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

Li Hou¹, Daisuke Mori², Yukari Takase¹, Rahmawati Minhajat¹, Fumio Yamasaki¹, Kohji Miyazaki³ and Osamu Tokunaga*,¹

Departments of ¹Pathology and Biodefense and ³Surgery, Faculty of Medicine, Saga University, Saga, Japan
²Clinical Laboratory, Pathology Division, Saga Prefectural Hospital Koseikan, Saga, Japan

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-CD105 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 conclusion, 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. 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-
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°C 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°C 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

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Pretreatment</th>
<th>Dilution</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD31</td>
<td>Mono</td>
<td>Proteinase K</td>
<td>1:20</td>
<td>Dako</td>
</tr>
<tr>
<td>CD105</td>
<td>Mono</td>
<td>MW-EDTA</td>
<td>1:50</td>
<td>Novocastra</td>
</tr>
<tr>
<td>vWF</td>
<td>Mono</td>
<td>Proteinase K</td>
<td>1:100</td>
<td>Dako</td>
</tr>
<tr>
<td>VEGF</td>
<td>Mono</td>
<td>MW-AC</td>
<td>1:100</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>PCNA</td>
<td>Mono</td>
<td>MW-EDTA</td>
<td>1:50</td>
<td>Dako</td>
</tr>
</tbody>
</table>

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, moderate 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 x² test. SYSTAT 10.2 (Systat Software Inc., Chicago,
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.
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 mean 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-CD105 or vWF between HCC and non-tumorous liver tissue.

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

<table>
<thead>
<tr>
<th>Variable</th>
<th>Positive Microvessel Density</th>
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<tbody>
<tr>
<td></td>
<td>MVD-CD105</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male (n=31)</td>
<td>14.6±12.5</td>
</tr>
<tr>
<td>Female (n=7)</td>
<td>32.0±26.9</td>
</tr>
<tr>
<td>Tumor Size</td>
<td></td>
</tr>
<tr>
<td>≤3 cm (n=13)</td>
<td>14.4±12.5</td>
</tr>
<tr>
<td>&gt;3 cm (n=25)</td>
<td>19.3±18.7</td>
</tr>
<tr>
<td>Histology Type</td>
<td></td>
</tr>
<tr>
<td>Well (n=7)</td>
<td>15.1±14.9</td>
</tr>
<tr>
<td>Moderate (n=27)</td>
<td>15.9±14.1</td>
</tr>
<tr>
<td>Poor (n=4)</td>
<td>35.5±14.1</td>
</tr>
<tr>
<td>IM</td>
<td></td>
</tr>
<tr>
<td>With (n=14)</td>
<td>26.8±19.8</td>
</tr>
<tr>
<td>Without (n=24)</td>
<td>11.9±11.1</td>
</tr>
<tr>
<td>Vascular Invasion</td>
<td></td>
</tr>
<tr>
<td>With (n=18)</td>
<td>18.2±17.4</td>
</tr>
<tr>
<td>Without (n=20)</td>
<td>17.4±17.3</td>
</tr>
</tbody>
</table>

*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).
HCC with IM than those without IM. *P = 0.0214. Twenty five trahepatic metastasis (IM).

MVD-CD105 is significantly higher in Fig. (6).

MVD-CD105 in large HCCs (> 3cm) with or without in-

portal canal area. There was also a significant difference in

and consequently be able to reflect a neoangiogenesis in ma-

lignant 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 be-

cause 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 melano-

ma [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 controver-

sial opinions about its specificity in that the higher expres-

sion 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-CD31 was a significant and inde-

pendent 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 stain-
ing 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.

Metastasis is the most lethal attribute of malignant tu-
mors. HCC often gives rise to IM, which would lead to fail-
ure of the cure. CD105 is a homodimeric membrane glyco-

protein expressed on endothelial cells that can bind to TGF-

β1 and β3. It is only expressed on active endothelial cells

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Among the angiogenesis factors, VEGF is the most im-
portant 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 tis-
sue 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.

**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 facilita-
tes 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 exten-
sively investigated and found to be a useful prognostic
indicator in many cancers [13-15]. CD105 has been demon-
strated to be a good tumor angiogenesis marker
in breast cancer [22], colorectal cancer [10, 23] and melano-
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IM of HCC. As CD105 is expressed on new blood vessels,
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**Table 3. Multivariate Analysis of the Contributing Factors to
the Intrahepatic Metastasis**

<table>
<thead>
<tr>
<th>Factor</th>
<th>F</th>
<th>P Value</th>
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<tbody>
<tr>
<td>Diameter</td>
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<td>0.5584</td>
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<tr>
<td>Histology type</td>
<td>1.51</td>
<td>0.9590</td>
</tr>
<tr>
<td>Capsule invasion</td>
<td>5.28</td>
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</tr>
<tr>
<td>Venous invasion</td>
<td>2.15</td>
<td>0.9889</td>
</tr>
<tr>
<td>MVD-CD105</td>
<td>9.69</td>
<td>0.0214</td>
</tr>
<tr>
<td>MVD-CD31</td>
<td>2.21</td>
<td>0.5291</td>
</tr>
<tr>
<td>Age</td>
<td>4.68</td>
<td>0.1964</td>
</tr>
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</table>

MVD: microvessel density.

* p-value between groups =0.0214
Expression of CD105 (Endoglin) in Hepatocellular Carcinoma

ACKNOWLEDGEMENTS

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REFERENCES


