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ORIGINAL ARTICLE

Absence of CD66a expression is associated with high microvessel density and high histologic grade in hepatocellular carcinoma

Chun-Chieh Wu [a], Sheau-Fang Yang [a,b], Wan-Tzu Chen [a], Hung-Pei Tsai [c], Chi-Wen Luo [a], Chee-Yin Chai [a,b,c], Hsin Ling Yin [a,b,*]

[a] *Department of Pathology, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung, Taiwan*
[b] *Department of Pathology, Faculty of Medicine, Kaohsiung Medical University, Kaohsiung City, Taiwan*
[c] *Graduate Institute of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan*

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Abstract Hepatocellular carcinoma (HCC) is a primary malignancy of the liver. Patients with HCC usually have poor prognosis and high mortality. It has been shown that carcinoembryonic antigen-related cell adhesion molecule 1 (CD66a) regulates cell signaling, proliferation, and tumor growth. The aim of this study is to analyze the expression and possible role of CD66a in HCC. Immunohistochemical staining of CD66a was performed on 86 HCC cases, and microvessel density was evaluated by CD34 immunostaining. The results were further correlated with clinicopathological parameters. For 47 of 86 HCC cases, the CD66a expression showed diffuse membrane or cytoplasmic staining. The other 39 HCC cases revealed loss of CD66a expression. Loss of CD66a expression was statistically significantly associated with large tumor size ($p = 0.016$), fatty change ($p = 0.039$), patients with transcatheter arterial embolization ($p = 0.007$), and high microvessel density ($p = 0.036$). CD34 expression had no significant association with tumor size, virus infection, histological grade, and capsular invasion. The diffuse and cytoplasmic expression of CD66a may involve the early stage of the HCC, and the loss of CD66a expression indicates tumor progression.

Conflicts of interest: All authors declare no conflicts of interest.

* Corresponding author. Department of Pathology, Kaohsiung Medical University Hospital, 100 Tzyou 1st Road, Kaohsiung 807, Taiwan.
E-mail address: fp7081@gmail.com (H.L. Yin).

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide [1]. Chronic infections of hepatitis B virus (HBV) and hepatitis C virus are associated with HCC, although the mechanism of the hepatocarcinogenesis is unclear. It has been shown that more than one-half of adult HCC patients worldwide are related to HBV infection [2]. Previous studies also showed that not only chronic inflammation and the effects of cytokines are major factors for the development of HCC [3], but an integration of HBV-DNA into the host cellular genome is also considered an important factor to enhance genomic instability and trigger specific oncogenic pathways [4]. HBV mutations in core promoter and precore regions have been reported to be linked to the severity of liver diseases and development of HCC [5]. In addition, different HBV genotypes have been found to be associated with different mutation rates of HBV and contribute to hepatocarcinogenesis [6].

Carcinoembryonic antigen-related cell adhesion molecule 1 (CD66a), so-called biliary glycoprotein, is a member of the carcinoembryonic antigen subfamily and belongs to the immunoglobulin superfamily. Therefore, CD66a consists of both structural features of the immunoglobulin superfamily and functional properties of the cadherin family of cell adhesion molecules [7,8]. It has been reported that CD66a regulates various cellular functions, including cell signaling, proliferation [9], apoptosis [10], angiogenesis [11], tumor growth [12], and epithelial cell polarization [10]. It has been demonstrated that CD66a expression in lung carcinoma [13] and malignant melanoma [14] is considered as a significant prognostic factor. By contrast, a loss or decrease of CD66a expression was found in various kinds of malignancies, including those of the breast [15], endometrium [16], colon [17], and liver [18]. However, the prognostic significance of CD66a in HCC is still unknown. In normal hepatocytes and low-grade HCC, the CD66a expression shows diffuse immunostaining, but most high-grade liver tumors reveal a loss or decrease of CD66a expression [18,19]. The aim of our study was to find out whether the expression of CD66a in HCC is associated with the prognosis, and to investigate the relationship between CD66a expression and angiogenesis in HCC.

Materials and methods

Patients

Eighty-six consecutive patients underwent curative partial hepatectomy as an initial treatment for HCC at the Department of Hepatobiliary in Kaohsiung Medical University Hospital (Kaohsiung, Taiwan) during the 10-year study period. Each specimen was examined, and clinicopathological parameters including age, sex, virus infection, alpha-fetoprotein, histological grade, vascular invasion, capsular invasion, inflammation, fibrosis, cirrhosis, and fatty change status of the adjacent liver were recorded. Clinical histories of the cases were obtained from their medical records. This study was performed after obtaining approval from the Kaohsiung Medical University Hospital Institutional Review Board (KMUH-IRB-20120054).

Tissue microarray preparation

Hematoxylin–eosin slides were examined under a light microscope. The representative regions were marked. A handmade paper mold with 40 (8×5) holes was prepared and taped on an open box previously. A 16-gauge bone marrow aspiration needle was used to punch paraffin wax cylinders (2 mm in diameter). The tissue core was carefully transferred using forceps to the prepared paper mold. After all the cylinders were implanted in the mold, the mold was embedded with paraffin. Prior to sectioning, the tissue array paraffin block was removed from the mold and cooled to $-10^{\circ}C$. Sections (4 μm thick) were cut using an ordinary microtome.

Immunohistochemical staining

One to four paraffin-embedded block(s) (median, 2 blocks) from each resected specimen were used for immunohistochemical (IHC) staining. Four serial sections, 4 μm thick, were cut from each microarray block: one for routine histologic examination using hematoxylin–eosin staining, two for IHC staining, and one for a negative control. Two independent surgical pathologists blinded to clinical details assessed each section.

A mouse monoclonal antibody against NCL-CD66a (VP-C363; Vector Laboratories Inc., Burlingame, CA, USA) and CD34 (M7165; Dako, Carpinteria, CA, USA), was used at a dilution of 1:50. The streptavidin–biotin immunoperoxidase method was used. The sections were deparaffinized and rehydrated, and then microwaved at 500 W for seven cycles of 3 minutes in 10 mmol/L citrate buffer (pH 6.0) to retrieve antigen activity. After blocking of endogenous peroxidase, sections were incubated overnight at $4^{\circ}C$ with anti-CD66a or CD34 antibody. They were then incubated at room temperature for 30 minutes with goat antimouse immunoglobulin conjugated to a peroxidase-labeled amino acid polymer, Simple Stain Max PO (M) (Nichirei, Tokyo, Japan). Diaminobenzidine was used as the chromogen, and the sections were counterstained with hematoxylin. Negative internal controls were treated the same way except for incubation with the primary antibody. Adjacent non-neoplastic liver tissue was used as a positive control.

Microvessel density (MVD) was defined as the number of CD34-positive staining vessels per field counted in the area of highest vascular density, as described previously [20]. Individual stained microvessels were counted at 400 magnifications. Random five fields, which appeared to contain the greatest number of microvessels in the areas per tumor section, were counted. The average of counts in these fields was considered in the analysis. All numbers in the text are quoted per mm^2.

Statistical analysis

CD66a expression was analyzed with clinicopathological parameters using chi-square test, and for survival time by the Kaplan–Meier method. CD34 expression was analyzed using independent t test. Statistical significance was considered when $p < 0.05$. All statistical analyses were performed using SPSS19 (SPSS, Chicago, IL, USA).

Results

Pattern of CD66a expression in hepatocytes and HCC

Nontumorous hepatocytes showed strong CD66a expression in the canalicular membrane (Figure 1A). For HCC, the CD66a expression was classified into three categories: diffuse expression, characterized by positive staining throughout the tumor specimen (Figure 1B); luminal expression, characterized by positive staining within the lumen-like structures in tumor cells (Figure 1C); and loss of expression (39 cases), in which there were distinct areas of negative staining within the tumor specimen (Figure 1D). Chi-square analysis showed that CD66a expression was not significantly correlated with age, sex, virus infection, serum alpha-fetoprotein level, stage, recurrence, histological grade, vascular invasion, capsular invasion, inflammation, fibrosis, and cirrhosis. CD66a was significantly associated with transcatheter arterial embolization and fatty change (Table 1). The mean tumor size of HCC with CD66a expression (47 cases) was 3.70 ± 2.13 cm, and the mean tumor size of the other 39 HCC cases without CD66a expression was 5.81 ± 4.95 cm. Independent *t* test also

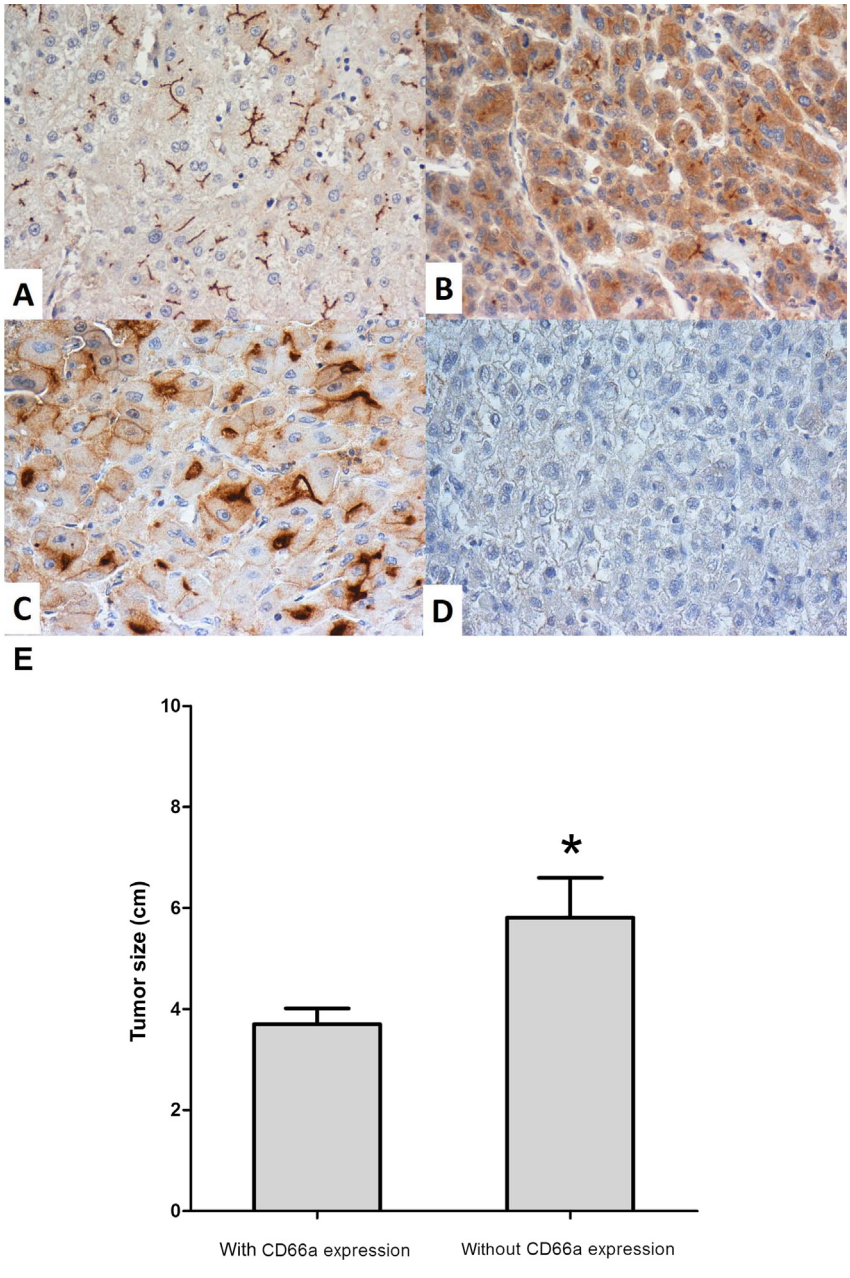

Figure 1. CD66a expression pattern in hepatocytes and HCC. CD66a showed (A) canalicular pattern in normal liver tissue, and (B) diffusely cytoplasmic staining, (C) luminal staining, and (D) loss of CD66a expression in HCC. (E) Tumor size in HCC is significantly associated with CD66a expression (mean $\pm$ SE). HCC = hepatocellular carcinoma; SE = standard error. $*$ $p < 0.05$, statistically significant.

Table 1 Relationship between CD66a expression and clinicopathological parameters.

	CD66a expression, *n* (%)			*p*
	1	2	3	
Age (y)				0.502
<50	3 (15.0)	8 (40.0)	9 (45.0)	
≥50	16 (22.6)	18 (28.1)	30 (46.9)	
Sex				0.274
Female	3 (17.6)	8 (47.1)	6 (35.3)	
Male	16 (23.9)	18 (35.3)	33 (49.3)	
Virus				0.305
None	1 (14.3)	4 (57.1)	2 (28.6)	
HBV	13 (28.3)	13 (28.3)	20 (43.5)	
HCV	2 (9.1)	6 (27.3)	14 (63.6)	
HBV + HCV	3 (33.3)	3 (33.3)	3 (33.3)	
Serum AFP (ng/dL)				0.429
≤20	4 (12.9)	12 (38.7)	15 (48.4)	
>20	10 (22.7)	12 (27.3)	22 (50.0)	
Unknown	5	2	2	
Stage				0.638
I/II	16 (21.0)	17 (33.9)	29 (45.2)	
III/IV	3 (9.1)	9 (40.9)	10 (50.0)	
Recurrence				0.066
Absent	9 (18.4)	12 (24.5)	28 (57.1)	
Present	10 (28.6)	14 (40.0)	11 (41.7)	
TAE				0.007*
Absent	14 (23.9)	17 (43.5)	14 (31.1)	
Present	5 (13.2)	9 (26.3)	24 (64.9)	
Unknown			1	
Histologic grade				0.733
Grade 1	2 (14.3)	5 (35.7)	7 (50.0)	
Grade 2	14 (24.1)	19 (32.8)	25 (43.3)	
Grade 3	3 (25.0)	2 (16.7)	7 (58.3)	
Vascular invasion				0.748
Absent	13 (22.8)	19 (33.3)	25 (43.9)	
Present	6 (22.2)	7 (25.9)	14 (51.9)	
Capsular invasion				0.436
Absent	16 (26.2)	18 (29.5)	27 (44.3)	
Present	3 (13.0)	8 (34.8)	12 (52.2)	
Inflammation				0.711
Grade 1	1 (33.3)	1 (33.3)	1 (33.3)	
Grade 2	0 (0)	2 (33.3)	4 (66.7)	
Grade 3	2 (40.0)	2 (40.0)	1 (20.0)	
Grade 4	16 (20.8)	21 (30.0)	33 (47.1)	
Fibrosis				0.568
Stage 1	0 (0)	0 (0)	1 (100.0)	
Stage 2	2 (9.5)	8 (38.1)	11 (52.4)	
Stage 3	6 (27.3)	5 (22.7)	11 (50.0)	
Stage 4	11 (27.5)	13 (32.5)	16 (40.0)	
Cirrhosis				0.462
Absent	8 (18.2)	13 (29.5)	23 (52.3)	
Present	11 (27.5)	13 (32.5)	16 (40.0)	
Fatty change				0.039*
≤5%	5 (16.1)	7 (22.6)	19 (61.3)	
>5%–≤33%	11 (28.2)	13 (33.3)	15 (38.5)	
>33%–≤66%	1 (8.3)	6 (50.0)	5 (41.7)	
>66%	2 (100.0)	0 (0)	0 (0)	
MVD				0.036*
Low density	9 (18.0)	12 (24.0)	29 (58.0)	
High density	10 (29.4)	14 (41.2)	10 (29.4)	

* $p < 0.05$, statistically significant.
AFP = alpha-fetoprotein; HBV = hepatitis B virus; HCV = hepatitis C virus; MVD = microvessel density; TAE = transcatheter arterial embolization.

showed that CD66a expression was significantly associated with tumor size ($p = 0.016$; Figure 1E). However, the Kaplan–Meier method showed that CD66a expression was not associated with survival time (data not shown).

Pattern of CD34 expression in HCC

CD34 immunostaining highlights the endothelial cells within HCC (Figure 2). We used the MVD to assess the total numbers of microvessels. Independent *t* test showed that CD34 expression had no significant association with tumor size, virus infection, histological grade, and capsular invasion. CD34 was associated with clinical stage and vascular invasion (Table 2).

Correlation between CD66a and MVD

The mean intratumor MVD of the total HCC cases was 82.23 ± 27.1 (range: 24.33–158.67). MVD was considered low when its value was ≤82.23 and high when the value was >82.23. Chi-square analysis was used to analyze the association between CD66a and MVD. The result showed that high MVD had loss of CD66a expression. By contrast, HCC with positive staining of CD66a usually showed low MVD. CD66a expression was significantly associated with MVD ($p = 0.036$; Table 1).

Discussion

CD66a is considered a tumor suppressor in various malignancies, including breast cancer [21] and prostate cancer [22]. However, upregulated CD66a expression is likely to be an adverse prognostic factor in lung carcinoma and malignant melanoma [14,23]. A previous study reported that loss of expression of CD66a is an independent factor for poor prognosis in HCC patients [24]. In our study, the expression of CD66a was significantly associated with post-transcatheter arterial embolization treatment, but not with histological grade or clinical stage. When embolization was performed before the surgical procedure, a wide range of tumor necrosis was present in the specimen. In this situation, tissue sampling for sectioning included necrotic area and its adjacent residual tumor tissue. We found that the cancer cells farther away from the tumor center were

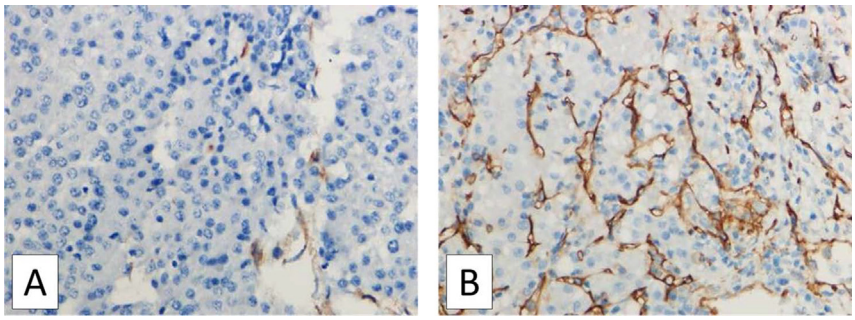

Figure 2. CD34 expression in HCC. CD34 immunostaining in HCC revealed (A) low MVD in low-grade HCC and (B) high MVD in high-grade HCC. HCC = hepatocellular carcinoma; MVD = microvessel density.

Table 2 Relationship between MVD and clinicopathological parameters.

Parameters	MVD, mean ± SD	*p*
Tumor size (cm)		
<5	87.98 ± 26.65	
≥5	79.15 ± 23.88	0.176
Virus infection		
None	79.91 ± 18.16	
HBV	82.74 ± 24.55	0.774
HCV	85.96 ± 27.78	0.597
HBV + HCV	98.83 ± 33.21	0.219
Clinical stage		
I/II	91.38 ± 25.67	
III/IV	68.84 ± 18.82	0.001*
Histological grade		
Grade 1	94.23 ± 28.62	
Grade 2	94.23 ± 24.66	0.230
Grade 3	70.88 ± 22.87	0.066
Vascular invasion		
Absent	90.34 ± 26.77	
Present	73.68 ± 19.38	0.010*
Capsular invasion		
Absent	83.98 ± 23.85	
Present	86.77 ± 30.23	0.685
Fatty change		
<5%	77.26 ± 25.44	
5—33%	85.65 ± 24.67	0.213
33—66%	98.55 ± 25.19	0.028*
>66%		

* $p < 0.05$, statistically significant.
HBV = hepatitis B virus; HCV = hepatitis C virus; MVD = microvessel density; SD = standard deviation.

able to survive and were less affected by embolization. In addition, cancer cells with high-grade histologic features were more resistant to hypoxia caused by embolization and survived.

It has been shown that tumor size after embolization treatment is significantly related to distant metastasis [25]. It indicates that residual cancer cells within a larger tumor that survive after embolization treatment are likely to cause distant metastasis. Our results also showed that the residual cancer cells were found to be absence of CD66a expression after embolization treatment. In addition, CD66a expression was significantly associated with tumor size. In fact, a larger tumor size will cause more hypoxic environment areas in the tumors. Cancer cells with higher histological grade and aggressive biological behavior usually tolerate and survive in the hypoxic environment. Therefore, it is consistent with our result that revealed loss of CD66a staining in larger HCC tumors.

In our study, CD66a expression was also associated with fatty change. The function of CD66a is to adjust the insulin clearance in the liver so that the insulin concentration in the blood is increased. Once the blood continues to maintain a high concentration of insulin, tissue insulin resistance and glucose tolerance will result; hence, fat easily accumulates in the organ [26,27]. Therefore, we speculate that CD66a may be involved in the development of fatty liver and may play an important role in hepatocarcinogenesis.

Angiogenesis is characterized by proliferation of new blood vessels from endothelial cells and occurs during wound repair, reproduction, and development. The angiogenesis process includes cell migration, proliferation, microvascular differentiation, extracellular matrix degradation, and structural reorganization [28]. It has been shown that tumor growth is associated with angiogenesis, by biological, pharmacological, and genetic evidence [29].

Endothelial progenitor cells from bone marrow are recruited to the vascular bed of tumors and contribute to tumor growth [30]. MVD is a reliable indicator for evaluating angiogenesis in tumors. IHC staining of vascular endothelial cell markers, such as CD34, CD31, and vascular endothelial growth factor, is used for highlighting the vascular channels in a tumor [31—34]. Previous studies have reported that MVD was an adverse predictor in several cancers [35], including HCC [33] and pancreatic cancer [36]. In our study, MVD was significantly compared with clinical stage, vascular invasion, and fatty change.

Loss of CD66a expression may predict poor survival after resection because it represents aggressive tumor biology characterized by large tumor size and higher histological grade. Our results showed that well-differentiated HCCs (Grades 1 and 2) were characterized by diffuse CD66a expression. As the tumor progressed, poorly differentiated foci (Grade 3) revealed loss of CD66a expression and emerged within the background tumor tissue that had diffuse CD66a expression [37]. Loss of CD66a expression in the foci always had a high histological grade, suggesting that loss of CD66a expression may reflect the

dedifferentiation of HCC cells. CD66a expression is down-regulated with increasing histological grade in malignant cells derived from the liver, endometrium, and prostate. Cervello et al. [38] showed loss of CD66a expression in 80% of poorly differentiated or undifferentiated HCCs, but not in well-differentiated tumors. The current study also revealed a high frequency of loss of CD66a expression in poorly differentiated HCCs (Grade 3). These findings suggest that loss of CD66a expression is usually a late event in the progression of HCC.

In colorectal adenocarcinoma, Neumaier et al. [39] reported that reduced CD66a expression of tumor cells was associated with regional lymph node metastasis. Pu et al. [40] found that in a transgenic mouse model of prostate carcinoma, expression of an epithelial cell adhesion molecule (a mouse homolog of CD66a) was lost in tumor cells involving lymph nodes. These data suggest that loss of CD66a expression in cells from a variety of tumors indicates a high metastatic potential. In conclusion, loss of CD66a expression of tumor cells may reflect the dedifferentiation of cancer cells in HCC. It also reflects aggressive tumor biology and thus indicates a poor prognosis for patients with HCC.

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