Expression of CD105 (Endoglin) in Hepatocellular Carcinoma and Correlation with Intrahepatic Metastasis: Analysis Using Tissue Microarrays and Comparison with Other Endothelial Marker

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Abstract: Neovascularization provides the route for nutrient supply to the tumor and the conduit for tumor cells to be shed into the circulation. CD31 is a pan-endothelial cell marker and CD105 is an active endothelial cell marker, but whether there is a link between CD105 expression and metastasis in Hepatocellular carcinoma (HCC) still remains unclear. A tissue microarray containing 38 HCCs and adjacent non-tumorous liver tissue samples was constructed. The microvessel density (MVD) of CD31, CD105, vWF and the expression of PCNA, VEGF were investigated in a HCC tissue microarray by immunohistochemistry. There was a significant difference between the score of MVD-CD31 in HCC (48.5 ± 29.7) and non-tumorous liver tissue (24.2 ± 22.3, P<0.01). The mean score of MVD-CD31 was higher in HCCs with high PCNA expression (68.4 ± 37.0) than in HCCs with low PCNA expression (37.9 ± 30.9, P = 0.012). MVD-CD105 and VEGF expression were significantly higher in HCC with intrahepatic metastasis (P < 0.01). Multivariate analysis confirmed that MVD- CD105 but not MVD-CD31 was an independent contributing factor to the intrahepatic metastasis. In conlusions, MVD-CD105 expression is associated with intrahepatic metastasis of HCC. Because CD105 is expressed in the activated endothelial cells of the newly formed blood vessels, neovascularization is important in the metastasis of HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers and is one of the leading causes of cancer death in Japan [1]. It is a tumor characterized by a propensity for vascular invasion and a high metastatic potential [2]. Angiogenesis is a prerequisite for tumor growth and metastasis [3]. Neovascularization provides not only the route for nutrient supply to the tumor but also the conduit for tumor cells to be shed into the circulation [4]. Newly formed capillaries have leaky basement membranes, making them more accessible to tumor cells than mature vessels. It has been demonstrated that increasing density of newly proliferating microvessels in growing tumors was correlated closely with increasing number of tumor cells shed into the bloodstream [5]. CD105 (endoglin) is a component of the transforming growth factor beta (TGF-β) receptor complex as it binds TGF-β1 and TGF-β3 with high affinity. CD105 has been reported as expressed by endothelial cells of proliferating capillaries [6]. However there are few reports on the expression of CD105 in hepatocellular carcinoma [7, 8]. The expression pattern of CD105 and a link between CD105 expressing capillaries and metastasis in HCC still remain unclear. Tissue microarray is a method which can detect the protein expression of a large number of samples simultaneously [9]. Therefore, we conducted a prospective study using tissue microarray methodology to evaluate the expression of CD105 in 38 resected HCCs.

MATERIALS AND METHODS

Patients and Tissue Samples

Thirty eight patients with curative resection of HCC were recruited into this prospective study at Saga University Hospital from 1999 to 2004 under the guideline of the Ethical Committee for Human Study, Saga University School of Medicine. The average age of patients was 64 years, ranging in age from 43 to 82 years. There were 7 women and 31 men. The average tumor size was 6.6 cm with 13 tumors less than 3 cm. Among the 38 patients with HCC, 27 had hepatitis C infection and 6 had hepatitis B infection. The fresh tissue specimens were fixed in 10% buffered formalin and embedded in paraffin. Intrahepatic metastasis (IM) was diagnosed as follows: near the main tumor, much smaller than main tumor and with the same histology type as the main tumor. During this study, we found that the number of surgically resected HCC cases larger than 3 cm in diameter was small and we added further 24 cases to match the number of HCC with large size for comparison by courtesy of our affiliated hospital Koseikan.

Construction of Tissue Microarrays of HCC

Tissue microarray was constructed using a Tissue Arrayer (Beecher Instruments Inc., Sun Prairie, WI, USA), providing microsections of 2 mm in diameter (Fig. **1**). The appropriate tumor areas were selected on HE paraffin sections. Three areas which were located in the central and pe-

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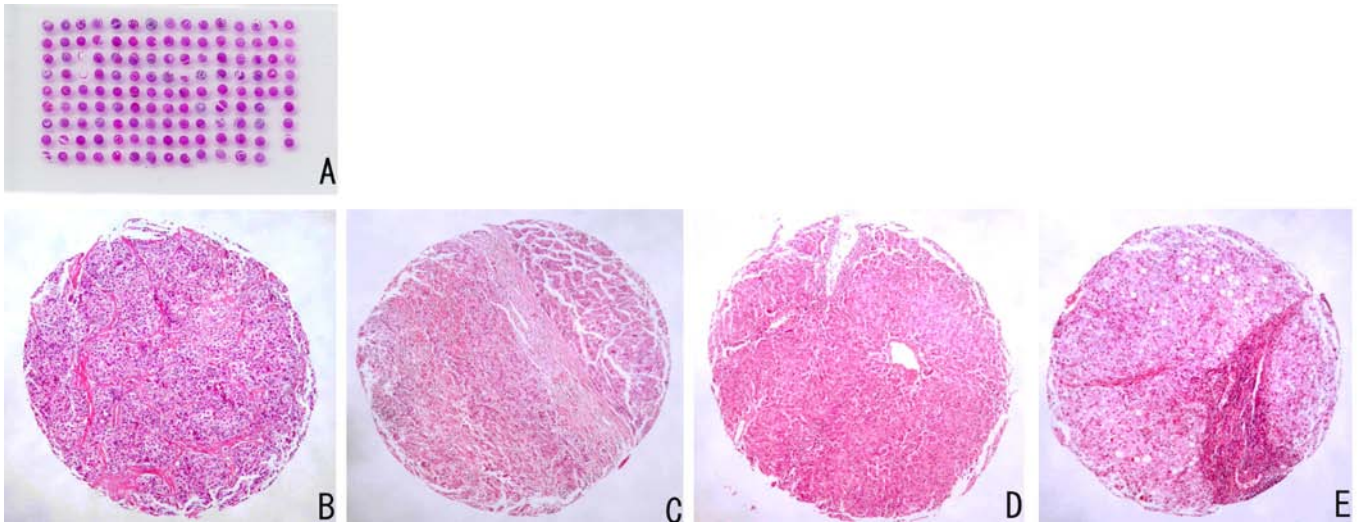

Fig. (1). The tissue microarray of HCC. (**A**) Slide of H.E. staining. (**B-E**) Representative tissue samples. (**B**) HCC. (**C**) Transitional zone of HCC. (**D**) Chronic hepatitis. (**E**) Cirrhosis.

ripheral part of the cancer, and non-cancerous liver tissue were selected in each sample. Cores were punched out from donor blocks and placed in recipient blocks. An array block of 119 cores was derived from normal liver tissue (n = 5), center part of the cancer (n = 38), peripheral part of the cancer (n = 38) and non-cancerous liver tissue (n = 38). The array blocks were then incubated for 30 min at 37℃ to improve adhesion between the cores and the recipient paraffin block. Immunohistochemical studies were performed on 4μm-thick paraffin sections.

Double-Immunofluorescence Staining

Double-immunofluorescence staining was performed following a previously reported method [10]. Briefly, the tissue array slides were deparaffinized and soaked in 0.01 M citrate buffer, pH 6.0, at 90°C for 40 min for antigen retrieval. The primary murine monoclonal antibody CD31, and then rhodamine-labeled secondary rabbit polyclonal antibody against murine IgG were allowed to react. After inactivation of the first step of primary and secondary antibodies by heating at 90°C for 15 min in citrated buffer, the second immunoreaction was carried out with another primary murine monoclonal antibody, CD68, and then with FITC-labeled secondary rabbit polyclonal antibody. Rhodamine and FITC-labeled samples were examined using a fluorescence microscope (Olympus BX60, Tokyo, Japan). To detect nonspecific-antibody binding, control sections were incubated with either normal murine or rabbit serum or phosphate-buffered saline instead of primary antibody. No staining was observed in these control samples.

Immunohistochemical Staining

Sections of tissue microarray were immunostained with human CD105, CD31, von Willebrand factor (vWF), proliferating cell nuclear antigen (PCNA), vascular endothelial growth factor (VEGF) monoclonal antibodies, respectively. Table **1** showed the details of antibody used in this research. The tissue sections were dewaxed, soaked in PBS (pH7.2), and then treated with 10% hydrogen peroxide to block endogenous peroxidase activity. Antigen retrieval was performed following a previously reported method [10]. The tissue microarray sections were then incubated with primary antibodies at 4℃ over night. The negative control was obtained by substituting the primary antibodies for PBS. Intensity of immunostaining for CD31, CD105 and vWF was assessed at ×200 magnification. The number of microvessels was counted from three areas and was expressed as microvessel density (MVD) on the average.

Table 1. Antibodies and Antigen Retrieval Methods

Antibody	Clone	Pretreatment	Dilution	Company
CD31	Mono	Proteinase K	1:20	Dako
CD105	Mono	MW-EDTA	1:50	Novocastra
vWF	Mono	Proteinase K	1:100	Dako
VEGF	Mono	MW-AC	1:100	Santa Cruz
PCNA	Mono	MW-EDTA	1:50	Dako

Antigen retrieval method for immunohistochemistry: MW:microwave, AC:citrate buffer, For immunofluorescence, see Materials and Methods.

The rate of PCNA-positive cancer cells was defined as the positively stained nuclei to the total cell count. The positive nuclei less than 1% was evaluated as 0, 1-20% evaluated as 1, 21-50% evaluated as 2, over 50% evaluated as 3. We regarded 2 and 3 as high expression of PCNA. The judgment of VEGF staining was adopted as the proper immunohistochemical score of the HCC on the basis of strength: 0, negative; 1, weak staining; 2, moderarate staining; 3, strong staining. We regarded 1-3 as positive VEGF staining. In this study, the staining pattern of VEGF was diffuse.

All of the immunostaining scores were calculated by two pathologists in a blinded manner.

Statistical Analysis

The clinical and pathologic characteristics of the patients in relation to MVD of CD105, CD31, vWF and the expression of VEGF, PCNA were compared by the student's *t* test and x^2 test. SYSTAT 10.2 (Systat Software Inc., Chicago,

IL, USA) was used. The P value of less than 0.05 was considered to be significant. For Multivariate analysis, JMP Statistics program (SAS Institute, Cary, NC, USA) was used.

RESULTS

The Expression Pattern of CD31, vWF, CD105, PCNA and VEGF in HCC and Non-Tumorous Liver

CD31 was universally expressed in endothelial cells of newly formed sinusoid in HCC (Fig. **2A**). In non-tumorous liver tissues, which were normal, cirrhotic or associated with chronic hepatitis, there was a limited staining at the portal area in the normal liver, and a sparse staining in the latter two (Fig. **2B**).

The pattern of staining by anti-vWF was different from that by anti-CD31, with staining of mainly larger vessels in the fibrous tissue within the tumor, rather than the capillary-like sinuoids between cancer cells (Fig. **3A**). CD105 was also expressed in endothelial cells of newly formed sinusoids in HCC (Fig. **3B**). PCNA was expressed in the nuclei (Fig. **3C**). VEGF was expressed mainly in the cytoplasm of HCC and non-tumorous liver tissues (Fig. **3D**).

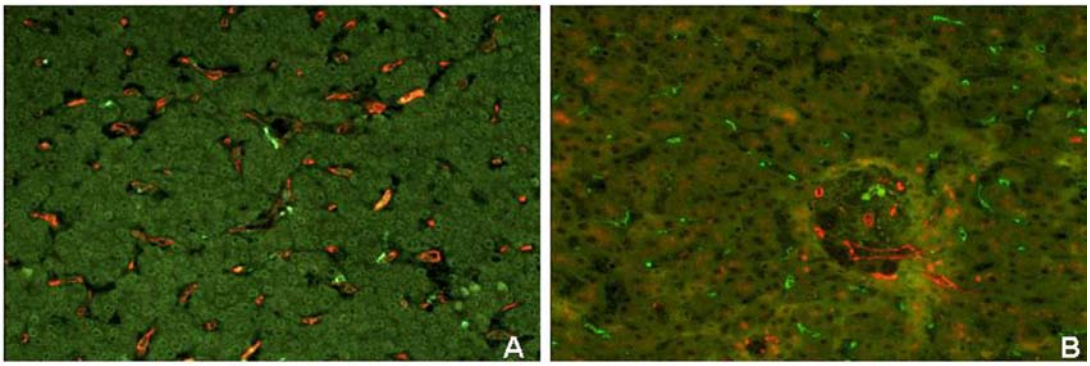

Fig. (2). CD31 expression in HCC and non-HCC liver tissue. (**A**) CD31 (red) was observed in endothelial cells of newly formed sinusoid in HCC. (**B**) CD31 was only positive in the vascular endothelial cells in portal area in non-HCC liver tissue. Green: CD68 positive kupffer's cells. x200.

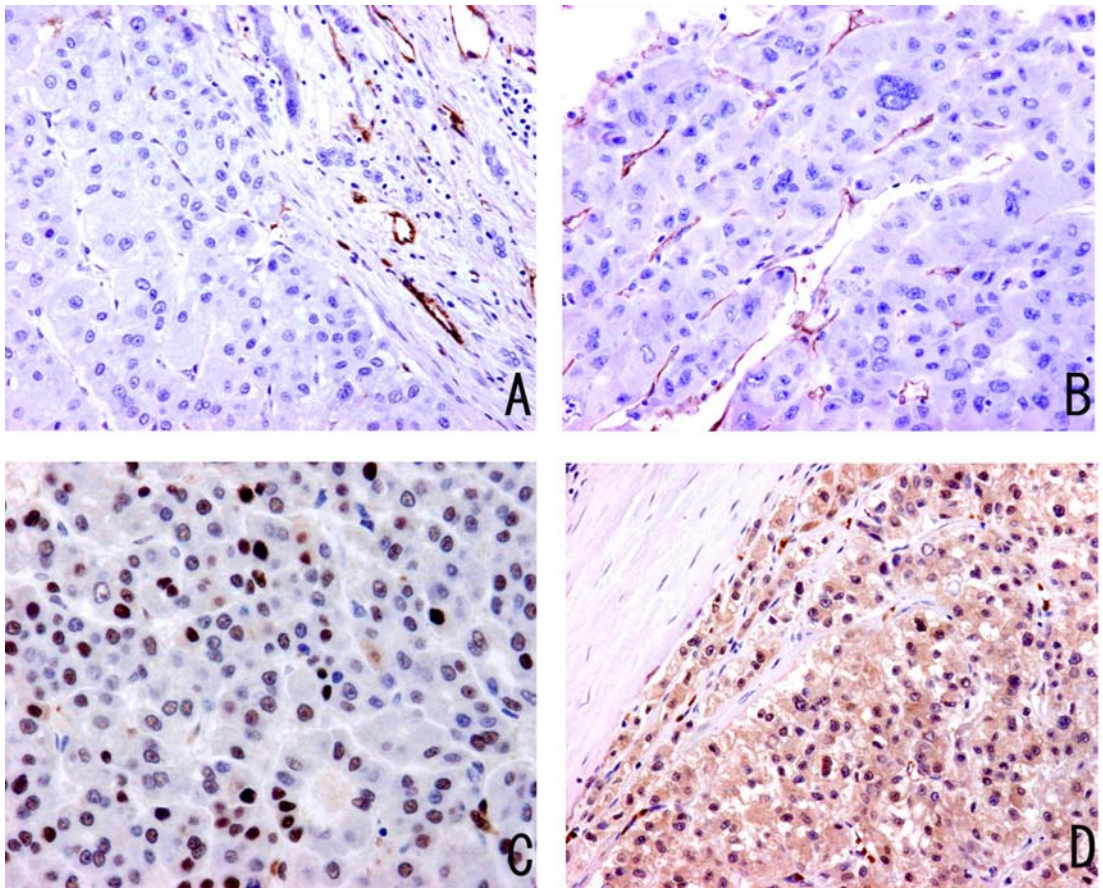

Fig. (3). The vWF, CD105, PCNA, and VEGF expression in HCC. (**A**) The vWF expression was observed in endothelial cells of larger vessels in the fibrous tissue of tumor. (**B**) CD105 expression was observed in endothelial cells of newly formed sinusoids in HCC. (**C**) PCNA expression was seen in nuclei of HCC cell. (**D**) VEGF expression was observed in HCC cell cytoplasm in a diffuse staining pattern. x200.

MVD in HCC and Non-Tumorous Liver

The mean score of MVD-CD31 was 48.5 ± 29.7 in HCC, whereas the mean score of MVD-CD31 was 24.2 ± 22.3 in non-tumorous liver tissue (Fig. **4**). There was a significant difference between the score of MVD-CD31 in HCC and non-tumorous liver tissue ($P < 0.01$). The mean score of MVD-CD31 was higher in HCC with high PCNA expression (68.4 ± 37.0) than in HCCs with low PCNA expression (37.9 ± 30.9, $P = 0.012$) (Fig. **5**).

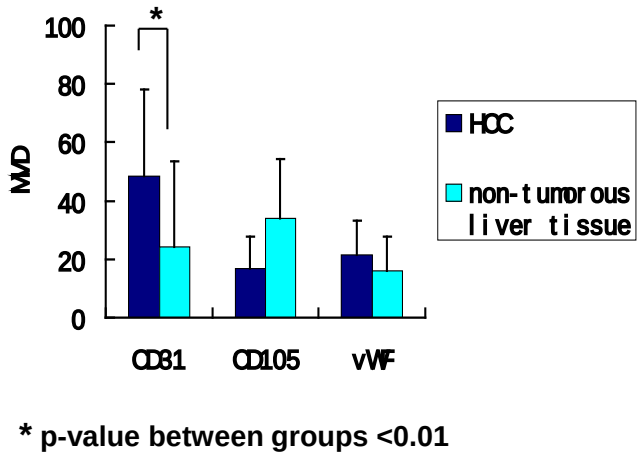

Fig. (4). Comparison of MVD in HCC and non-tumorous liver tissue. There was a significant difference on the score of MVD-CD31 between HCC and non-tumorous liver tissue(* P<0.01). But there was no significant difference on the score of MVD-105 or vWF between HCC and non-tumorous liver tissue.

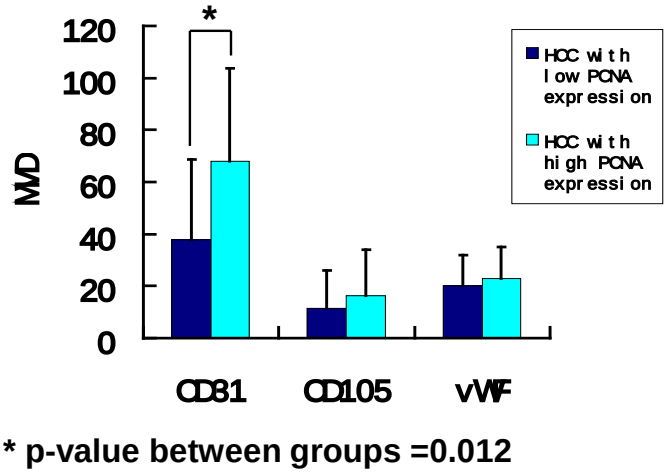

Fig. (5). The relationship between MVD-CD31, -CD105, and -vWF and the expression of PCNA in HCC. The mean score of MVD-CD31 was higher in HCCs with high PCNA expression than with low PCNA expression. * P=0.012. There was no significant difference between the score of CD105 and vWF.

The MVD-CD105 was significantly lower than MVD-CD31 in the same tissue. There was a diffuse staining pattern of CD105 in adjacent non-tumorous livers in some specimens. There was no significant difference between the score of MVD-105 or vWF in HCC and non-tumorous liver tissue.

Correlation Between MVD and Expression of PCNA and VEGF and Clinicopathological Parameters

The analysis of MVD-CD31, MVD-CD105, MVD-vWF in relation to various clinicopathological parameters is summarized in Table **2**. Significantly higher MVD-CD105 was only associated with HCC with intrahepatic metastasis (IM) ($p < 0.01$). Multivariate analysis showed that a high MVD-CD105 was a significant ($p = 0.0214$) and independent contributing factor to intrahepatic metastasis (IM, Table **3**). Furthermore, in large HCCs (> 3cm) with IM, the MVD-CD105 was 27.09 ± 18.35, while in large HCCs without IM, the MVD-CD105 was 7.27 ± 6.07. It revealed that CD105 was significantly stronger expressed in HCC with IM even in large tumours (Fig. **6**). No significant differences, however, were found between MVD-CD31 or MVD-vWF and each clinicopathological parameter such as gender, tumor size, venous invasion and IM.

Table 2. Relationship Between MVD of CD105, CD31, vWF and Clinicopathological Parameters in HCCs

Variable	Positive Microvessel Density		
	MVD-CD105	MVD-CD31	MVD-vWF
Sex			
Male(n=31)	14.6±12.5	56.5±35.5	22.6±11.7
Female(n=7)	32.0±26.9	32.0±36.6	16.8±14.2
Tumor Size			
≤3 cm(n=13)	14.4±12.5	65.3±32.0	22.8±8.0
>3 cm(n=25)	19.3±18.7	49.6±37.1	21.5±13.5
Histology Type			
Well(n=7)	15.1±14.9	45.2±32.3	14.8±10.7
Moderate(n=27)	15.9±14.1	56.8±39.2	22.4±12.6
Poor(n=4)	35.5±14.1	34.8±7.8	26.8± 8.3
IM			
With (n=14)	26.8±19.8*	66.6±39.4	25.2±15.2
Without (n=24)	11.9±11.1*	44.9±32.9	19.7±9.9
Vascular Invasion			
With(n=18)	18.2±17.4	54.7±39.8	22.7±15.4
Without(n=20)	17.4±17.3	50.5±33.7	20.6±8.2

*$P < 0.01$.
IM:Intrahepatic metastasis (IM).

There was no significant difference in the intensity of VEGF expression between the HCC cells and the non-tumorous liver cells. However, VEGF was expressed in 92.3% (12/13) of HCCs with IM and in 45.8% (11/24) of HCCs without IM. VEGF expression was significantly higher in HCC with IM ($p < 0.01$).

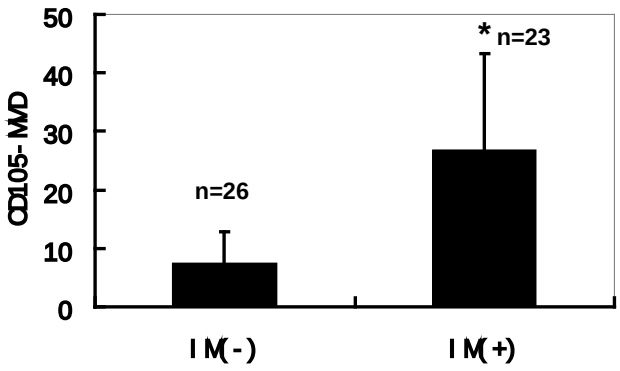

Fig. (6). MVD-CD105 in large HCCs (> 3cm) with or without intrahepatic metastasis (IM). MVD-CD105 is significantly higher in HCC with IM than those without IM. $^{*}P = 0.0214$. Twenty five HCC cases larger than 3cm were combined with 24 those cases from Koseikan Hospital.

Table 3. Multivariate Analysis of the Contributing Factors to the Intrahepatic Metastasis

Factor	F	P Value
Diameter	2.07	0.5584
Histology type	1.51	0.9590
Capsule invasion	5.28	0.1527
Venous invasion	2.15	0.9889
MVD-CD105	9.69	0.0214
MVD-CD31	2.21	0.5291
Age	4.68	0.1964

MVD: microvessel density.

DISCUSSION

The growth and metastasis of solid tumors are dependent on the formation of new blood vessels [11]. Angiogenesis is closely associated with the cancer development and facilitates tumor progression and metastasis [12]. Different from that in normal physiological condition, the balance of pro-angiogenesis and anti-angiogenesis is disturbed in tumor microenvironment, thus leading to abnormal vessel growth. Intratumoral microvessel density (IMVD) has been extensively investigated and found to be a useful prognostic marker in many cancers [13-15]. CD31 is a pan-endothelial cell marker, which is expressed in both of immature tumor blood vessels and mature blood vessels [16]. IMVD-CD31 may reflect the exact number of tumor blood vessels than other endothelial cell marker. We found that the mean score of MVD-CD31 in HCC was markedly higher than that in non-tumorous liver tissue. The frequency of MVD was in accordance with the results of previous studies on HCC [17]. And in non-tumorous liver, CD31 positive vessels existed on portal canal area. There was also a significant difference in the score of MVD-CD31 between HCC with different PCNA expression. These results suggested that increased angiogenesis prompted the proliferation of HCC, which might be related to the carcinogenesis of HCC.

Metastasis is the most lethal attribute of malignant tumors. HCC often gives rise to IM, which would lead to failure of the cure. CD105 is a homodimeric membrane glycoprotein expressed on endothelial cells that can bind to TGF-β1 and -β3. It is only expressed on active endothelial cells and consequently be able to reflect a neoangiogenesis in malignant tumors [10, 18]. Neovascularization provides not only the route for nutrient supply to the tumor but also the conduit for tumor cells to be shed into the circulation because newly formed vessels are often absent of basement membrane [19]. Recent research has shown that CD105 is only weakly expressed in normal tissues, but it is strongly expressed in tumor endothelial cells [10, 20, 21]. CD105 has been demonstrated to be a good tumor angiogenesis marker in breast cancer [22], colorectal cancer [10, 23] and melanoma [24]. Higher expression and the superiority of CD105 over CD31 in active angiogenesis in HCC were recognized by many investigators [7, 8, 25, 26], but there are controversial opinions about its specificity in that the higher expression was not only present in microvessels in HCC but also in hepatic sinus endothelium in non-tumorous tissue with or without cirrhosis [26]. CD105 expression as a prognostic indicator is also controversial in microvessels of HCC [7, 8]. Benetti reported that TGF-β1 promotes the migration of CD105 expressing HCC-derived endothelium [27]. But the direct link between CD105 expression and IM still remains unclear; we found that CD105 expression was higher in HCCs with IM than those without IM. Multivariate analysis showed that a high MVD-CD105 was a significant and independent contributing factor to intrahepatic metastasis. And CD105 was significantly expressed stronger in HCC with IM even in same size tumors. In our knowledge, we reported the close relationship between MVD-CD105 and IM for the first time. These results suggested that neovascularization might promote IM in HCC. Interestingly, we found a diffuse staining of CD105 in non-tumorous livers adjacent to HCC, which was previously reported [26]. It was not surprising because most of the HCC patients in this study were infected with hepatitis C virus (27/38) or B virus (6/38), which leads to chronic hepatitis or cirrhosis. The state of virus infection will induce a biological activity to the liver cell and enhance the CD105 expression.

Among the angiogenesis factors, VEGF is the most important one and a hot field of the study at present. A high serum level of VEGF is a predictor of poor outcome after resection of HCC [28]. We found that VEGF was expressed in 92.3% (12/13) of HCCs with IM and in 45.8% (11/24) of HCCs without IM. This result suggested that up-regulation of VEGF was related to IM of HCC.

In conclusion, we detected the MVD of CD31, CD105, vWF and the expression of PCNA, VEGF using a HCC tissue microarray. A higher MVD-CD105 was associated with IM of HCC. As CD105 is expressed on new blood vessels, neovascularization might be important in the metastasis of HCC. Further studies are needed to clarify the mechanisms.

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