	-	-	-	-	-	-	
6	-	-	±	-	-	-	-	-	
7	++	+	-	-	-	-	-	-	2
8	++	-	-	-	-	-	-	-	
12	++	-	-	-	-	-	-	-	
13	++	-	-	-	-	-	-	-	
14	+++	-	-	+	-	-	++	-	3
15	+++	++	-	-	-	-	-	-	2
17	-	-	±	-	-	+	-	-	2
19	-	++	-	-	-	-	-	-	
21	+++	-	±	±	-	-	-	-	3
22	-	-	±	++	+	-	-	-	3
25	-	++	-	±	-	-	-	-	2
28	-	++	-	-	-	-	-	-	
29	-	+++	-	-	-	-	-	-	
32	++	-	-	-	-	-	-	-	
34	±	±	-	-	-	-	-	-	2
35	-	-	-	-	-	-	++	-	
37	-	-	-	-	+	-	-	-	
42	-	++	-	-	-	-	-	-	
43	++	++	-	+	-	-	+	-	4
44	-	-	++	-	-	-	-	-	
45	-	++	-	-	+	-	-	-	2
46	-	++	-	-	-	-	-	-	
49	-	+++	-	-	-	-	-	-	
50	++	-	-	-	-	-	-	+	

All samples were negative for 5HT, MEL, PGP, PAP and BOM. The samples 9-11, 16, 18, 20, 23, 24, 26, 27, 30, 31, 33, 36, 38-41, 47 and 48 gave negative results for all markers. M, number of positive markers per given case.

Discussion

Sixty percent of hepatocellular carcinomas (HCCs) analyzed in the present study expressed reactivity to antisera directed against one or more «neuroendocrine» antigens, and multireactivity was noted in 24%. Positivity was most frequently encountered with Leu-7 and S100, whereas NSE, SYN, SOM, CG, NEU and VIP were seen in 10% or less of these tumors. Bireactivity, as visualized by double immunostaining, was found for the combinations Leu-7/S100, or NSE/SOM, respectively. In contrast, no reactivity was observed for HMG-45, 5-HT, PGP, PAP, and BOM. In all reactive HCCs, positive staining results were distinct, localized in the cytoplasm or nuclei of typical carcinoma cells, and clearly different from non-specific background staining.

In a previous study (Wang et al., 1991) it has been shown that 6/6 FL-HCCs showed positive immunostaining for PGP 9.5, three for NSE, three for MEL and one for S-100 protein; thus further illustrating the particular phenotype of this tumor, which has been shown by EM to contain neurosecretory granules (Payne et al., 1986). However, also 5/10 «classical» HCCs revealed positive immunostaining for PGP 9.5, three for VIP, three for MEL, and two for calcitonin (Wang et al., 1991).

The aim of the present study was threefold. Firstly, we were interested in determining the presence of «neuroendocrine» markers in a larger group of HCCs covering a wide spectrum of subtypes, grades and distinct cellular features, including HCCs containing

Table 5. Neuroendocrine immunohistochemistry with double staining.

PATIENTS	Abs	INTENSITY	PATTERN	POSITIVE STAINING		COMMENT
				CELL TYPE	IN SITU	
15	LEU	+++	G,D	Gr, Eo, Gi	Cytoplasm	APAAP and ABC reaction in same and different tumor cells
	S100	+++	D	Gr, Eo	Cytoplasm and nuclei	
22	NSE	++	D	Gr	Nuclei	APAAP and ABC reaction in same tumor cells
	SOM	+++	D	Gr	Cytoplasm and nuclei	

G, granular reaction product. D, diffuse reaction product. APAAP, alkaline phosphatase-anti-alkaline-phosphatase. ABC, avidin-biotin complex. Gr, granular cell. Eo, eosinophilic cell. Gi, giant cell.

clear, oxyphilic and giant cells; secondly, we extended the set of antibodies to detect a broader array of potential markers, covering both «pan-neuroendocrine» markers and several GIT peptides; thirdly, we employed methods under highly controlled conditions, including improved blocking procedures and clearly positive tissue controls processed in parallel. Special care was taken to apply a four-step blocking procedure to avoid non-specificity of reactions. Thus, it appears that about 50% of «classical» HCCs express reactivity for markers considered to be typical for cells of the neuroendocrine lineage; 14% of the HCCs reacted with antisera directed against GIT peptides. Double immunostaining revealed the presence of a general «neuroendocrine» marker (NSE) together with that of a specific peptide (somatostatin). Positivity of several markers was detected in both trabecular and pseudoglandular (acinar) components of HCCs, and half of the positive tumors were shown to produce bile.

These observations suggest that «neuroendocrine» and morphological differentiation in HCCs may go in line, and may even occur in situations where cells with a high degree of morphological polarity are formed. However, no particular grade predominated among the positive cases even though, as in other series, grades 2 and 3 were most frequently observed. There was, in contrast, a difference in immunoreactivity with regard to tumor cell types involved. The highest yield of positivity was obtained with cells morphologically most close to hepatocytes (85%), whereas positivity for cells clearly deviating from the hepatocyte phenotype (clear cells, oxyphilic cells, giant cells) was seen in about 10% only. Irrespective of the overall type and grade, it thus appears that the HCC cell type most likely to express reactivity for «neuroendocrine» markers is a hepatocyte-like differentiated cell with abundant, granular cytoplasm, frequently polarized and producing bile, and not a poorly

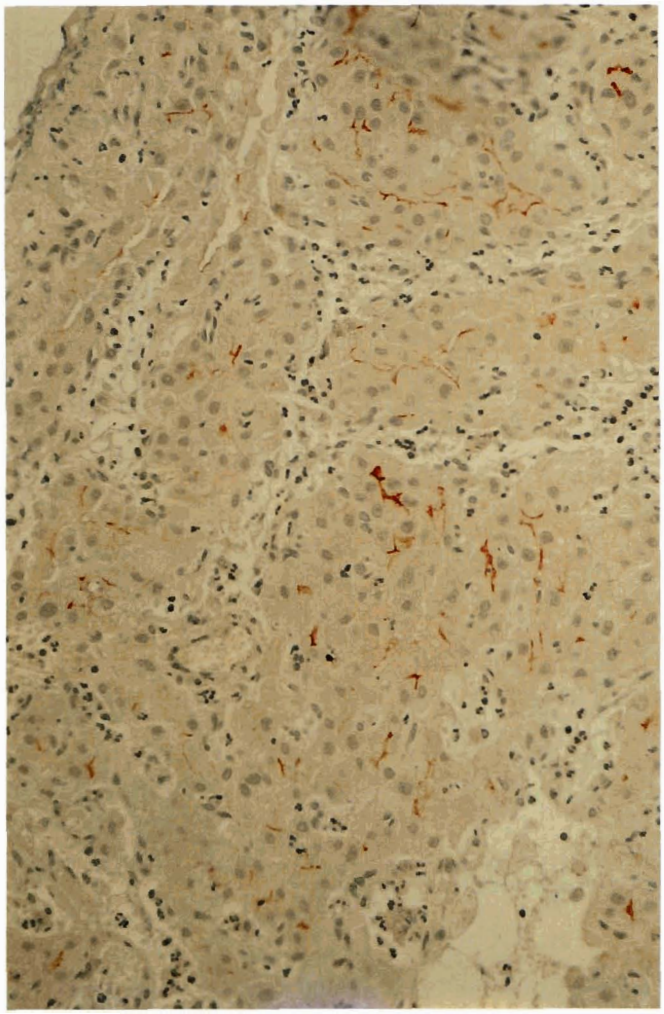

Fig. 3. Synaptophysin immunostaining of a trabecular HCC, grade 2, with bile formation (case 44), yielding a linear pattern along the surface of some tumor cells. This staining pattern may indicate apical membrane differentiation. APAAP immunostain. x 250

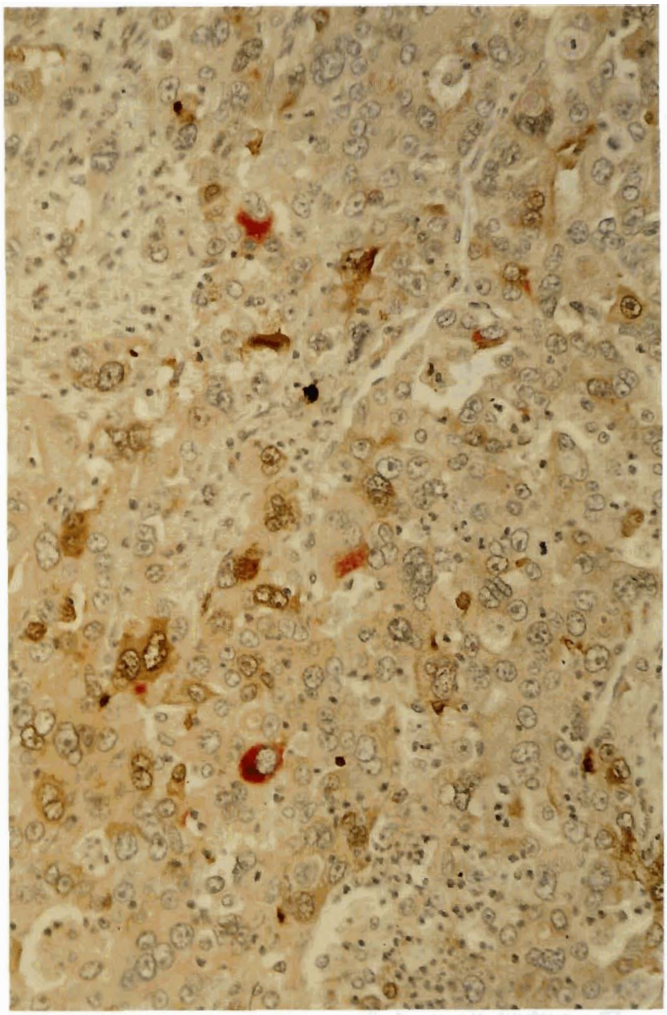

Fig. 4. Double immunostaining of an HCC (same case as in Fig. 1). The pair of antisera Leu-7/S-100 protein was used. Reaction products are brown for S-100 and red for Leu-7. x 350

Table 6. Immunohistochemical findings of neuroendocrine differentiation in 30/50 hepatic neoplasms: cell types involved.

			CELL TYPE						STAINING		
PATIENT	TUMOR	Abs	Gr	Eo	Cl	Ox	Fa	Gi	Intensity	Pattern	In Situ
1	HCC	LEU	Gr						±	G	(2) paranuclear
		S100	Gr						++	D	cytoplasm+nuclei
2	HCC	NEU	Gr						+	D	(1) cytoplasm
3	HCC	NEU			Cl				+	D	cytoplasm
4	HCC	SOM	Gr						+	D	cytoplasm
5	HCC	LEU	Gr					Gi	+	G,D	cytoplasm
6	HCC	SYN	Gr						±	D,L	(4) apical membrane
7	HCC	LEU	Gr	Eo					++	G,D,L	paranuclear
		S100	Gr						+	D	cytoplasm
8	HCC	LEU	Gr						++	G,D	paranuclear
12	HCC	LEU	Gr						++	G,D	paranuclear
13	HCC	LEU	Gr						++	G,D	paranuclear
14	HCC	LEU	Gr						+++	G	cytoplasm + canaliculi
		NSE	Gr						+	D	(3) nuclei
		CG	Gr						++	G	paranuclear
15	HCC	LEU	Gr	Eo		Ox		Gi	+++	G,D	cytoplasm
		S100	Gr	Eo					++	D	cytoplasm
17	HCC	SYN	Gr						±	L	membrane
		NEU	Gr						+	D	cytoplasm
19	HCC	S100	Gr	Eo					++	D	cytoplasm>nuclei
21	HCC	LEU	Gr						+++	G	cytoplasm
		SYN	Gr						±	L	membrane
		NSE	Gr						±	D	nuclei
22	HCC	SYN	Gr						±	L	membrane
		NSE	Gr						++	D	nuclei
		SOM	Gr						+	D	cytoplasm+nuclei
25	HCC	S100	Gr		Cl				++	D	nuclei>cytoplasm
		NSE	Gr						±	D	nuclei+cytoplasm
28	HCC	S100	Gr						++	G,D	nuclei+cytoplasm
29	HCC	S100	Gr						+++	D	nuclei
32	HCC	LEU	Gr						++	G	cytoplasm
34	HCC	LEU	Gr						±	D	cytoplasm
		S100	Gr						±	D	cytoplasm
35	HCC	CG	Gr						++	D	cytoplasm+paranuclear
37	HCC	SOM	Gr						+	D	cytoplasm
42	HCC	S100	Gr						++	G,D	nuclei+cytoplasm
43	HCC	LEU	Gr						++	D,L	apical membrane
		S100	Gr						++	D	nuclei>cytoplasm
		NSE	Gr						+	G	cytoplasm
		CG	Gr						+	D	cytoplasm
44	HCC	SYN	Gr						++	D	cytoplasm
45	Fa-HCC	S100				Ox		Gi	++	D	nuclei+cytoplasm
		SOM				Ox		Gi	+	D	nuclei
46	Fa-HCC	S100	Gr					Gi	++	D	cytoplasm
49	Fl-HCC	S100							+++	D	nuclei>cytoplasm
50	Fl-HCC	LEU							++	G,D	cytoplasm
		VIP							+	D	cytoplasm

Abbreviations: see Tables 1-3. G, granular reaction product. L, reaction product linear/apical. D, diffuse reaction product.

differentiated cell.

The pathogenesis of these phenomena has not been clarified, but several points may be raised. As previously suggested (Wang et al., 1991), «endocrine» characteristics may be expressed independently of the morphological phenotype: thus, «endocrine» differentiation may be far more widespread than originally appreciated, giving rise to diagnostic pitfalls. Therefore, the problem of endocrine cells in non-endocrine tumors has been recently addressed (Bosman,

1989; Wright, 1990). The phenomenon of «neuroendocrine» features in HCCs may depend either on a unique property of some HCC cells to differentiate along such an axis, or on the reliability of markers thought to be characteristic for «true» neuroendocrine cells.

Leu-7 (Abo and Balch, 1981), which was the most frequently encountered general marker observed in the present series, has formerly been shown to be expressed by cells of the central and peripheral nervous system, (apparently through its relation to myelin-associated glycoproteins), and has been found in neuroendocrine cells, in some carcinomas, including small and large cell carcinomas of the lung, and in mesotheliomas (Schuller-Petrovic et al., 1983; Nobile-Orazio et al., 1984; Cole et al., 1985; Perentes and Rubinstein, 1986; May and Perentes, 1987; Mayall et al., 1991). A similarly broad immunoreactivity has been demonstrated for another group of «neuroendocrine» markers, such as NSE (Dhillon et al., 1985; Haimoto et al., 1985; Cras et al., 1986; Pahlmann et al., 1986; Kayser et al., 1988; Mayall et al., 1991), CG (Helman et al., 1988), and SYN (Gould et al., 1986; Gould, 1987). In contrast to a previous study (Wang et al., 1991), expression of PGP 9.5 (Thompson et al., 1983) was not detected in the present series of HCCs, and mammalian bombesin (Sunday et al., 1988) could not be demonstrated. In addition to these so-called «pan-neuroendocrine» markers, some HCCs appear to produce substances known to be expressed by endocrine cell systems of the gastrointestinal tract (Gould, 1982; Chejfec et al., 1988). Neurotensin, which has previously been shown to be produced by fibrolamellar HCCs (Collier et al., 1984), was present in 6% of our cases, but restricted to granular or clear tumor cells. Among the members of GIT peptides, we noted positivity for somatostatin and vasoactive intestinal peptide (Buffa et al., 1977; Arimura et al., 1978; Mendelson, 1982), but not for pancreatic polypeptide. Thus, some HCCs may not only express general «neuroendocrine» markers, but appear to mimick some neuroendocrine characteristics of the gut and the pancreas (Debas and Mulvihill, 1991). This phenomenon is not related to preexisting hepatobiliary endocrine cells, because normal and neoplastic cells of this type have been described in the bile duct system, but not in the hepatic parenchyma (Dancygier et al., 1984; Kurumaya et al., 1989; Hsu et al., 1991; O'Hara et al., 1992). «Neuroendocrine» differentiation in HCCs may thus be related to the unique property of hepatocytes to change differentiation under certain circumstances, a phenomenon known as metaplasia. Cells of several tissues in adult organisms are capable of multi-potent development (Scarpelli, 1985), and this potential is a striking feature of liver tissue. Intestinal metaplasia of the liver has been induced in rats exposed to 2-acetylaminofluorene (Tatematsu et al., 1985), and intestinal-type cells are known to appear in experimentally produced hepatomas (Yoshida et al., 1978). «Neuroendocrine» differentiation of HCCs, and intestinal metaplasia of hepatic parenchyma are perhaps related since intestinal endocrine cells appear to originate from the same precursor as epithelial cells (Cheng and Leblond, 1974). On the other hand, extensive pancreatic metaplasia of the liver may either occur «spontaneously» in rats and humans (Rao et al., 1988; Wolf et al., 1990) or under experimental conditions in the rat liver (Kimbrough, 1973) and in liver tumors (Lee et al., 1989). Duct cells in regenerating hamster pancreas can transdifferentiate into hepatocytes (Makino et al., 1990). This unique differentiation plasticity in two organs originating from a common primordium may be transmitted to their neoplastic offspring, and may also relate to «neuroendocrine» differentiation in HCCs.

References

Abo T. and Balch C.M. (1981). A differentiation antigen of human NK and K cells identified by a monoclonal antibody (HNK-1). J. Immunol. 127, 1024-1029.

Arends J.W., Wiggers T., Verstijnen C. and Bosman F.T. (1986). The occurrence and clinicopathological significance of serotonin immunoreactive cells in large bowel carcinoma. J. Pathol. 149, 97-101.

Arimura A., Coy D.H., Chihara M., Fernández-Durango R., Samols E., Chihara K., Meyers C.A. and Schally A.V. (1978). Somatostatin. In: Gut hormones. Bloom S.R. (ed). Churchill Livingstone. Edinburgh. pp 437-445.

Barsky S.H., Linnoila I., Triche T. and Costa J. (1984). Hepatocellular carcinoma with carcinoid features. Human Pathol. 15, 892-894.

Bosman F.T. (1989). Endocrine cells in non-endocrine tumours. J. Pathol. 159, 181-182.

Buffa R., Capella C., Solcia E., Frigerio B. and Said S.I. (1977). Vasoactive intestinal peptide (VIP) cells in the pancreas and gastrointestinal mucosa: An immunohistochemical and ultrastructural study. Histochemistry 50, 217-227.

Chejfec G., Falkmer S. and Askenstein U. (1988). Neuroendocrine tumors of the gastrointestinal tract. Pathol. Res. Pract. 183, 143-154.

Cheng H. and Leblond C.P. (1974). Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. V. Unitarian theory of the origin of the four epithelial cell types. Am. J. Anat. 141, 537-562.

Cole S.P.C., Mirski S., McGarry R.C., Cheng R., Campling B.G. and Roder J.C. (1985). Differential expression of the Leu-7 antigen on human lung tumor cells. Cancer Res. 45, 4285-4290.

Collier N.R., Weinbren K., Bloom S.R., Lee Y.C., Hodgson H.J. and Blumgart L.H. (1984). Neurotensin secretion by fibrolamellar carcinoma of the liver. Lancet I, 538-540.

Cras P., Federsppiel S.S., Gheuens J., Martin J.J. and Lowenthal A. (1986). Demonstration of neuron-specific enolase in nonneuronal tumors using a specific monoclonal antibody. Ann. Neurol. 20, 106-107.

Dancygier H., Klein U., Leuschner U., Hubner K. and Classen M. (1984). Somatostain-containing cells in the extrahepatic biliary tract of humans. Gastroenterology 86, 892-896.

Debas H.T. and Mulvihill S.J. (1991). Neuroendocrine design of the gut. Am. J. Surg. 161, 243-249.

Dhillon A.P., Rode J., Dhillon D.P. Moss E., Thompson R.J., Spiro S.G. and Corrin B. (1985). Neural markers in carcinoma of the lung. Br. J.

Cancer 51, 645-652.

Edmondson H.A. and Steiner P.E. (1954). Primary carcinoma of the liver. A study of 100 cases among 48900 necropsies. Cancer 7, 462-503.

Gibson J.B. and Sobin L.H. (1978). Histological typing of tumours of the liver, biliary tract and pancreas. International Histological Classification of tumours, 20. World Health Organization, Geneva.

Gould V.E. (1982). Neuroendocrine tumors in «miscellaneous» primary sites: clinical, pathologic, and histogenetic implications. Progr. Surg. Pathol. IV. 181-198.

Gould V.E., Lee I. and Wiedenmann B. (1986). Synaptophysin: A novel marker for neurons, certain neuroendocrine cells, and their neoplasms. Hum. Pathol. 17, 979-983.

Gould V.E. (1987). A new and promising pan-NE marker. Arch. Pathol. Lab. Med. 111, 791-794.

Haimoto H., Takahashi Y. and Koshikawa T. (1985). Immunohistochemical localization of gamma-enolase in normal human tissues other than nervous and neuroendocrine tissues. Lab. Invest. 52, 257-263.

Helman L.J., Gazdar A.F., Park J.G., Cohen P.S., Cotelingam J.D. and Israel M.A. (1988). Chromogranin. A expression in normal and malignant human tissues. J. Clin. Invest. 82, 686-690.

Hsu W., Deziel D.J., Gould V.E., Warren W.H., Gooch G.T. and Staren E.D. (1991). Neuroendocrine differentiation and prognosis of extrahepatic biliary tract carcinomas. Surgery 110, 604-611.

Kayser K., Schmid W., Ebert W. and Wiedenmann B. (1988). Expression of neuroendocrine markers (neuronspecific enolase, synaptophisin and bombesin) in carcinoma of the lung. Pathol. Pract. 183, 4120417.

Kimbrough R.D. (1973). Pancreatic-type tissue in livers of rats fed polychlorinated biphenyls. J. Natl. Cancer Inst. 51, 677-681.

Kuramaya H., Ohta G. and Nakanuma Y. (1989). Endocrine cells in the intra-hepatic biliary tree in normal livers and hepatolithiasis. Arch. Pathol. Lab. Med. 113, 143-147.

Lee B.C., Hendricks J.D. and Bailey G.S. (1989). Metaplastic pancreatic cells in livers tumors induced by diethylnitrosamine. Exp. Mol. Pathol. 50, 104-113.

Makino T., Usuda N., Rao S., Reddy J.K. and Scarpelli D.G. (1990). Transdifferentiation of ductular cells into hepatocytes in regenerating hamster pancreas. Lab. Invest. 62, 552-561.

May E.E. and Perentes E. (1987). Anti-Leu 7 immunoreactivity with human tumours, its value in the diagnosis of prostatic adenocarcinoma. Histopathology 11, 295-304.

Mayall F.G., Jasani B. and Gibbs A.R. (1981). Immunohistochemical positivity for neuron-specific enolase and Leu-7 in malignant mesotheliomas. J. Pathol. 165, 325-326.

Mendelson G. (1982). Vasoactive intestinal polypeptide (VIP) and the spectrum of tumors producing the watery diarrhea syndroms. Progr. Surg. Pathol. IV, 199-216.

Nakashima T. and Kojiro M. (1987). Hepatocellular carcinoma. An atlas of its pathology. Springer Verlag. Tokyo, Berlin, Heidelberg, New York, London, Paris.

Nobile-Orazio E., Hays A.P., Latov N., Perman G., Golier J., Shy M.E. and Freddo L. (1984). Specificity of mouse and human monoclonal antibodies to myelin-associated glycoprotein. Neurology (Cleveland) 34, 1336-1342.

O'Hara B.J., McCuye P.A. and Miettinen M. (1992). Bile duct adenomas with endocrine component. Immunohistochemical study and comparison with conventional bile duct adenomas. Am. J. Surg. Pathol. 16, 21-25.

Pahlmann S., Escher T. and Nilsson K. (1986). Expression of gamma subunit of enolase, neuron-specific enolase, in human non-neuroendocrine tumors and derived cell lines. Lab. Invest. 54, 554-560.

Payne C.M., Nagle R.B., Paplanus S.H. and Graham A.R. (1986). Fibrolamellar carcinoma of liver: a primary malignant oncocytic carcinoid? Ultrastruct. Pathol. 10, 539-552.

Perentes E. and Rubinstein L.J. (1986). Immunohistochemical recognition of human neuroepithelial tumors by anti-Leu 7 (HNK-1) monoclonal antibody. Acta. Neuropathol. (Berl) 69, 227-233.

Rao M.S., Dwivedi R.S., Subbarao V., Usman M.I., Scarpelli D.G., Nemali M.R., Yeldandi A., Thangada S., Kumar S. and Reddy J.K. (1988). Almost total conversion of pancreas to liver in the adult rat: a reliable model to study transdifferentiation. Biochem. Biophys. Res. Commun. 156, 131-136.

Roskams T., van den Oord J.J., DeVos R. and Desmet V.J. (1990). Neuroendocrine features of reactive bile ductules in cholestatic liver disease. Am. J. Pathol. 137, 1019-1025.

Ruck P., Harms D. and Kaiserling E. (1990). Neuroendocrine differentiation in hepatoblastoma: an immunohistochemical investigation. Am. J. Surg. Pathol. 14, 847-855.

Sarsfield P. and Anthony P.P. (1990). Small cell undifferentiated (neuroendocrine) carcinoma of the colon. Histopathology 16, 357-363.

Scarpelli D.G. (1985). Multipotent developmental capacitiy of cells in the adult animal. Lab. Invest. 52, 331-333.

Schuller-Petrovic S., Gebhart W., Lassmann H., Rumpold H. and Kraft D. (1983). A shared anti-genic determinant between natural killer cells and nervous tissue. Nature 306, 179-181.

Sunday M.E., Kaplan L.M. and Motoyama E. (1988). Biology of disease. Gastrin-releasing peptide (mammalian bombesin) gene expression in health and disease. Lab. Invest. 59, 5-13.

Tatematsu M., Kaku T., Medline A. and Farber E. (1985). Intestinal metaplasia as a common option of oval cells in relation to cholangiofibrosis on liver of rats exposed to 2-acetylaminofluorene. Lab. Invest. 52, 354-362.

Thompson R.J., Doran J.F., Jackson P., Dhillon A.P. and Rode J. (1983). PGP 9.5-a new marker for vertebrate neurons and neuroendocrine cells. Brain Res. 278, 224-228.

Wang J.H., Dhillon A.P., Sankey E.A., Wightman A.K., Lewin J.F. and Scheuer P.J. (1991). Neuroendocrine differentiation in primary neoplasms of the liver. J. Pathol. 163, 61-67.

Wolf H.K., Burchette J.L., García J.A. and Michalopoulos G. (1990). Exocrine pancreatic tissue in human liver: a metaplastic process? Am. J. Surg. Pathol. 14, 590-595.

Wright N.A. (1990). Endocrine cells in non-endocrine tumours. J. Pathol. 161, 85-87.

Yoshida Y., Kaneko A., Chisaka O. and Onoe T. (1978). Appearance of intestinal type of tumor cells in hepatoma tissue induced by 3'-methyl-4-dimethylaminoazobenzene. Cancer Res. 38, 2753-2756.

Accepted April 19, 1993