J Hepatobiliary Pancreat Sci (2016) 23:414–421
DOI: 10.1002/jhbp.356

ORIGINAL ARTICLE

Immunohistochemical study of hepatocyte, cholangiocyte and stem cell markers of hepatocellular carcinoma: the second report: relationship with tumor size and cell differentiation

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Published online: 12 June 2016

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DOI: 10.1002/jhbp.356

Abstract

Background The purpose of this study is to investigate whether ordinary hepatocellular carcinomas (HCCs) show positivity of stem/progenitor cell markers and cholangiocyte markers during the process of tumor progression.

Methods Ninety-four HCC lesions no larger than 8 cm from 94 patients were immunohistochemically studied using two hepatocyte markers (Hep par 1 and α-fetoprotein), five cholangiocyte markers (cytokeratin CK7, CK19, Muc1, epithelial membrane antigen and carcinoembryonic antigen) and three hepatic stem/progenitor cell markers (CD56, c-Kit and EpCAM). The tumors were classified into three groups by tumor size: S1, < 2.0 cm; S2, 2.0–5.0 cm; S3, 5.0–8.0 cm. The tumors were also classified according to tumor differentiation: well, moderately and poorly differentiated. The relationship between the positive ratios of these markers, tumor size and tumor differentiation was examined.

Results The positive ratios of cholangiocyte markers tended to be higher in larger sized and more poorly differentiated tumors (except for CK7). The positive ratios of stem/progenitor cell markers tended to be higher in larger sized and more poorly differentiated tumors (except for c-Kit).

Conclusion Ordinary HCC can acquire the characteristic of positivity of cholangiocyte and stem/progenitor cell markers during the process of tumor progression.

Keywords Hepatocellular carcinoma · Immunohistochemistry · Progenitor cell · Stem cell · Transdifferentiation

Introduction

Owing to the recent advance of studies of primary liver cancer with stem/progenitor cell features and/or biliary differentiation [1–8], classification of combined hepatocellular-cholangiocarcinoma (CH-CC) was newly created [9]. CH-CC is now classified into classical type and subtypes with stem cell features. At present, the formation mechanism of CH-CC can be classified into two types: (i) hepatocellular carcinoma transdifferentiating into CH-CC, and (ii) malignant transformation of stem/progenitor cells. Of these two, the classical type can be an example of the first pathway, and the subtypes with stem cell features can be examples of the second pathway. After this dramatic revision of the classification, several important works of CH-CC with stem cell features were published [10–12].

Studies of transdifferentiation have greatly progressed, as well as those of stem/progenitor cells [13–17]. In a former study [18], we examined the expression of cholangiocyte and stem

cell markers in ordinary hepatocellular carcinoma (HCC) tissues. The results suggested that this expression could be interpreted as transdifferentiation. However, the relationship between the positive ratio of these markers, tumor size and tumor differentiation was not examined. Various stem cell/progenitor cell markers were not examined, except for c-Kit.

In the present study, we examined the positive ratios of various markers of hepatocyte, cholangiocyte and stem/progenitor cells in ordinary HCC cases. The data were then compared with tumor size and tumor differentiation. We investigated whether ordinary HCCs show positivity of various markers during the process of tumor progression.

Methods

Patients and tissue specimens

Tissue samples from 94 patients with ordinary HCC were obtained by surgical resection at the Department of Surgery, Teikyo University Hospital and Toranomon Hospital in 2001–2009. Their clinico-pathological data are shown in Table 1. These HCC patients consisted of 73 men and 21 women ranging in age from 44 to 82 years (mean, 67 years). Thirteen patients were positive for serum hepatitis B surface antigen, 66 were positive for serum anti-hepatitis C virus antibody, and 15 were negative for both. The large diameters of these 94 tumors ranged from 0.9 to 8.0 cm (mean, 3.1 cm).

Histological diagnosis and classification of differentiation of these HCCs were made according to the World Health Organization (WHO) classification [19]. As for tumor cell differentiation, 16 cases were well-differentiated, 74 cases were moderately differentiated, and four cases were poorly differentiated. Histological type was predominantly the ordinary trabecular type in all 94 cases; pseudoglandular type was found as a minor component. Special types, such as scirrhous, fibrolamellar and sarcomatous were not found.

Table 1 Clinico-pathological data

Age	44–82 years (mean, 67 years)
Gender	Male, 73 cases; female, 21 cases
Causative factors of liver disease	HBsAg+, 13 cases; HCAb+, 66 cases
Non-cancerous liver tissue	NL, 3 cases; CH, 46 cases; LC, 45 cases
Tumor size	0.9–8.0 cm (mean, 3.1 cm)
Cell differentiation	Well, 16 cases; moderate, 74 cases; poor, 4 cases

CH chronic hepatitis, *HBsAg* hepatitis B surface antigen, *HCAb* hepatitis C antibody, *LC* liver cirrhosis, *NL* normal liver

The diagnostic criteria of ordinary HCC and differentiation from CH-CC were based on the mucin staining. We defined ordinary HCC as those cases whose mucin-positive area is less than 10%. The lesion whose mucin-positive area was larger than 10% was classified as a CH-CC. In our previous study, 68 HCC cases were studied [18]. One case was deleted from these 68 cases because the mucin-positive area was larger than 10%. The rest of 67 cases were included in the present study.

The surrounding non-tumorous liver tissues showed chronic hepatitis in 46 patients and cirrhotic change in 45 patients. The other three patients showed normal liver.

This study was approved by the Research Ethics Review Board of the Teikyo University School of Medicine and Toranomon Hospital.

This study was supported in part by grants from The Vehicle Racing Commemorative Foundation.

Immunohistochemistry

Tissue samples were fixed in 10% formalin solution and embedded in paraffin for histological diagnosis and immunohistochemistry.

The primary antibodies are summarized in Table 2. Hep par 1 and α-fetoprotein (AFP) were used as hepatocyte markers. Cytokeratin 7 (CK7), cytokeratin 19 (CK19), Muc 1, carcinoembryonic antigen (CEA) and epithelial membrane antigen (EMA) were used as cholangiocyte markers. CD56, c-Kit, and EpCAM were used as stem cell/progenitor cell markers.

Formalin-fixed paraffin-embedded tissue samples, cut at 3-μm thickness, were deparaffinized with xylene and rehydrated with graded ethanol. Antigen retrieval was performed by boiling at 98°C for 40 minutes in 0.01mol/L sodium citrate buffer (pH 6.0), or in Tris/EDTA buffer (pH9.0). Immunohistochemistry was performed using Dako Autostainer plus (Dako, Glostrup, Denmark). Endogenous peroxidase was quenched with 3% H_2O_2 in distilled water for 5 min. After treatment with protein blocking solution (Dako) for 10 min, the slides were incubated with primary antibodies for 30 min at room temperature. The sections were then stained by the detection method using EnVision kit (Dako ChemMate) according to the manufacturer's protocol and counterstained with hematoxylin.

The expression of these markers was evaluated semiquantitatively as follows:

Negative (Score 0); No positive staining, or tumor cells were not stained with a clustered pattern e.g. less than five definitively positive cells in a cluster.

Table 2 Primary antibodies used in this study

	Primary antibody	Clone	Source	Dilution	Antigen retrieval
Hepatocyte markers	Hep par 1	OCH1E5	Dako	1:60	Citrate buffer pH6.0
	AFP	C3	Leica	1:60	None
Cholangiocyte markers	CK7	OV-TL12/30	Dako	1:60	EDTA pH9.0
	CK19	RCK108	Progen Biotechnik GmbH	1:60	Citrate buffer pH6.0
	Muc1	Ma695	Leica	1:125	Citrate buffer pH6.0
	CEA	12-140-10	Leica	1:100	EDTA pH9.0
	EMA	E29	Dako	1:125	Citrate buffer pH6.0
Hepatic stem/progenitor cell markers	c-Kit	Polyclonal	Dako	1:100	Citrate buffer pH6.0
	CD56	1B6	Leica	1:60	EDTA pH9.0
	EpCAM	VU-1D9	Calbiochem	1:250	EDTA pH9.0

AFP α-fetoprotein, *CK7* cytokeratin 7, *CK19* cytokeratin 19, *CEA* carcinoembryonic antigen, *EMA* epithelial membrane antigen

1+ (Score 1); Tumor cells were stained with a clustered pattern e.g. at least five definitively positive cells in a cluster. Positive cells were less than 5% of the specimen.

2+ (Score 2); 5–10%, 3+ (Score 3); 10–50%, 4+ (Score 4); 50–100%.

Positive ratio was calculated by the following formula:

The number of positive lesions/ the number of the all lesions ×100 (%).

Because semiquantititive evaluation of membranous pattern (canalicular staining) of CEA and EMA was very difficult, we evaluated only cytoplasmic pattern in CEA and EMA.

Statistical analysis

The tumors were classified into three groups by tumor size (long axis) and cell differentiation. The groups of tumor size were classified as follows: S1, < 2.0 cm (29 cases); S2, 2.0–5.0 cm (54 cases); S3, 5.0–8.0 cm (11 cases). The groups of tumor cell differentiation were classified as follows according to WHO classification: well-differentiated (16 cases), moderately differentiated (74 cases), and poorly differentiated (four cases).

Data of simple positive ratio were analyzed using Fisher's exact test to evaluate the significance of differences among the groups by tumor size and cell differentiation, respectively. Semiquantitative immunohistochemical score (0/1/2/3/4) of these groups were analyzed using Kruskal–Wallis analysis. $P < 0.05$ was considered statistically significant.

Results

The simple positive ratio and semiquantitative immunohistochemical score of the groups are summarized in Table 3 (correlation with tumor size) and Table 4 (correlation with cell differentiation).

Correlation between the immunohistochemical positive ratio of hepatocyte, cholangiocyte, stem/progenitor cell markers and tumor size

Hepatocyte markers: Hep par 1 and AFP

Hep par 1 showed cytoplasmic staining with coarsely granular patterns in HCC cells (Fig. 1a), which was more heterogeneous than that of hepatocytes both in normal and non-neoplastic liver tissues with chronic liver disease. All 94 HCC cases were positive for Hep par 1 regardless of tumor size (Table 3). Positive staining of AFP was found in none of 29 (0%) cases of S1, 7/54 (12.9%) cases of S2, and 2/11 (18.2%) cases of S3 (Table 3) (Fig. 1b). Although not statistically significant, the positive ratio of AFP was the lowest in the S1 HCC group, and the highest in the S3 HCC group.

Cholangiocyte markers: CK7, CK19, Muc1, CEA and EMA

CK7 was positively stained in 24/29 (82.8%) cases of S1, 38/54 (70.4%) cases of S2, and 9/11 (81.8%) cases of S3 (Table 3) (Fig. 1c). While the positive ratio of CK7 varied, more than 75% (71/94, 75.5%) cases in this study were positive for this cholangiocyte marker.

By contrast, the positive ratio of CK19 was lower than that of CK7 in each HCC group. The positive ratio was 4/29 (13.8%) cases of S1, 10/54 (18.5%) cases of S2, and 4/11 (36.4%) cases of S3 (Table 3) (Fig. 1d). Although it was not statistically significant, the group with larger HCCs tended to show a higher positive ratio than the group with smaller HCCs.

Muc1 was positively stained in 2/29 cases (6.9%) of S1, 5/54 (9.3%) cases of S2, and 5/11 (45.5%) cases of S3 (Table 3) (Fig. 1e). The positive ratio of Muc1 in the S3 HCC group was significantly higher than that of the S1 ($P < 0.01$) and S2 HCC groups ($P < 0.05$). Semiquantitative scores of these groups were significantly different ($P < 0.01$).

Table 3 Semiquantitative evaluation of hepatocyte, cholangiocyte, and stem/progenitor cell markers; the correlation with tumor size

	Group of tumor size	Number of positive cases	Positive ratio (%)	*P*-value (S1 vs. S2/ S1 vs. S3/ S2 vs. S3)	Score of immunohistochemistry (0/1/2/3/4)[a]	*P*-value
Hep par 1	S1 ($n = 29$)	29	100	N/A	0/0/5/0/24	0.143
	S2 ($n = 54$)	54	100		0/0/0/4/50	
	S3 ($n = 11$)	11	100		0/0/0/0/11	
AFP	S1 ($n = 29$)	0	0	0.090/0.071/0.642	29/0/0/0/0	0.098
	S2 ($n = 54$)	7	12.9		47/1/1/3/2	
	S3 ($n = 11$)	2	18.2		9/1/0/0/1	
CK7	S1 ($n = 29$)	24	82.8	0.292/1.000/0.713	5/7/10/6/1	0.539
	S2 ($n = 54$)	38	70.4		16/9/12/13/4	
	S3 ($n = 11$)	9	81.8		2/0/5/3/1	
CK19	S1 ($n = 29$)	4	13.8	0.761/0.182/0.232	25/2/2/0/0	0.253
	S2 ($n = 54$)	10	18.5		44/7/3/0/0	
	S3 ($n = 11$)	4	36.4		7/2/2/0/0	
Muc1	S1 ($n = 29$)	2	6.9	1.000/0.011[b]/0.009[c]	27/2/0/0/0	0.002[c]
	S2 ($n = 54$)	5	9.3		49/3/2/0/0	
	S3 ($n = 11$)	5	45.5		6/2/3/0/0	
CEA	S1 ($n = 29$)	0	0	0.087/<0.001[c]/0.015[b]	29/0/0/0/0	< 0.001[c]
	S2 ($n = 54$)	6	11.1		48/4/1/1/0	
	S3 ($n = 11$)	5	45.5		6/3/2/0/0	
EMA	S1 ($n = 29$)	2	6.9	0.316/0.125/0.412	27/2/0/0/0	0.193
	S2 ($n = 54$)	9	16.7		45/5/3/1/0	
	S3 ($n = 11$)	3	27.3		8/1/1/1/0	
CD56	S1 ($n = 29$)	1	3.4	0.653/0.015[b]/0.023[b]	28/1/0/0/0	0.004[c]
	S2 ($n = 54$)	4	7.4		50/3/0/1/0	
	S3 ($n = 11$)	4	36.4		7/2/0/2/0	
c-kit	S1 ($n = 29$)	0	0	N/A	29/0/0/0/0	N/A
	S2 ($n = 54$)	0	0		54/0/0/0/0	
	S3 ($n = 11$)	0	0		11/0/0/0/0	
EpCAM	S1 ($n = 29$)	1	3.4	0.412/0.479/1.000	28/0/1/0/0	0.512
	S2 ($n = 54$)	6	11.1		48/4/1/1/0	
	S3 ($n = 11$)	1	9.1		10/0/0/0/1	

AFP α-fetoprotein, *CK7* cytokeratin 7, *CK19* cytokeratin 19, *CEA* carcinoembryonic antigen, *EMA* epithelial membrane antigen, *N/A* not available

[a] Score 0, negative; score 1, a clustered pattern (at least five definitively positive cells in a cluster) < 5% positive cells; score 2, 6% to 10% positive cells; score 3, 10% to 50% positive cells; score 4, 50% to 100% positive cells

[b] $P < 0.05$

[c] $P < 0.01$

A cytoplasmic staining pattern of CEA was found in 11/94 (11.7%) examined HCCs (Fig. 1f), and its appearance was correlated with tumor size: none of 29 (0%) cases of S1, 6/54 (11.1%) cases of S2, and 5/11 (45.5%) cases of S3 (Table 3). The positive ratio of CEA expression in the S3 HCC group was significantly higher than those of the S1 ($P < 0.001$) and S2 HCC groups ($P < 0.05$). Semiquantitative analysis of the scores was also significant ($P < 0.001$).

A cytoplasmic staining pattern of EMA was found in 14/94 (14.9%) examined HCCs (Fig. 1g), and its appearance was observed in 2/29 (6.9%) cases of S1, 9/54 (16.7%) cases of S2, and 3/11 (27.3%) cases of S3 (Table 3). The positive ratio of EMA expression with cytoplasmic patterns in the group with large HCCs tended to be higher than that of the group with small HCCs, although the difference was not significant.

Stem/progenitor cell markers: CD56, c-Kit and EpCAM

CD56 was found in 9/94 (9.6%) HCCs, and they were correlated with tumor size; 1/29 (3.4%) cases of S1, 4/54 (7.4%) cases of S2, and 4/11 (36.4%) cases of S3 showed positive staining (Table 3) (Fig. 1h). The positive ratio of CD56 expression in the S3 HCC group was significantly higher than those of the S1 ($P < 0.05$) and S2 HCC groups ($P < 0.05$). Semiquantitative analysis was also proved to be significant ($P < 0.01$).

Table 4 Semiquantitative evaluation of hepatocyte, cholangiocyte, and stem/progenitor cell markers; the correlation with cell differentiation

	Cell differentiation	Number of positive cases	Positive ratio (%)	*P*-value (well vs. moderately/ well vs. poorly/ moderately vs. poorly)	Score of immunohistochemistry (0/1/2/3/4)[a]	*P*-value
Hep par 1	Well ($n = 16$)	16	100	N/A	0/0/4/0/12	0.024[b]
	Moderately ($n = 74$)	74	100		0/0/1/3/70	
	Poorly ($n = 4$)	4	100		0/0/0/1/3	
AFP	Well ($n = 16$)	0	0	0.342/0.200/0.394	16/0/0/0/0	0.236
	Moderately ($n = 74$)	8	10.8		66/2/1/2/3	
	Poorly ($n = 4$)	1	25.0		3/0/0/1/0	
CK7	Well ($n = 16$)	14	87.5	0.508/0.032[b]/0.057	2/6/5/3/0	0.272
	Moderately ($n = 74$)	56	75.7		18/10/22/18/6	
	Poorly ($n = 4$)	1	25.0		3/0/0/1/0	
CK19	Well ($n = 16$)	3	18.8	1.000/0.249/0.165	13/2/1/0/0	0.378
	Moderately ($n = 74$)	13	17.6		61/7/6/0/0	
	Poorly ($n = 4$)	2	50.0		2/2/0/0/0	
Muc1	Well ($n = 16$)	0	0	0.353/0.004[c]/0.011[b]	16/0/0/0/0	< 0.001[c]
	Moderately ($n = 74$)	9	12.2		65/5/4/0/0	
	Poorly ($n = 4$)	3	75.0		1/2/1/0/0	
CEA	Well ($n = 16$)	0	0	0.202/ N/A /1.000	16/0/0/0/0	0.190
	Moderately ($n = 74$)	11	14.9		63/7/3/1/0	
	Poorly ($n = 4$)	0	0		4/0/0/0/0	
EMA	Well ($n = 16$)	0	0	0.206/0.035[b]/0.146	16/0/0/0/0	0.036[b]
	Moderately ($n = 74$)	12	16.2		62/7/3/2/0	
	Poorly ($n = 4$)	2	50.0		2/1/1/0/0	
CD56	Well ($n = 16$)	1	6.2	1.000/0.088/0.050	15/1/0/0/0	0.017[b]
	Moderately ($n = 74$)	6	8.1		68/4/0/2/0	
	Poorly ($n = 4$)	2	50.0		2/1/0/1/0	
c-kit	Well ($n = 16$)	0	0	N/A	16/0/0/0/0	N/A
	Moderately ($n = 74$)	0	0		74/0/0/0/0	
	Poorly ($n = 4$)	0	0		4/0/0/0/0	
EpCAM	Well ($n = 16$)	0	0	0.342/ N/A/1.000	16/0/0/0/0	0.311
	Moderately ($n = 74$)	8	10.8		66/4/2/3/1	
	Poorly ($n = 4$)	0	0		4/0/0/0/0	

AFP α-fetoprotein, *CK7* cytokeratin 7, *CK19* cytokeratin 19, *CEA* carcinoembryonic antigen, *EMA* epithelial membrane antigen, *N/A* not available

[a] Score 0, negative; score 1, a clustered pattern (at least five definitively positive cells in a cluster) < 5% positive cells; score 2, 6% to 10% positive cells; score 3, 10% to 50% positive cells; score 4, 50% to 100% positive cells

[b] $P < 0.05$

[c] $P < 0.01$

The expression of c-Kit was not found in any of the examined 94 HCC cases (Table 3). Although our former paper reported that eight cases of HCC showed positive staining [18], the number of positive cells was very few. These findings were not sufficient for the criteria of positivity in the present study.

The positive ratios of EpCAM of the S1, S2 and S3 groups were 1/29 (3.4%), 6/54 (11.1%), and 1/11 (9.1%), respectively (Table 3) (Fig. 1i). The positive ratio of EpCAM did not show the highest value in the largest S3 HCC group. However, the positive ratio of the smallest S1 group showed the smallest value. In addition, a case of S3 group showed the highest score 4 in the semiquantitative evaluation.

Correlation between the immunohistochemical positive ratio of hepatocyte, cholangiocyte, stem/progenitor cell markers and tumor cell differentiation

Hepatocyte markers: Hep par 1 and AFP

All of 94 HCC cases were positive for Hep par 1 regardless of cell differentiation (Table 4). AFP was positively stained in none of 16 (0%) cases of the well-differentiated HCC group, 8/74 (10.8%) cases of the moderately differentiated HCC group, and 1/4 (25%) cases of the poorly differentiated HCC group (Table 4). The positive ratios

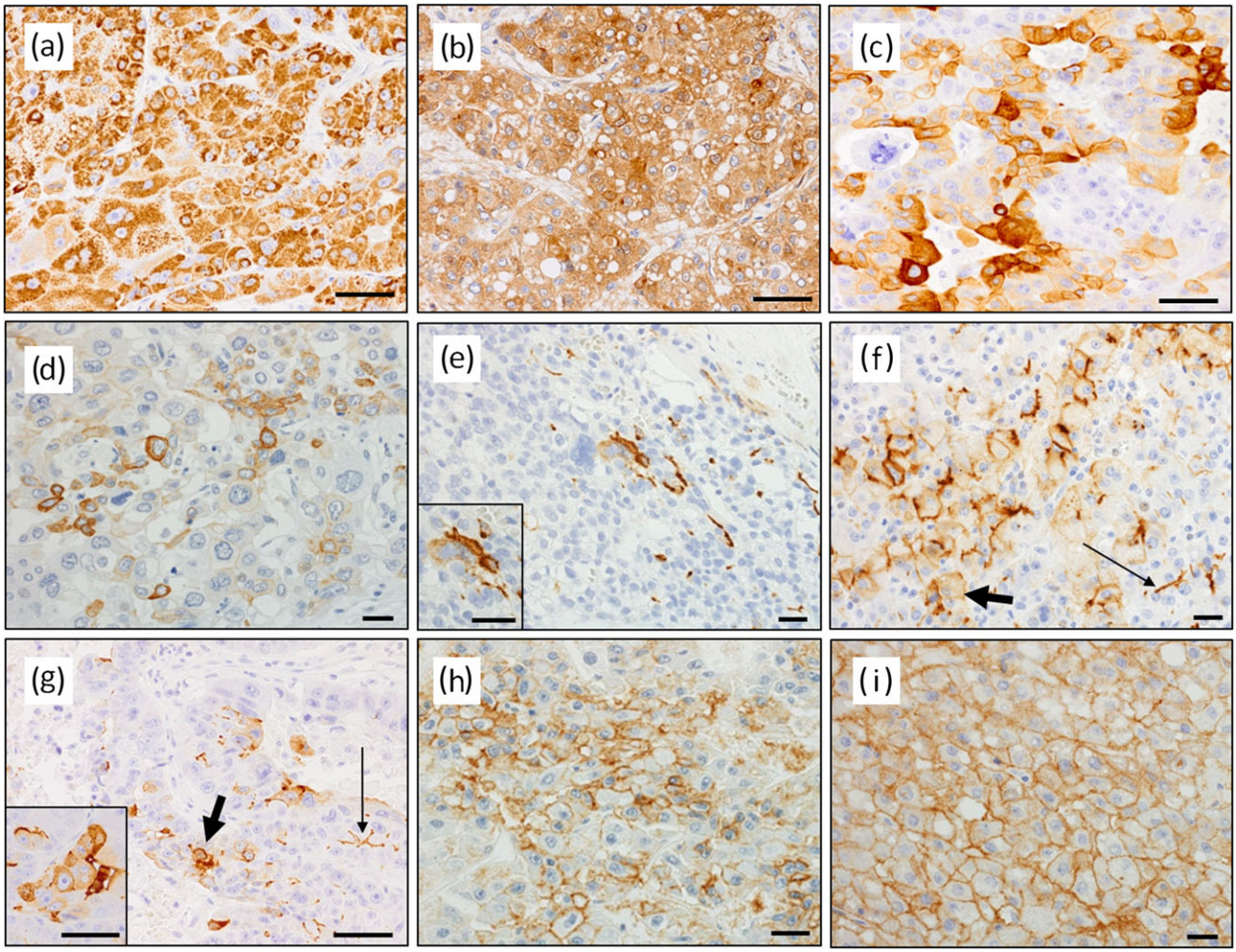

Fig. 1 Immunohistochemical profiles of hepatocellular carcinomaPositive immunoreactivity for two hepatocyte markers: Hep par 1 (**a**), AFP (**b**), five cholangiocyte markers: CK7 (**c**), CK19 (**d**), Muc1 (**e**), CEA (**f**) and EMA (**g**) and three hepatic stem/progenitor cell markers: CD56 (**h**) and EpCAM (**i**) was detected. In order to show small positive cell clusters clearly, a high magnification figure was inserted as an inset of (**e**) and (**g**), respectively. Immunoreactivity for CEA and EMA showed the two staining patterns, i.e. intraluminal canalicular pattern (thin long arrows) and/or cytoplasmic (thick short arrows). Only cytoplasmic pattern was semiquantitatively evaluated. Many specimens of CK19, Muc1, CEA, EMA, CD56 and EpCAM, the positive pattern was found as "small positive clusters in a large negative background area". Bar 50μm (**a**–**c**, **g** and inset), bar 20 μm (**d**, **e** and inset, **f**, **h** and **i**).

of AFP-expressing HCCs were higher in the poorer differentiated HCCs, although this was not statistically significant.

Cholangiocyte markers: CK7, CK19, Muc1, CEA and EMA

CK7 was positively stained in 14/16 cases (87.5%) of the well-differentiated, 56/74 (75.7%) cases of the moderately differentiated, and 1/4 (25%) cases of the poorly differentiated HCC groups (Table 4). The positive ratio of CK7 expression in the poorly differentiated HCC group was significantly lower than those of the well-differentiated ($P < 0.05$). However, semiquantitative analysis of the scores was not significant.

CK19 was positively stained in 3/16 (18.8%) cases of the well-differentiated, 13/74 (17.6%) cases of the moderately differentiated, and 2/4 (50%) cases of the poorly differentiated HCC groups (Table 4). The positive ratio of CK19 in the poorly differentiated HCC group was higher than those of the well-differentiated and moderately differentiated HCC groups, although not to a statistically significant degree.

Muc1 was positively stained in none of 16 cases (0%) of the well-differentiated, 9/74 (12.2%) cases of the moderately differentiated, and 3/4 (75%) cases of the poorly differentiated HCC groups (Table 4). The positive ratio of Muc1 of the poorly differentiated HCC group was significantly higher than those of the well-differentiated ($P < 0.01$) and moderately differentiated HCC groups ($P < 0.05$). Semiquantitative analysis

was also proved to be significant with a very low *P*-value ($P < 0.001$).

A cytoplasmic staining pattern of CEA was found in 11/74 (14.9%) cases of the moderately differentiated HCC group but not in the well-differentiated and poorly differentiated HCC groups (Table 4).

A cytoplasmic staining pattern of EMA was found in none of 16 (0%) cases of the well-differentiated, 12/74 (16.2%) cases of the moderately differentiated, and 2/4 (50%) cases of the poorly differentiated HCC groups. The positive ratio of EMA with cytoplasmic patterns in the poorly differentiated HCC group was significantly higher than that of the well-differentiated HCC group ($P < 0.05$). Semiquantitative analysis was also significant ($P < 0.05$).

Stem/progenitor cell markers: CD56, c-Kit and EpCAM

CD56 was positively stained in 1/16 cases (6.2%) of the well-differentiated, 6/74 (8.1%) cases of the moderately differentiated, and 2/4 (50%) cases of the poorly differentiated HCC groups (Table 4). The positive ratio of CD56 in the poorly differentiated HCC group was higher than those of the moderately and well-differentiated HCC groups, although the difference was not statistically significant. The expression of c-Kit was not found in any of the 94 HCC cases (Table 4). The positive ratios of EpCAM of the well-, moderately-, and poorly-differentiated group were 0/16 (0%), 8/74 (10.8%), and 0/4 (0%), respectively (Table 4).

Discussion

The WHO classification of CH-CC has been entirely revised [9] on the basis of recent advances in stem/progenitor cell studies [1–5]. It is speculated that some of these CH-CC are of stem/progenitor cell origin [11]. They are now classified as subtypes with stem cell features. Evaluation of stem cell features and biliary differentiation is truly important because these stem/progenitor cell and/or biliary characteristic of the primary liver cancer is closely related to their clinical behavior [6–8, 10, 12]. However, it is still controversial whether they actually originated from stem/progenitor cells. Their stem/progenitor cell features could be acquired phenotypes during the process of transdifferentiation. Studies of transdifferentiation have also progressed considerably [13–17]. According to those studies, cholangiocellular carcinoma phenotype can be derived from HCC component [13–15]. In animal models, non-neoplastic hepatocytes are able to transdifferentiate into cholangiocytes [16, 17].

Those studies provide evidence of CH-CC of the classical type, which is considered to be formed by transdifferentiation. Our previous study also showed that ordinary HCC includes cholangiocyte marker-positive areas [18]. At present, however, we have little data as to the expression of stem/progenitor cell phenotypes caused by transdifferentiation. Our previous study did not deal with various stem cell/progenitor cell markers except for c-Kit.

In the present study, we added CD56 and EpCAM as stem/progenitor cell markers as well as c-Kit, as these markers were described as representative stem/progenitor cell markers by the WHO classification [9]. We added small and large HCCs to the former materials. And then, we examined the positive ratios of various markers of hepatocyte, cholangiocyte and stem/progenitor cells in ordinary HCC cases. In addition, we evaluated immunostaining semiquantitatively. The data were compared with tumor size and tumor cell differentiation.

The positive ratio of cholangiocyte markers CK19, Muc1, CEA and EMA tended to be higher in larger sized and more poorly differentiated tumors. These results support the formation mechanism of the classical type of CH-CC, which is formed by transdifferentiation. According to animal model studies, non-neoplastic hepatocytes can also transform into cholangiocytes [16, 17].

The positive ratio of the stem/progenitor cell markers also showed a similar tendency. CD56 tended to show a higher positive ratio in larger sized and more poorly differentiated tumors. EpCAM was positive only in the moderately differentiated HCC group. None of the well-differentiated HCCs was positively stained. These results suggest the possibility that expressions of stem/progenitor cell markers are acquired phenotypes during the process of tumor progression. The expression of stem cell features itself does not necessarily mean a stem/progenitor cell origin. Transdifferentiation can be another type of the formation mechanism of subtypes of stem cell features.

The significance of stem/progenitor cell markers should also be considered. In fact, CD56 and EpCAM are sometimes positively stained in non-neoplastic bile ducts. Interlobular bile ducts also show positivity of c-Kit [20]. These stem/progenitor cell markers can be interpreted as bile duct markers. Therefore, CH-CC with stem cell features might have been formed by bile duct transformation.

The distribution pattern of new phenotype foci within the tumor nodule should be carefully examined when speculating about the tumor cell origin [18]. If CH-CC foci emerged within a pre-existing stem/progenitor cell tumor nodule, stem cell marker-negative foci must exist as small areas within a large positive nodule. If stem/progenitor cell marker-positive foci emerged within a pre-existing ordinary HCC, stem cell marker-positive foci must exist as small areas within a large negative nodule. In evaluating the new phenotypic foci, the cutoff value of the immunostaining must be settled at a very low level. By this reasoning, in the present study a small clustered positive area was evaluated as a positive area. In this study, all the stem/progenitor cell marker-positive foci were found as "small positive foci in a large negative area" [18].

The cholangiocyte markers were also found with the same pattern. These distribution patterns suggested that the stem/progenitor cell markers as well as the cholangiocyte markers emerged as a result of transdifferentiation. These results must be attributed to the methods of this study, in which various phenotypes were examined within ordinary HCC tissues.

In the future, we will need to search for other types of the tumors that show small stem/progenitor cell marker-negative areas within a large positive area. If we find such types of tumors, they must be recognized as being from stem/progenitor cell origin.

As far as the present HCC cases are concerned, stem/progenitor cell features appeared as acquired phenotypes during the process of tumor progression. We should think of the possibility of transdifferentiation when we find stem cell features within HCC tissues besides the possibility of stem cell origin. Of course, tumors of stem/progenitor cell origin should also be pursued.

Conflict of interest None declared.

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