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Abstract

Hematoxylin and eosin (H-E) stained liver sections of 47 autopsy cases of hepatic malignancies were examined. There were 43 cases of hepatocellular carcinoma (subtypes of 30 trabecular, 7 solid, 5 pseudoglandular, and one scirrhous carcinoma), 3 of cholangiocellular carcinoma and one of mixed carcinoma. After immunohistochemical staining, benign hepatocytes reacted positively with anti-epithelial membrane antigen (EMA). Hepatocellular carcinoma cells reacted more weakly than benign hepatocytes. It was noted that the microtubular structure, which could not be demonstrated even by alcian blue or cationic ferric hydroxide colloid stabilized with cacodylate (Fe-CaC), was clearly detected with anti-EMA. The EMA-positive microtubular structures may indicate terminal cholangiolar differentiation. Based on EMA, seven more cases formerly classified as hepatocellular carcinoma by H-E were reclassified as mixed carcinoma, totaling eight (17.0%). The histologic classification of "mixed carcinoma" has been 1.5 to 2.0% of primary liver cancers in Japan, but we suggest there may be more cases of "mixed carcinoma" identified in the future. In conclusion, we emphasize that EMA staining is useful for more accurate classification of hepatic tumors.

KEYWORDS: primary liver carcinoma, immunohistochemistry, histochemistory, epithelial membrane antigen

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Histochemical and Immunohistochemical Analyses of Primary Carcinoma of the Liver

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Hematoxylin and eosin (H-E) stained liver sections of 47 autopsy cases of hepatic malignancies were examined. There were 43 cases of hepatocellular carcinoma (subtypes of 30 trabecular, 7 solid, 5 pseudoglandular, and one scirrhous carcinoma), 3 of cholangiocellular carcinoma and one of mixed carcinoma. After immunohistochemical staining, benign hepatocytes reacted positively with anti-epithelial membrane antigen (EMA). Hepatocellular carcinoma cells reacted more weakly than benign hepatocytes. It was noted that the microtubular structure, which could not be demonstrated even by alcian blue or cationic ferric hydroxide colloid stabilized with cacodylate (Fe-CaC), was clearly detected with anti-EMA. The EMA-positive microtubular structures may indicate terminal cholangiolar differentiation. Based on EMA, seven more cases formerly classified as hepatocellular carcinoma by H-E were reclassified as mixed carcinoma, totaling eight (17.0 %). The histologic classification of "mixed carcinoma" has been 1.5 to 2.0 % of primary liver cancers in Japan, but we suggest there may be more cases of "mixed carcinoma" identified in the future. In conclusion, we emphasize that EMA staining is useful for more accurate classification of hepatic tumors.

Key words : **primary liver carcinoma, immunohistochemistry, histochemistory, epithelial membrane antigen**

According to the "Japanese Liver Cancer Study Group" (1), 2,286 of 4,658 cases with the primary malignant liver tumor autopsied were histologically composed of 2,038 hepatocellular carcinoma (HCC)(89.2 %), 146 cholangiocellular carcinoma (CCC)(6.4 %), 33 mixed carcinoma (1.4 %), 30 hepatoblastoma (1.3 %), 6 sarcoma (0.3 %), and 33 others (1.4 %). Liver cirrhosis or liver fibrosis coexisted in 88.3 % of HCC and in 21.2 % of CCC. In "The General Rules for Clinical and Pathological Study of Primary Liver Cancer (2nd Edition)"(2), primary liver cancers are classified histologically as HCC, CCC, mixed HCC and CCC, hepatoblastoma and undifferentiated carcinoma. HCC consists of four subtypes: trabecular, pseudoglandular, solid and scirrhous, and is subclassified as types I, II, III, and IV based on cell atypism (Edmondson (3)).

Histochemically, cancer cell differentiation toward the bile ducts has only been recognized with mucin production using alcian blue or mucicarmin staining (2). Therefore, pseudoglandular or microtubular structures without mucin are excluded from the mixed HCC and CCC

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group. Serologically, alpha fetoprotein (AFP)(4, 5) and CA 50 (6) have been used as tumor markers of HCC. Immunohistochemically, positivity for AFP, carcinoembryonic antigen (CEA), CA 19-9 or lysozyme (LZM) has been reported in HCC (7-11). Immunohistochemical response for anti-LZM is parallel with anti-AFP response, and LZM has recently been suggested as a useful marker for immunohistochemical diagnosis of HCC (12). In practice, AFP is an accepted marker for HCC, although it was also reported positive in benign hepatic cells (13). We report, herein, our results of a histochemical and immunohistochemical analyses in the subtypes of HCC and CCC.

Materials and Methods

Liver tissues were obtained from 47 autopsied cases of the primary liver cancers from the Okayama University Medical School Hospital, Kurashiki Central Hospital, and Himeji Red Cross Hospital from May 1987 to April 1989. The patients' ages ranged from 25 to 86 years with the peak incidence at 50 to 59, and with a 5:1 ratio of men to woman (Table 1). The liver samples were fixed with 10% buffered formalin and embedded in paraffin. Serial sections were cut to 4μm and stained with hematoxylin and eosin (H-E), Azan Mallory (AM), alcian blue and PAS (AB-PAS), and cationic ferric hydroxide colloid stabilized with cacodylate (Fe-CaC)(14). Immunohistochemical examinations were made with antibodies to AFP, epithelial membrane antigen (EMA)(15) and LZM, using the avidin-biotin-peroxidase complex (ABC) method of Hsu (16). Antibodies against AFP (polyclonal), EMA (monoclonal) and LZM (polyclonal) from Dako Japan Co. Ltd. were used with dilution of 1:300, 1:100 and 1:300, respectively.

Based on "The General Rules for Clinical and Pathological Study of Primary Liver Cancer (2nd Edition)" (2), primary liver cancers were classified as HCC with subclasses of trabecular, pseudoglandular, solid and scirrhous types, mixed carcinoma and CCC. The grading for atypism was made according to Edmondson (3) with H-E.

Table 1 Age distribution in the 47 autopsied cases

	<40	40~49	50~59	60~69	70~79	80~89
Male	1*	3	19(3)	7	7(1)	1(1)
Female	0	0	3	1	4(1)	1(1)
Total	1	3	22	8	11	2

*Patient was 25 years old.
( ): Without cirrhosis

Results

The histological classification of our cases is summarized in Fig. 1 and Table 2. There were 43 HCC (91.5%, Fig. 1A to 1F), 3 CCC (6.4%,

Table 2 Histologic classification of primary liver cancers

	Classified by H-E (%)	Edmondson's grading				Reclassified by H-E and EMA
		I	II	III	IV	
HCC						
Trabecular type	30(63.8)	2	19	9		28(59.6)
Pseudoglandular type	5(10.7)		5			1 (2.1)
Solid type	7(14.9)		4	2	1	6(12.8)
Scirrhous type	1 (2.1)		1			1 (2.1)
HCC and CCC	1 (2.1)					8(17.0)
CCC	3 (6.4)					3 (6.4)
Total	47	2	29	11	1	47

EMA: Immunohistochemistry by epitherial membrane antigen, H-E: Hematoxylin and eosin staining
HCC: Hepatocellular carcinoma
CCC: Cholangrocellular carcinoma

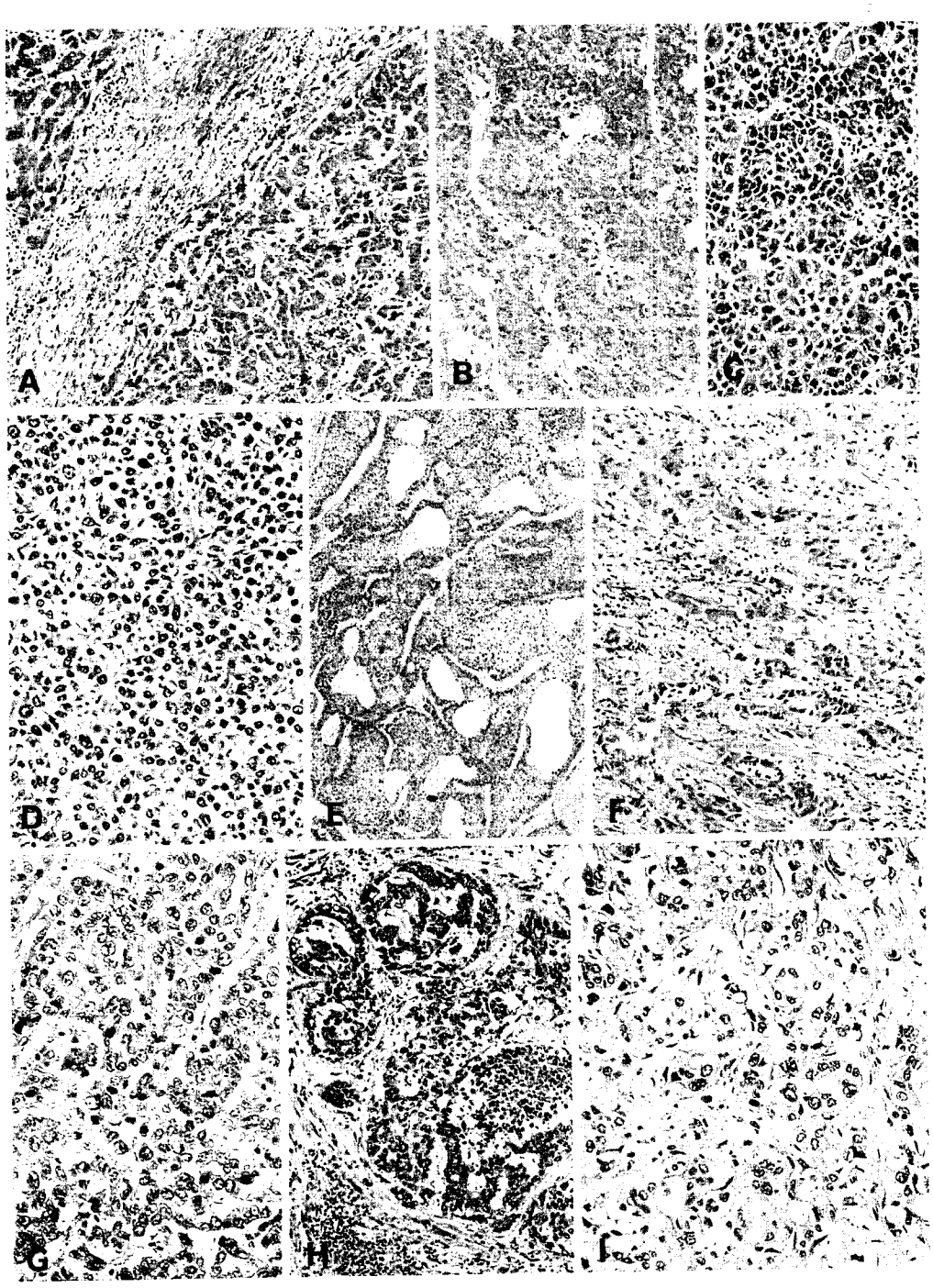

Fig. 1 Histological grading (Edmondson) and types of hepatoma with H-E stainings.
A: Grade I; B: Grade II; C: Grade III; D: Grade IV. A and B: trabecular type; C and D: solid type; E: pseudoglandular type; F: scirrhous type; G and H: mixed carcinoma; I: cholangiocecellular carcinoma. A–D and F-H: × 90; E: × 36; I: × 180.

Fig. 1I), and 1 mixed carcinoma (2.1 %, Fig. 1G, H) with H-E. Thirty-nine cases of HCC (90.7 %) and one case of CCC (33.3 %) were combined with liver cirrhosis. The 43 cases with HCC were subdivided into 30 trabecular (69.8 %, Fig. 1A, B), 7 solid (16.3 %, Fig. 1C, D), 5 pseudoglandular (11.6 %, Fig. 1E), and 1 scirrhous type (2.3 %, Fig. 1F). With immunohistochemical stainings, 2 of 30 trabecular, 4 of 5 pseudoglandular and one of 7 solid types were reclassified retrospectively as mixed carcinoma (Tables 2 and 3). The frequency of HCC atypism was; types I (Fig.1A) in 2 cases; II (Fig. 1B) in 29 cases; III (Fig. 1 C) in 11 cases; and IV (Fig. 1D) in 1 case.

The correlation of histological classification with H-E and histochemical and immunohistochemical reactivity in tumorous and non-tumorous foci is summarized in Tables 3 and 4. When stained with AB-PAS, the amounts of intracytoplasmic glycogen granules in tumor and non-tumor cells in HCC varied in every type of carcinoma (Table 3). In mixed carcinoma and CCC, glycogen granules were rarely encountered. Glandular structures were AB-positive, but pseudoglandular structures were AB-negative. Fine-granular and diffuse staining was observed in the cytoplasm of hepatic cells in the cytoplasm of hepatic cells in the non-tumorous and tumorous areas after treatment with anti-EMA. Hepatocellular carcinoma cells reacted more weakly than benign hepatocytes (Fig. 2C, F). The stability of cholangiolar cells was quite different from that of hepatic cells, and EMA was strongly positive along the luminal surface of the cholangiolar ducts. In the hepatocellular carcinoma group, four cases of pseudoglandular type, six cases of trabecular type, and one case of solid type were strongly positive for EMA, among which seven cases showed EMA-positive microtubular structures similar to the staining pattern of chorangiolar ducts and were reclassified into mixed carcinoma on the basis of EMA stainability (Fig. 3H, Table 3). In CCC, duct structures were

Table 3 Histologic classification, and histochemical and immunohistochemical response

Reagent	Staining response	Histologic classification with H-E: Trabecular (30 cases) T	Trabecular (30 cases) Non-T	Pseudogland. (5 cases) T	Pseudogland. (5 cases) Non-T	Solid (7cases) T	Solid (7cases) Non-T	Scirrhous (1 case) T	Scirrhous (1 case) Non-T	Mixed (1 case) T	Mixed (1 case) Non-T	CCC (3 cases) T	CCC (3 cases) Non-T	Total (47 cases) T	Total (47 cases) Non-T
PAS	−	19	14	2	3	2	3	1	1	1	0	1	3	26	24
	±	3	3	2	0	2	0	0	0	0	0	1	0	8	3
	+	4	5	0	0	1	0	0	0	0	1	1	0	6	6
	++	3	5	1	1	2	2	0	0	0	0	0	0	6	8
	+++	1	3	0	1	0	2	0	0	0	0	0	0	1	6
Anti-EMA	−	10	0	1	0	4	1	0	0	0	0	0	0	15	1
	±	8	0	0	1	0	0	0	1	0	0	1	1	9	3
	+	6	6	0	2	2	0	0	0	0	1	0	1	8	10
	++	3	12	1*	1	0	4	0	0	0	0	1	1	5	18
	+++	3(2)*	12	3*	1	1*	2	1	0	1	0	1	0	10	15
Anti-AFP	−	8	2	1	0	2	0	0	0	0	0	1	1	12	3
	±	5	5	0	1	2	2	1	1	0	0	2	1	10	10
	+	9	15	0	2	2	3	0	0	1	1	0	1	12	22
	++	7	6	2	1	1	1	0	0	0	0	0	0	10	8
	+++	1	2	2	1	0	1	0	0	0	0	0	0	3	4

*Cases with microtubular structures, strongly positive to EMA and reclassified as mixed carcinoma.
Mixed: Mixed carcinoma; CCC: cholangio cellular carcinoma; T: tumor cells; non-T: non-tumor cells.
−: negative; ±: weakly positive; +: positive; ++: strongly positive; +++: very strongly positive.

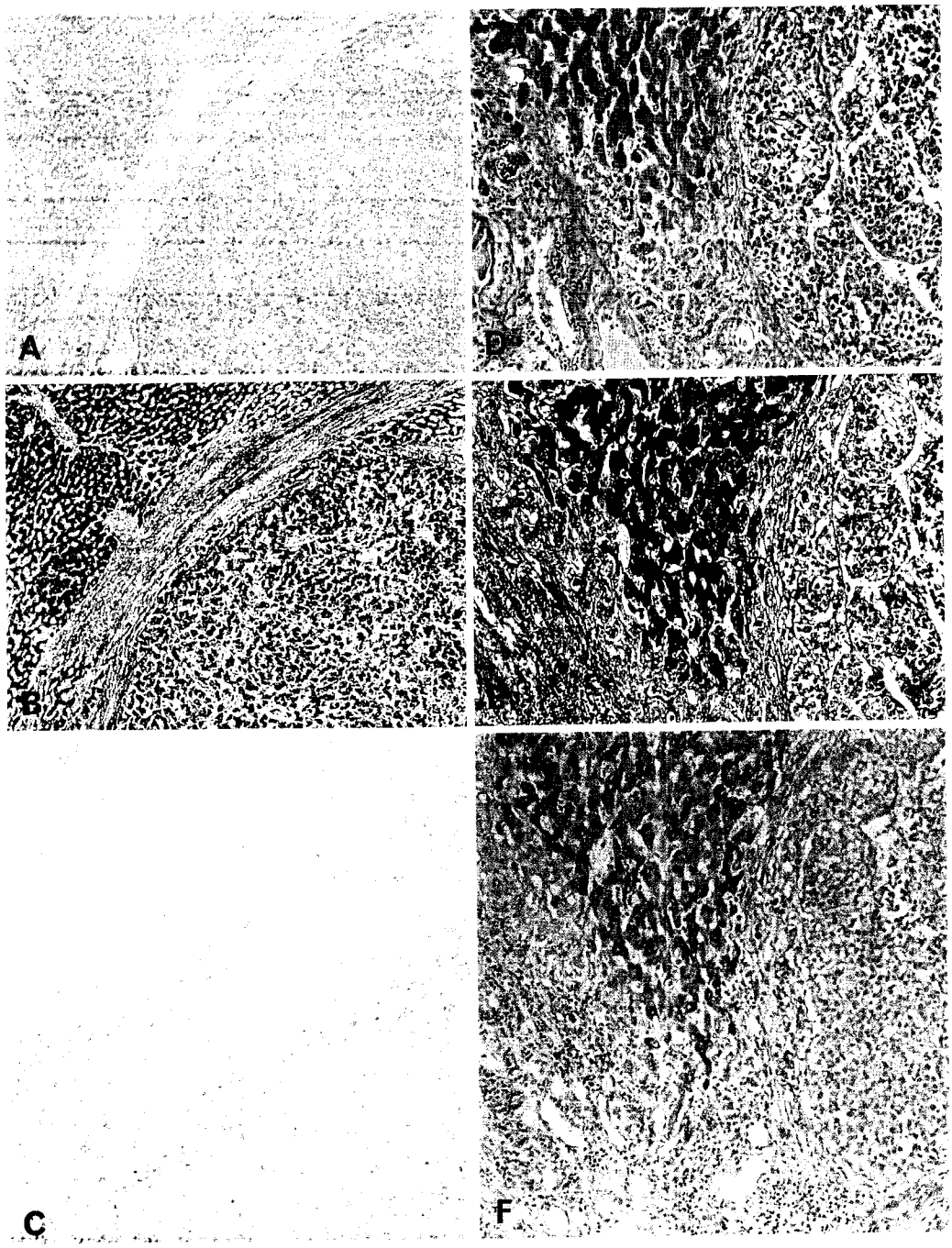

Fig. 2 Differences in the histochemical and immunohistochemical stainings of hepatoma and benign hepatic cells.
A, B, and C (Edmondson I) and D, E, and F (Edmondson IV) in the same fields of serial sections. AM responses of hepatoma are reduced considerably in Edmondson I (B), and obviously in Edmondson IV (E). A and D: H-E; B and F: AM; C and F: anti-EMA. A and B: ×36; C–F: ×90.

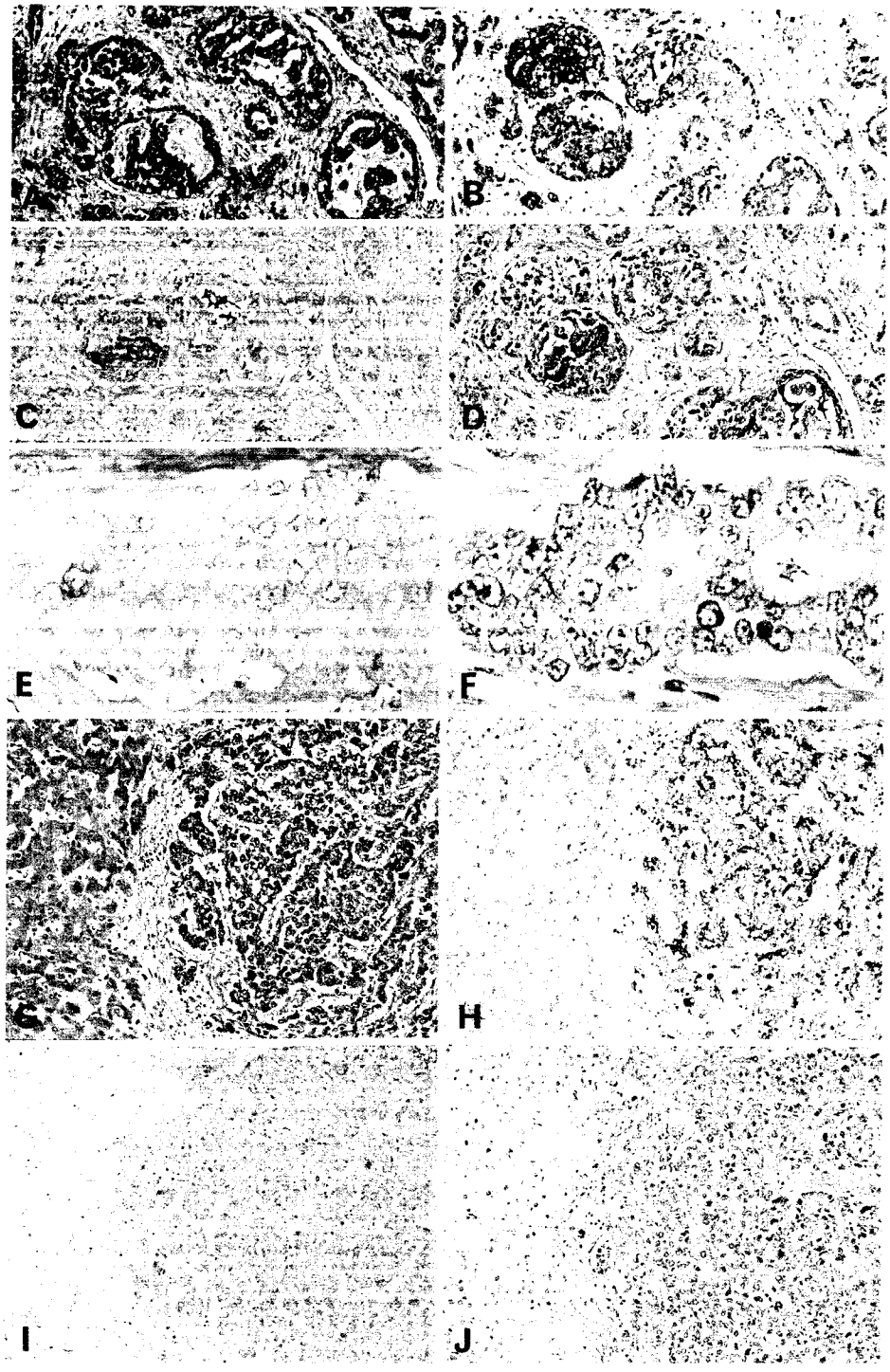

Fig. 3 Identification of duct or ductule structures with histological and immunohistochemical staining.
A–D, E and F, and G–J: in the same fields on serial sections. A and G: H-E; B and H: EMA; C, E, and I : Fe-CaC; D, F, and J: AB-PAS. A–D and G–J: ×90; E and F: ×360.

Table 4 Edmondson's grading with H-E, and histochemical and immunohistochemical response in HCC.

Reagent	Staining response	Histlogic classification with H-E							
		Edmondson I (2 cases)		Edmondson II (29 cases)		Edmondson III (11 cases)		Edmondson IV (1 cases)	
		T	Non-T	T	Non-T	T	Non-T	T	Non-T
PAS	−	1	1	18	17	6	4	0	0
	±	0	0	5	1	1	1	0	0
	+	1	0	3	2	1	2	0	0
	++	0	1	3	6	2	1	1	0
	+++	0	0	0	3	1	3	0	1
Anti-EMA	−	0	0	10	3	5	1	1	0
	±	1	0	4	1	1	1	0	0
	+	1	0	7	5	3	1	0	0
	++	0	2	2	10	0	4	0	1
	+++	0	0	6	10	2	4	0	0
Anti-AFP	−	1	0	6	5	3	1	1	0
	±	0	1	6	3	3	3	0	1
	+	1	1	9	12	2	4	0	0
	++	0	0	7	7	3	2	0	0
	+++	0	0	1	2	0	1	0	0

T: tumor cells; non-T: non-tumor cells.
− ~ +++: the same as in Table 3

strongly positive to anti-EMA. Twenty-nine of 47 primary liver cancers were positive with anti-AFP in non-tumor cells: positive both in tumor and non-tumor cells in 10 cases, and only in non-tumor cells in 19 cases. AFP was positive in 23 of 43 with HCC (types I in 1/2 cases; II in 17/29; III in 5/11; and IV in 0/1), positive in all mixed carcinoma cases (7/7), and positive in benign cells of only one CCC (1/3). With anti-LZM, LZM was strongly positive in 2 of 43 HCC, although only one of these two was parallel with AFP. Mixed carcinoma and CCC were totally negative to anti-LZM. With AM, the tumor was more weakly stained in red color than non-tumorous areas (Fig. 2B, E). This tendency was more remarkable in mixed carcinoma and CCC.

Figs. 3A to 3D are serial sections of one CCC case to demonstrate the histochemical and immunohistochemical characteristics of the tubular structures. In this case, tubular structures were stained positive not only with anti-EMA (Fig. 3B), but also with Fe-CaC (Fig. 3C). and AB (Fig. 3D). Differences in staining between Fe-CaC and AB was not seen in the larger tubular structures. Fe-CaC was positive on the luminal surface or the cytoplasm of the cholangioles, but AB was negative in the microtubular structure. Pseudoglandular structures were negatively stained with AB and were occasionaly positive with Fe-CaC (Figs. 3E, F).

Figs. 3G to 3J are serial sections of a trabecular type of HCC. EMA was clearly positive on the surface of microtubular structure and cytoplasm of tumor cells (Fig. 3H). Both Fe-CaC (Fig. 3I) and AB (Fig. 3J) were negative in such an extremely small microtubular structure. But, microtubular structures that were positively stained only with Fe-CaC were occasionally detected in trabecular pattern of HCC.

Table 5 summarizes the relationship between AFP level in serum and immunohistochemical responses of AFP. The cases with a high level of serum AFP tended to be positive to anti-AFP,

Table 5 Relationship between types of primary liver cancers and alpha fetoprotein (AFP)

Patient no.	Histologic pattern	Edmodson's grading	AFP		Serum AFP (mg/ml)	Post mortem period(h)
			Tumor	Non-tumor		
1	Trabecular	I	+	±	4	3.5
2	Trabecular	I	−	+	2,010	6.7
3	Trabecular	II	+	++	18	2.0
4	Trabecular	II	−	−	19	9.0
5	Trabecular	II	+	+	4,260	9.2
6	Trabecular	II	++	++	9,635	2.0
7	Trabecular	II	+++	++	37,500	2.0
8	Trabecular	II	++	++	64,600	2.0
9	Trabecular	II	++	++	70,000	2.0
10	Pseudogland.	II	±	+	5	9.0
11	Solid	II	−	±	3	2.0
12	Solid	II	+	+++	700	2.0
13	Solid	II	++	++	3,000	8.5
14	Trabecular	III	−	±	244	9.5
15	Trabecular	III	−	±	8,200	7.5
16	Trabecular	III	−	±	8,200	7.5
17	Trabecular	III	++	+	17,407	1.5
18	Solid	IV	−	±	779	5.0
19	Mixed		−	+	334	2.0

− ~ +++: the same as in Table 3

and also tended to be positive in non-tumor foci.

Discussion

According to the "Japanese Liver Cancer Study Group" (1), mixed carcinoma was found only in 1.4 % of primary liver cancer patients. In our samples, using H-E, mixed carcinoma was present in 2.1 % of cases. But with Fe-CaC and anti-EMA stainings, 8 of 47 cases (17.0 %) were mixed carcinoma (Table 2). In 43 HCC, which were classified with H-E, seven cases were finally determined as mixed carcinoma with EMA and Fe-CaC (Table 2). Microtubular structures were not detected even in AB stainings but were sometimes stained with Fe-CaC (Figs. 3E, F). However, it is very important to note that the microtubular structures, even those negative with Fe-CaC (Fig. 3 I), were positive with anti-EMA (Fig. 3H), indicating that a careful examination to find microtubular structures is required. These anti-EMA-positive microtubular structures may indicate a terminal cholangiolar cell differentiation associated with mixed carcinoma. Thus, histochemical and immunochemical examinations are necessary to determine more precise classification of primary liver cancers, especially of mixed carcinoma.

EMA was believed to be stained negatively in both benign and malignant hepatocytes (15). However, they stained clearly positive with anti-EMA in our study (Fig. 2C, F, 3H and Tables 3, 4). The above facts were very interesting (Fig. 2C, F), although the biological significance of EMA is still unknown. Based on our results, anti-EMA together with Edmondson's typing would constitute more accurate methods for histological typing of primary liver cancers. Differences in staining between tumor and non-tumor cells in AM (Fig. 2B, E) were obvious, but the biological significance was quite unknown.

Allen *et al.* (17) proposed to classify mixed carcinomas of HCC and CCC into three types

according to development: (a) separated neoplastic masses may be comprised entirely of a liver cell type and of a bile duct type; (b) contiguous masses with different characteristics may intermingle as they grow; and (c) individual masses may display both features and be so intimately associated that they can be interpreted only as arising from the same site. Experimentally, hepatocytes and cholangiocytes can differentiate from terminal cholangiolar cells, which are considered to be stem cells with a biphasic capacity of differentiation to hepatic or cholangiolar cells (18). Allen's proposal in (c) appears to be the most acceptable, especially in a tumor mass consisting of cholangiolar and hepatic cells as in HCC and mixed carcinoma. Based on our observations, the cholangiolar cells forming relatively larger ducts (Fig. 3A) were positively stained with anti-EMA (Fig. 3B), Fe-CaC (Fig. 3C), and AB (Fig. 3D), but some cells differentiating towards the terminal cholangioles failed to respond to AB (Fig. 3F) and Fe-CaC (Fig. 3I) in that order. We emphasize again that anti-EMA-positive cells with microtubular structures, which were negative to AB-PAS and Fe-CaC, are considered to be stem cells with a biphasic differentiation to hepatic and cholangiolar cells.

The relationship between serum AFP level and immunohistochemical reactivity to anti-AFP (Table 5) suggests that AFP in serum was produced by tumor cells. In these cases benign hepatic cells were also positively stained with anti-AFP, indicating that both tumor and non-tumor cells participate to elevate the serum AFP level. On the other hand, AFP was negative in tumor cells but positive in non-tumor cells in some cases. The non-tumor cells with AFP production in these cases might contribute to an increased serum AFP level.

In conclusion, we emphasize that anti-EMA staining is necessary for the definite diagnosis of mixed carcinoma.

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