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Hepatocyte Expressions in Hepatocellular Carcinomas, Gastrointestinal Neoplasms, and Non-neoplastic Gastrointestinal Mucosa: its Role as a Diagnostic Marker

We performed immunohistochemical staining against Hepatocyte (Hep) and CD10 antibodies in 75 hepatocellular carcinoma (HCC), 50 cholangiocarcinomas, 49 colorectal adenocarcinomas, and 308 gastric adenocarcinomas by tissue array method. We also evaluated the various non-neoplastic adult tissues and fetal digestive organs. Hep was expressed in 80% of HCCs, and HCCs without Hep expression were more likely to have a higher Edmondson & Steiner grade than HCCs with Hep expression (p=0.004). In non-HCCs, 16% of cholangiocarcinomas, 8.2% of colorectal carcinomas, and 44.2% of gastric carcinomas expressed Hep. Gastric carcinomas with Hep expression were significantly associated with early gastric carcinomas (p<0.001). In non-neoplastic tissues, Hep was found expressed in normal hepatocytes, small intestinal mucosa, and intestinal metaplasia of the stomach. Fetal hepatocytes expressed Hep after 19 weeks of gestation. CD10 was detected in 46.7% (35/75) of HCCs, and canalicular staining pattern was predominant in HCCs. In conclusion, the expression of Hep and CD10 may help to distinguish HCCs from non-HCCs.

Key Words : *Carcinoma, Hepatocellular; Hepatocytes; Hepatocyte-Paraffin 1; CD10 Antigen; Immunohistochemistry*

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INTRODUCTION

The liver is a common site of metastasis from many primary sites, particularly the lung, breast, and the gastrointestinal tract, and hepatocellular carcinoma (HCC) may show a variety of histologic patterns (1). The pathologic diagnosis of HCC may be difficult using routine histologic stains, particularly when judgement must be rendered based on a small biopsy specimen. Immunohistochemistry has been applied extensively to hepatic neoplasms for the differential diagnosis of HCC, cholangiocarcinoma, and metastatic carcinoma. The marker most commonly used is alpha-fetoprotein (AFP) (2), but unfortunately, its sensitivity is low in tumor tissue. Only 25-45% of HCC cases are positive for AFP by immunohistochemistry, and even then, its expression is often patchy and weak (1, 3, 4). Other markers, including polyclonal carcinoembryonic antigen (pCEA), ferritin, albumin, fibrinogen, and alpha-1-antitrypsin, are also used (1, 5, 6, 8).

It has been reported that normal adult liver cells contain cytokeratin (CK) 8 and CK18, whereas bile duct epithelial cells contain CK7, 8, 18, and 19, and metastatic carcinomas express sets of CKs that are derived from their primary sites of origin. Therefore, an immunostaining panel of CK7, CK19, and CK20 is helpful for the differential diagnosis of HCC; however, these CK expression sets often overlap. Moreover, it has been found that the expressions of CKs may change when malignancy develops, and additionally, 5-20% of cases of HCCs cannot be diagnosed by CK immunostaining (9-12).

In 1993, Wennerberg et al. reported the development of a new monoclonal antibody named hepatocyte paraffin 1 (Hep), which reacts with paraffin-embedded normal and neoplastic liver tissue (13). A number of subsequent studies then confirmed Hep as a relatively specific marker for HCC, though some gastric adenocarcinomas, neuroendocrine carcinomas, and cholangiocarcinomas were also found to be positive for Hep (14-16). Because previous studies have not examined large numbers of cases of each tumor, it is uncertain how Hep expression correlates to the histologic patterns of HCC or non-HCC. In addition, Hep expressions in normal adult or fetal tissues other than liver have not been extensively studied. In this study, we evaluated the expression of Hep in 75 HCCs, and in 407 non-HCCs. Because Hep was expressed in the intestinal metaplasia of the stomach and mucosal columnar cells of small intestine, but not in colorectal mucosa, by the test procedure using human control slide for immunohistochemistry (Superbiochips Laboratories, Seoul, Korea), we evaluated Hep expressions in colonic type and small intestinal type of intestinal metaplasia. For analysis of the correlation between the degree of cell differentiation and Hep-positivity, the digestive organs of the fetus of various gestational ages were included in this study.

MATERIALS AND METHODS

Cases

Seventy-five cases of primary HCCs from the files of the Department of Pathology, Seoul National University Hospital, Seoul, Korea were examined in this study. To evaluate immunoreactivity in non-HCCs, we studied 50 primary cholangiocarcinomas, 49 primary colorectal adenocarcinomas, and 308 primary gastric adenocarcinomas. All of these tumors had been surgically resected, fixed in 10% neutral formalin and embedded in paraffin. Glass slides were reviewed for histologic classification and differentiation using the Edmondson & Steiner nuclear grading system (17) and histological pattern categorization (18) for HCCs, and the WHO classification (19, 20) for non-HCCs.

Non-neoplastic adult tissues were obtained from; 15 cases of intestinal metaplasia of the stomach (resected specimens from gastric adenocarcinomas), 5 duodenum samples (resected specimens from gastric adenocarcinomas), and 4 colorectum samples (resected specimens from colorectal adenocarcinomas). In addition, digestive organs, including the tongue, liver, esophagus, stomach, small intestine, and colorectum, of variable gestational ages (i.e., of 15, 16, 19-22, 24, 28, and 30 weeks of gestation) were selected.

Tissue-Array Methods

All of the above tissues, except 17 cases of HCCs which were stained using whole section slides, were evaluated using the tissue-array methods. Core tissue biopsies (2 mm in diameter of tumor and fetal tissue, and 4 mm in diameter for non-neoplastic tissues) were taken from individual paraffin-embedded tissues (donor blocks) and were arranged in a new recipient paraffin block (tissue array block) using a trephine apparatus (Superbiochips Laboratories, Seoul, Korea) (21). Each tissue array block contained up to sixty cases, and the study involved a total of 11 blocks of tissue-array blocks. An adequate case was defined as a tumor occupying more than 10% of the core area.

Immunohistochemistry

Four-μm thick sections were cut from whole tissue blocks or tissue-array blocks, deparaffinized and dehydrated. Immunohistochemical stainings against Hep (1:100, DAKO Corporation, Glostrup, Denmark) were performed using a streptavidin peroxidase based procedure after an antigen retrieval using microwaves. The immunolabeling pattern of each case was scored as positive (strong labeling), weakly positive (faint staining), or negative (absence of staining), and the extent of immunolabeling were scored as diffusely positive ($\geq$90%), focally positive (5-89%), or negative (<5%). For the statistical analysis, the results of immunostaining were considered positive if 5% or more of the cells were positive (strong labeling).

We were able to perform the test procedure using human control slide for immunohistochemistry (Superbiochips Laboratories). Hep was found to be expressed only in hepatocytes, HCCs, the intestinal metaplasia of the stomach, and in the mucosal columnar cells of small intestine. Other neoplastic or non-neoplastic tissues, including the skin, breast, pancreas, lymph node, stomach, lung, salivary gland, bile duct, spleen, gallbladder, colorectum, kidney, prostate, seminal vesicle, testis, uterus, placenta, adrenal, thyroid, brain and their various tumors, were almost all negative for Hep reactivity.

In addition, immunohistochemical stainings against CD10 (1:80, Novocastra Laboratories Ltd., Newcastle upon Tyne, U.K.) were performed in HCCs and non-HCCs, as described above. The results of immunostaining were considered to be positive, if $\geq$5% of the cells were positive and they could be categorized as canalicular, non-canalicular (membranous) or cytoplasmic in pattern (22).

HID-AB2.5 Staining

High iron diamine and alcian blue (pH 2.5) (HID-AB2.5) staining was used, to stain sulphated (brown) and acidic non-sulphated (blue) mucosubstances simultaneously, in 15 cases of intestinal metaplasia of the stomach. Intestinal metaplasia was classified, as previously described (23), as follows: type I, mature absorptive cells and goblet cells, the latter secreting sialomucins; type II, few or absent absorptive cells, presence of columnar "intermediate" cells in various stages of differentiation secreting neutral and acid sialomucins and goblet cells secreting sialomucins or, occasionally, sulfomucins, or both; and type III, columnar "intermediate" cells secreting predominantly sulfomucins and goblet cells secreting sialomucins or sulfomucins, or both.

Statistical Analyses

Either the chi-square test or Fisher's exact test (2-sided) was used to determine the correlation between the histologic grade of HCCs or non-HCCs and Hep expression status. Results were considered as statistically significant at p values of less than <0.05. All statistical analyses were conducted using the SPSS 11.0 (SPSS, Chicago, IL).

RESULTS

Hepatocyte Expressions in HCCs and Other Carcinomas

Of the 75 cases of HCCs, 60 were positive for Hep (80%) (Table 1). The Hep staining pattern is distinctly granular and diffusely cytoplasmic (Fig. 1). Hep expression was found in 16% (8/50) of cholangiocarcinomas, in 8.2% (4/49) of colo-

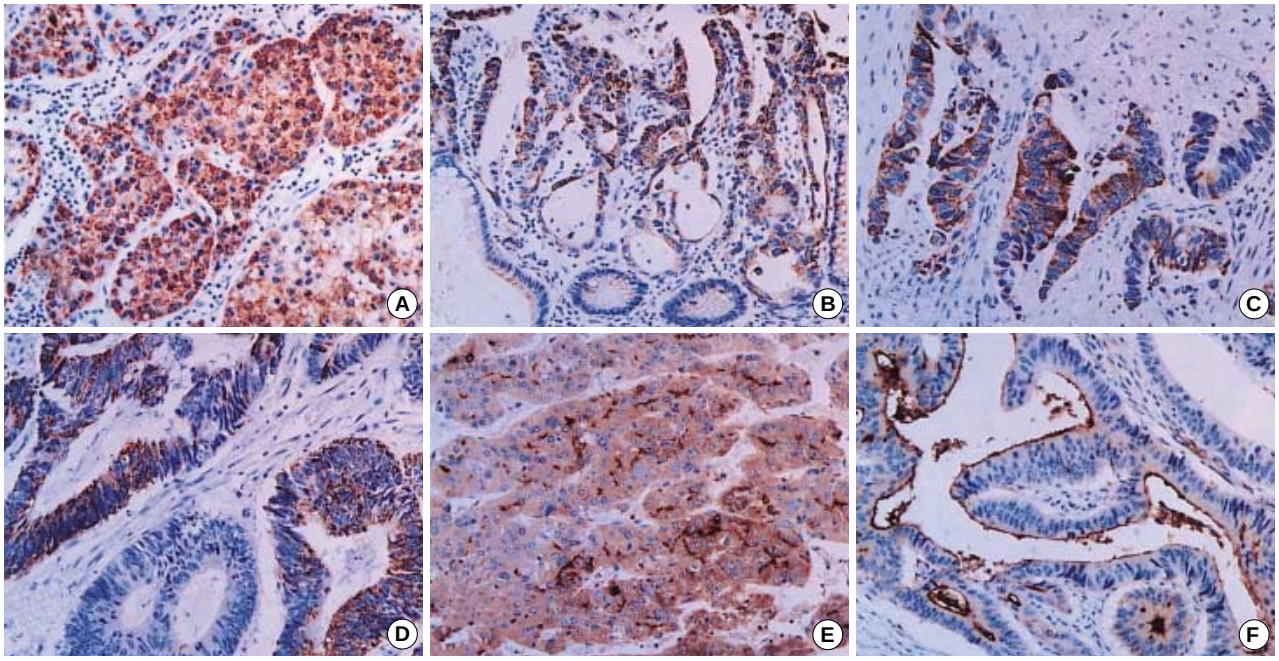

Fig. 1. The immunostaining of Hep was found in HCCs (A, ×200), early gastric carcinoma (B, ×200), cholangiocarcinoma (C, ×200), and colonic adenocarcinoma (D, ×200). A canalicular staining pattern of CD10 is only found in HCCs (E, ×200) and a non-canalicular membranous staining pattern of CD10 is seen in colonic adenocarcinoma (F, ×200).

rectal adenocarcinomas, and in 44.2% (136/308) of gastric adenocarcinomas. Hep staining in some of the HCCs and non-HCCs was restricted to focal areas, and the density of such staining varied from cell to cell. A diffuse staining pattern was more frequently seen in Hep-positive HCCs (36/60, 60%) than in Hep-positive non-HCCs (40/148, 27%), of which difference was statistically significant (p<0.001). In this series, Hep staining had a sensitivity of 80.0% and specificity of 63.6% in detecting HCCs.

Table 2 shows the correlation between Hep expression and the histologic grade of HCCs or non-HCCs. HCCs without Hep expression were more likely to have higher Edmondson & Steiner grades than HCCs with Hep expression (p=0.004). All 4 cases of HCCs with compact or scirrhous pattern were negative for Hep. In non-HCCs, Hep expressions was more frequently found in well-differentiated adenocarcinomas rather than in poorly differentiated type, and this was statistically significant in cholangiocarcinomas (p=0.035) and gastric adenocarcinomas (p<0.001). Signet ring cell carcinomas and mucinous carcinomas of the stomach frequently showed Hep expression, and Hep expression was more frequently seen in early gastric carcinomas than in advanced carcinomas (p< 0.001). Histologic classification of colorectal adenocarcinoma was not associated with Hep expression status (p=0.155).

Table 1. Hepatocyte expressions in HCCs and non-HCCs

	*HCCs	Non-HCCs		
		Cholangio-carcinoma	Colorectal †adenoca.	Gastric adenoca.
Positive				
Diffuse (≥90%)	36	3	1	36
Focal (5-89%)	24	5	3	100
Total	60 (80%)	8 (16%)	4 (8.2%)	136 (44.2%)
Negative (<5%)	15 (20%)	42 (84%)	45 (91.8%)	172 (54.8%)
Total cases	75 (100%)	50 (100%)	49 (100%)	308 (100%)

*HCC, hepatocellular carcioma; †adenoca., adenocarcinoma.

Hepatocyte Expressions in Non-Neoplastic Human Tissues

As a result of a human control slide in the test procedure, normal hepatocytes, mucosal columnar cells of the small intestine and intestinal metaplasia of stomach were found to express Hep. Therefore, we constructed a tissue-array slide, consisting of 15 cases of intestinal metaplasia of the stomach, 5 of the duodenum and 4 of the colorectum, and performed HID-AB2.5 staining and immunohistochemical staining against Hep. All 5 duodenal mucosa were positive for Hep, while the 4 colorectal samples were negative. HID-AB2.5 staining, showed that 15 cases of intestinal metaplasia were classifiable as; 7 cases of intestinal metaplasia type I, 5 cases of type II, and 3 cases of type III. All cases of type I and type III showed Hep expression, but only 2 cases out of 5 type II showed Hep expression. Hep expression in types II and III was restricted to focal areas (Fig. 2A-J).

Fetal hepatocytes with 15 and or 16 weeks of gestation were negative for Hep, while only 5% of hepatocytes were positive

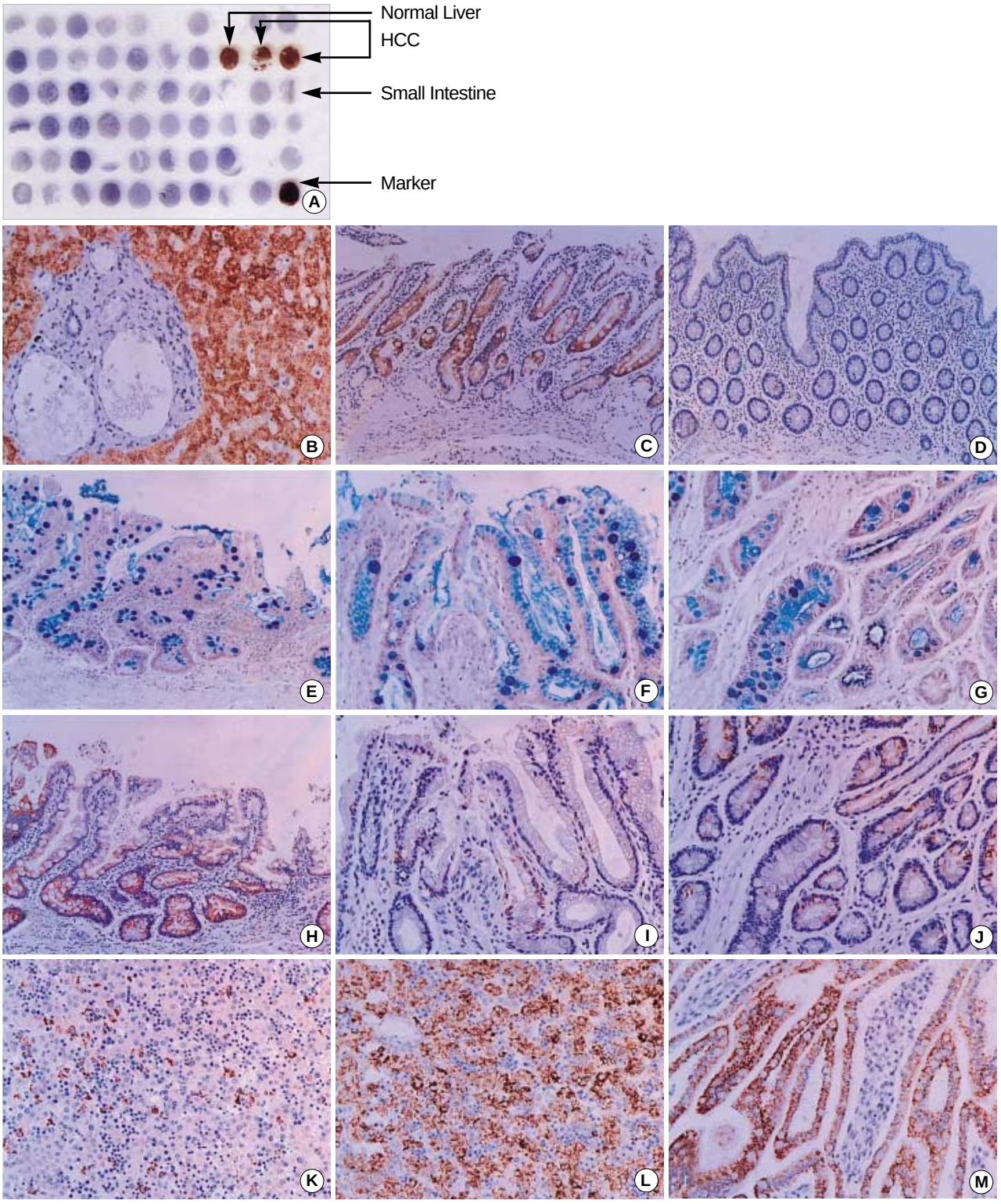

Fig. 2. According to the staining results of human control slide, hepatocytes, HCC and small intestinal mucosa are positive for Hep (A). Hep is positive in normal hepatocytes (B, ×200) and duodenum (C, ×200), but negative in colon (D, ×200). HID-AB2.5 histochemical stains divide gastric mucosa with intestinal metaplasia into type I (E, ×200), type II (F, ×200), or type III (G, ×200). Type I is positive for Hep (H, ×200), but type II (I, ×200) and type III (J, ×200) are focal positive. Only 5% of hepatocytes are positive in fetus at 19 weeks of gestation (K, ×200), and diffusely positive in fetus at 20 weeks of gestation (L, ×200). Fetal small intestine at 20 weeks of gestation show diffuse Hep expression (M, ×200).

in the fetus at 19 weeks of gestation, and were diffusely positive in the fetus with 20, 21, 22, 24, 28 and 30 weeks of gestation. No staining of the bile ducts or other nonparenchymal cells in the fetus was observed. Fetal mucosa of the small intestine with 20, 21, 24, 28 and 30 weeks of gestation was also observed expressing Hep. Another fetal digestive organs, such as the tongue, esophagus, stomach and colon with at any gestational age, did not show Hep expression (Fig. 2K-M).

Table 2. The correlation between Hepatocyte expression and histologic grade in HCCs and non-HCCs

Tumor	No. of cases	Positive cases	*p* value
Hepatocellular carcinoma			
Edmondson-Steiner grade			0.004
I	9	9 (100%)	
II	29	27 (93.1%)	
III	20	15 (75%)	
IV	17	9 (52.9%)	
Architectural patterns			0.001
Trabecular	57	46 (80.7%)	
Pseudoglandular	1	1 (100%)	
Mixed	13	13 (100%)	
Compact	3	0 (0%)	
Scirrhous	1	0 (0%)	
Cholangiocarcinoma (differentiation)			0.035
Well differentiated	6	3 (50%)	
Moderate differentiated	27	2 (7.4%)	
Poor differentiated	17	3 (17.6%)	
Gastric adenocarcinoma			
WHO classification			<0.001
Well differentiated	29	20 (69%)	
Moderate differentiated	82	29 (35.4%)	
Poor differentiated	133	47 (35.3%)	
Signet ring cell carcinoma	15	12 (80%)	
Mucinous carcinoma	49	28 (57.1%)	
Tumor progression			<0.001
Early gastric carcinoma	92	58 (63%)	
Advanced gastric carcinoma	216	78 (36.1%)	
Colorectal adenocarcinoma			
Well differentiated	2	1 (50%)	0.155
Moderate differentiated	41	3 (7.3%)	
Poor differentiated	5	0 (0%)	
Mucinous carcinoma	1	0 (0%)	

CD10 Expressions in HCCs and Non-HCCs

CD10 was detected in 46.7% (35/75) of HCCs, 36% (18/50) of cholangiocarcinomas, 40.8% (20/49) of colorectal adenocarcinomas, and in 14% (42/300) of gastric adenocarcinomas (Fig. 1, Table 3). The staining pattern was either canalicular, non-canalicular (membranous), or cytoplasmic (22). The canalicular staining pattern was predominated in HCCs (22 out of 38 CD10-positive HCCs), but this pattern was not found in non-HCCs. The canalicular staining was only seen in trabecular, and mixed trabecular and pseudoglandular patterns of HCCs, and not in the compact, scirrhous or pseudoglandular patterns (data not shown).

DISCUSSION

Hep is a monoclonal antibody that reacts with a hepatocyte-specific epitope resistant to formalin fixation and tissue processing. The granular intracytoplasmic staining pattern suggests organelle localization, possibly mitochondrial, but the target antigen has been remains unknown (13). Unlike AFP, the sensitivity of Hep in HCCs is relatively high. Hep sensitivity in the previously reported and present study was 80% or more (13, 14, 16, 24-26). AFP and Hep expressions in HCCs are associated with histologic grade, and poorly differentiated HCCs are more likely to be AFP positive (16). Well differentiated tumors are also more likely to be Hep positive. Immunostaining with another diagnostic markers, such as a set of CKs, could result in more accurate diagnoses. However, CK expressions may change when malignancy develops, and the CK staining patterns of gastric adenocarcinomas are very heterogenous (12). Therefore, immunostaining with Hep and AFP in combination with a set of CKs in HCCs would increase diagnostic sensitivity and specificity.

In their original report, Wennerberg et al. found that nine of 205 cases of non-HCCs (5%) were Hep positive (13). All nine of these cases were from the gastrointestinal tract, and 3 of 10 gastric adenocarcinomas (all were poorly differentiated, signet ring, or mixed intestinal/signet ring) showed Hep expression. A number of subsequent studies revealed that 30-45% of gastric adenocarcinomas were positive for Hep, whereas only 7-15% of cholangiocarcinomas and 0-5% of colorectal adenocarcinomas showed Hep expression (14, 16, 24). Because

Table 3. CD10 expressions in HCCs and non-HCCs

	HCCs	Non-HCCs		
		Cholangiocarcinoma	Colorectal adenocarcinoma	Gastric adenocarcinoma
Canalicular	21/75 (28%)	0/50 (0%)	0/49 (0%)	0/300 (0%)
Non-canalicular	10/75 (13.3%)	13/50 (26%)	15/49 (30.6%)	32/300 (10.7%)
Cytoplasm	4/75 (5.4%)	5/50 (10%)	5/49 (10.2%)	10/300 (3.3%)
Total positive	35/75 (46.7%)	18/50 (36%)	20/49 (40.8%)	42/300 (14%)

non-HCCs in our study mainly consisted of gastric adenocarcinomas, Hep specificity in the present study (63.6%) was lower than that previously reported (about 94%) (13, 14, 16, 24-26). However, the distinct histopathologic features of Hep-positive gastric adenocarcinomas may be helpful in differential diagnosis. Gastric adenocarcinomas with Hep expression showed a tendency of having well differentiated tubular, signet ring and mucinous histologic types, and were associated with early gastric carcinomas, which had exceptionally rare liver metastasis. Additionally, Hep staining in gastric carcinoma was more frequently restricted to focal areas than in HCCs. However, well differentiated HCCs more frequently showed Hep expression, tumor cells of HCCs did not produce mucin, and diffuse staining was more frequent than in non-HCCs.

In this study, fetal hepatocytes at 15 and 16 weeks of gestation did not show Hep expression, while fetal hepatocytes showed Hep expression after 19 weeks of gestation. Hep expression in normal hepatocytes was more intense and diffuse than in HCCs, and Hep positivity was significantly associated with the differentiation of HCCs. These results suggest that Hep immunoreactivity is a reflection of the degree of hepatocyte differentiation. Fasano et al. (27) reported that the staining pattern of Hep in hepatoblastomas was more focal in distribution, more variable and less intense than in the surrounding normal liver, and that Hep staining was generally less intense in embryonal- than in fetal-type hepatoblastoma. They also suggest the existence of a correlation between Hep-positivity and the degree of hepatocyte differentiation.

Intestinal metaplasia appears to take two major forms, complete and incomplete, based on similarities in structure and function to the normal mucosa of the small intestine and of the colon (28-30). In the complete type (type I) of intestinal metaplasia, the lesion most closely resembles the small intestine and reveals numerous columnar absorptive cells. In contrast, the incomplete type (types II and III) consists of a mixture of gastric foveolar and colonic type goblet cells. According to the results of the human control slide (Superbiochips Laboratories, Seoul, Korea), small intestinal mucosa and intestinal metaplasia of the stomach were positive for Hep, and showed a distinct granular cytoplasmic expression pattern, but colorectal mucosa was negative for Hep. Therefore, it has been suggested that Hep expression may be restricted to intestinal metaplasia of type I. By using HID-AB2.5 staining, 15 intestinal metaplasia were divided into 7 of the complete type and 8 of the incomplete type, but this typing was not entirely consistent with the Hep expression status. This was perhaps because HID-AB2.5 staining could not strictly categorize the types of intestinal metaplasia, and because this typing of intestinal metaplasia had a spectrum with considerable heterogeneity (28, 31).

In this study, we investigated using the tissue array method, and found that this method enabled us to analyze a large number of HCCs and non-HCCs, especially gastric adenocarcinomas. Accordingly, we could evaluate the correlation between Hep expression status and the histopathologic patterns of HCCs or non-HCCs statistically. The potential limitations of this method are mainly associated with the acquisition of information from only a tiny area in each tumor. Because intratumoral heterogeneity of the Hep antigen expression has been reported, and was also found observed in this study, it might be that the Hep expression status in our study was under or overestimated. In order to address the influence of tumor heterogeneity, multiple replicate tissue array blocks were constructed, both by ourselves and by other researchers (18, 32, 33). In all such research, the results from each replica array were almost identical, statistically meaningful. In this study, the positivity of Hep in HCCs using whole section slides was 82.4% (14/17) and positivity using tissue-array slides was 79.3% (46/58), which was not statistically significant (data not shown).

CD10 is a 100-kD cell surface metalloproteinase, which was originally identified on tumor cells of acute lymphoblastic leukemia, and was thus named as common acute lymphoblastic leukemia antigen (CALLA) (34). It was shown recently that CD10 is expressed in both normal and neoplastic liver tissue, where it exhibits a canalicular distribution pattern. Thus, CD10 may be a serve as an useful additional marker for differentiating HCCs and non-HCCs (22, 35, 36). In the present study, only HCCs showed a canalicular CD10 expression pattern, which also suggests that CD10 may be helpful for in the differential diagnosis of HCCs from non-HCCs.

In summary, Hep expression was found to be highly sensitive in detecting HCCs. However, about 40% of gastric adenocarcinomas also showed Hep expression, and the specificity of Hep expression was low at detecting HCCs. Because gastric adenocarcinomas with Hep expression had somewhat different histopathologic features from HCCs with Hep expression, Hep expression status could be helpful for the diagnosis of HCC, especially by using a panel approach in combination with AFP and CKs. In non-neoplastic tissues other than liver, small intestinal mucosa and intestinal metaplasia also expressed Hep with a distinct granular cytoplasmic staining.

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