Endoglin (CD105) Expression in Angiogenesis of Primary Hepatocellular Carcinomas: Analysis using Tissue Microarrays and Comparisons with CD34 and VEGF

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Abstract. Few studies about angiogenesis in hepatocellular carcinoma (HCC) have been conducted and little is known about the significance of angiogenesis in HCC. In this study, the clinicopathological significance of tumor microvessel density (MVD) was assessed in 105 patients with HCC by immunohistochemical staining of CD105, CD34, and vascular endothelial growth factor (VEGF). Moreover, the use of the tissue microarray technique in evaluating angiogenesis of HCC was appraised. The MVD by CD105 immunostaining (MVD-CD105) was significantly lower in larger tumors (5 cm diameter as a cutoff point, p = 0.001), more aggressive tumors, as indicated by venous infiltration (present vs absent, p = 0.001), and tumors with advanced TNM stage (stage I & II vs stage III, p = 0.011). A lower score of MVD by CD34 immunostaining (MVD-CD34) showed significant association only with venous invasion (p <0.001), whereas the MVD by CD105 immunostaining in tissue microarray (MVD-MA) was significantly lower only in larger sized tumors (p = 0.043). Moreover, MVD-CD105 was positively associated with the expression intensity of VEGF (p = 0.009), but not for MVD-CD34 (p = 0.088). When median scores of MVD were used as cut-off points, the patients with higher score of MVD-CD105 had a significantly poorer prognosis in either disease-free or overall survival analysis (p = 0.002 and p = 0.009, respectively), whereas similar prognostic significance of MVD-CD34 was not observed in overall survival analysis (p = 0.052) but was observed in disease-free survival analysis (p = 0.022). No prognostic significance of MVD-MA was found in either disease-free or overall survival analysis (p = 0.277 and p = 0.712, respectively). These data demonstrate the superiority of CD105 over CD34 as a marker of angiogenesis in HCC and indicate that the tissue microarray technique is unsuitable for evaluating angiogenesis in HCC.

Keywords: liver carcinoma, angiogenesis, endoglin, CD105, CD34, VEGF, tissue microarray

Introduction

Tumor angiogenesis, the formation of new blood vessels associated with a neoplasm, is essential for tumor growth and metastasis, and is one of the most important events occurring in the neoplastic process [1,2]. Tumor microvessel density (MVD), reflecting angiogenesis in tumor areas, was shown to be an independent factor of prognosis and metastasis in many tumors [3-6], although some studies reported contrary findings, with nil or negative correlation between MVD and tumor recurrence or survival [7,8]. A possible reason for these discrepancies may be the different methods used in the evaluation of MVD.

Hepatocellular carcinoma (HCC) is a common and aggressive human tumor in southeast Asia, and is one of the major causes of morbidity and mortality. Even after careful surgical excision and chemotherapy, this tumor shows a high percentage of recurrence and metastasis, and the mean survival is very short compared to other major solid tumors.
HCC is a typical hypervascular tumor; intrahepatic and lung metastases suggest its hematogenous dissemination, and angiogenesis may be an important factor identifying those patients with high risk of recurrence or distant metastasis. However, little is known about the mechanism and contribution of angiogenesis in different stages of HCC development. Several studies have shown that certain endothelial markers are expressed diffusely in microvessels of HCC, and that their levels of expression correlate with the prognosis of the patients [9-11].

Most studies have used antibodies against von Willebrand factor, CD31, and CD34. However, these are pan-endothelial cell markers that react not only with proliferating vessels but also with

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Fig. 1. Immunohistochemical staining of consecutive histological sections of a hepatocellular carcinoma show the expression of CD105 (panel A) and CD34 (panel B), as indicated by the brown staining of the vasculature (x200). Several vessels that are highlighted by anti-CD34 (B) are not highlighted by anti-CD105 (A).

Fig. 2. Scatter plots show: (A) correlation between scores of MVD-CD105 and MVD-CD34, and (B) correlation between scores of MVD-CD105 and MVD-MA.
established vessels in the tumor. Therefore, these may not be ideal markers of tumor angiogenesis in HCC. Endoglin (CD105) is an integral membrane glycoprotein and an important component of the transforming growth factor-β (TGF-β) receptor complex [12]. Endoglin antibody binds preferentially to activated endothelial cells in angiogenesis [13,14], and thus is potentially a more specific marker for tumor angiogenesis than the pan-endothelial markers [15,16].

In this study, we investigated the expressions of CD105, CD34, and vascular endothelial growth factor (VEGF) in HCC using tissue microarray and immunohistochemical staining techniques. We examined 105 cases of surgically resected HCC in attempts to define a putative role for CD105 expression in tumor angiogenesis and to employ the tissue microarray technique to evaluate the angiogenesis in HCC.
Materials and Methods

Patients. A group of 105 consecutive patients with HCC, 89 men and 16 women, received curative resection in the Department of Hepatobiliary Surgery of Nanjing Drum Tower Hospital. The mean age of the patients was 50.0 ± 12.5 yr. In 72 patients (68.6%), the HCC was associated with liver cirrhosis, and 85 of the patients (81.0%) were positive for serum HBsAg. The maximum diameter of the HCC in this series averaged 7.3 cm. There were 66 patients with large tumors (maximum diameter >5 cm), 39 with small tumors (maximum diameter ≤5 cm), and 48 with multiple tumors (more than 2 nodules).

Before operation, no chemotherapy or other treatment was given and no patient was found to have extrahepatic metastasis. All patients were followed-up by monitoring serum α-fetoprotein levels and by ultrasonography every 3 mo after resection; for suspicious cases, computed tomography and/or magnetic resonance imaging were used to verify the recurrence. Patients who died from liver failure within 1 mo after operation were excluded from this study. No patients underwent re-resection for recurrence.

Tissue samples. Surgical specimens were fixed in 10% formalin and embedded in paraffin. Sections (4 μm thick) were cut from the blocks and stained with hematoxylin and eosin (H&E) for study of the pathological features of HCC in accordance with the Classification of Carcinomas of the Liver (Modified) proposed by UICC [17]. Sections (4 μm) were also prepared for immunohistochemical study.

Constructing tissue microarrays. Representative regions of the blocks were delineated by the H&E-stained sections and cylindrical tissue cores (6 mm diameter) were prepared with a tissue microarray constructor (Beecher Instruments MTA-1, Silver Spring, MD). Five cores were removed from each donor block and arrayed in a recipient block. Thereafter, sections (4 μm) were cut from the array blocks for immunohistochemical staining.

Immunohistochemical staining. HCC tissue sections were immunostained with CD105 and CD34, and tissue microarray sections were immunostained with CD105 and VEGF by the Elivision technique. Briefly, these sections were dewaxed in xylene, dehydrated in ethanol, and then treated with 3% hydrogen peroxide for 30 min to block endogenous peroxidase activity. After incubation with 10% normal mouse serum for 20 min to reduce non-specific antibody binding, the sections were incubated with rabbit polyclonal anti-CD105 antibody (diluted 1:200, Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-CD34 antibody (diluted 1:200, Santa Cruz Biotech.), or rabbit polyclonal anti-VEGF antibody (diluted 1:50, Santa Cruz Biotech.) at room temperature for 120 min. Then the sections were incubated with corresponding second antibody for 15 min, with 3,3'-diaminobenzidine (DAB) as the chromogen. Immunoglobulin-negative controls were used to rule out nonspecific binding. Immunohistochemical staining was assessed by 2 investigators who were unaware of the corresponding clinicopathological data. VEGF expression was categorized into 4 grades: negative, weak, moderate, and strong, according to the highest staining intensity of the five cores.

Microvessel counting. Tissue sections stained with CD105 and CD34 were used for evaluating MVD (designated MVD-CD105 and MVD-CD34, respectively), in accordance with Weidner’s [18] and Tanigawa’s [10] standards with a minor modification. Briefly, the slides were examined under x100 magnification for the hot spots rich in vessels and MVDs were counted under x200 magnification (0.708 mm²/field) according to the standards, so that every single brown-stained cell and cell cluster was calculated as a blood vessel, no matter whether a vessel lumen structure was seen. For vessels with large lumens, every 40 μm length of lumen was calculated as one vessel. Five different fields were chosen on each of the slides, and the stained vessels were counted simultaneously by 2 researchers under a multiocular microscope. The average of the 5 areas was recorded as the MVD score. Microarray sections stained with CD105 were totally screened for MVD (designated MVD-MA).

Statistics. Student’s t-test was used for comparison of continuous variables between groups. Pearson’s correlation coefficient was used to assess the relationship between continuous variables; Spearman’s correlation coefficient was used to assess the relationship between categorical variables. Overall survival was computed using the Kaplan-Meier method and comparison between groups was done by the log-rank test. Clinicopathological variables that were tested for correlation with MVD included gender, age, hepatitis B surface antigen (HBsAg) status, presence or absence of cirrhosis in non-tumorous liver, tumor size, tumor encapsulation, venous invasion, microsatellite nodules, and TNM stage. Statistical analyses were performed using the SPSS (version 12.0) statistical software; p <0.05 was considered statistically significant.

Results

Expressions of CD105 and CD34 and MVD scores in HCC. All of the HCC samples were immunoreactive with both anti-CD105 antibody and anti-CD34 antibody, highlighting microvessels and showing 3 patterns of expression: sinusoid-like, branching, and small without apparent lumina (endothelial sprouts) [19]. Fig. 1 shows typical immunostaining patterns of CD105 and CD34. Comparisons of consecutive sections showed that quite a few vessels highlighted by anti-CD34 were not highlighted by anti-CD105, whereas the vessels highlighted by anti-CD105 were almost all highlighted by anti-CD34. The mean score of MVD-CD105 was 141.0 ± 55.0, whereas the mean score of MVD-CD34 was 241.4 ±103.6, which was...
significantly higher (p <0.001). Significantly positive correlation was observed between the scores for the 2 markers (Pearson’s r = 0.774, p <0.001) (Fig. 2). In microarray sections (Fig. 3), CD105 was completely negative in 11/105 cases and the mean score of MVD-MA was only 57.2 ± 37.6, which was considerably lower than that of MVD-CD105, whereas significantly positive correlation was observed between them (Pearson’s r = 0.741, p <0.001) (Fig. 2).

**VEGF expression in HCC and the correlation between MVD and VEGF.** The expression intensity of VEGF in microarray sections were assessed in 4 grades: 14 cases negative, 35 cases weak, 33 cases moderate, and 23 cases strong (Fig. 3). Both MVD-CD105 and MVD-MA were positively associated with the expression intensity of VEGF, although MVD-CD105 showed a stronger correlation than MVD-MA (Spearman’s r = 0.254, p = 0.009 and r = 0.219, p = 0.025, respectively) whereas the correlation was not significant for MVD-CD34 (Spearman’s r = 0.167, p = 0.088) (Fig. 4).

**Correlation between MVD and clinicopathological parameters.** Table 1 summarizes the analyses of MVD-CD105, MVD-CD34, and MVD-MA in relation to various clinicopathological parameters. No significant correlations were found between either MVD-MA, MVD-CD105, or MVD-CD34 and gender, age, hepatitis B infection status, cirrhosis status of the adjacent non-tumorous liver, the status of tumor encapsulation, and microsatellite lesions of the patients. On the other hand, MVD-CD34, MVD-CD105, and MVD-MA were significantly related to several pathological variables. Both MVD-CD105 and MVD-MA were significantly lower in tumors >5 cm compared to tumors ≤5 cm in maximum diameter (p = 0.001 and p = 0.043, respectively), whereas lower scores of MVD-CD105 and MVD-CD34 both showed a statistically significant association with venous invasion (both p <0.001). However, only the score of MVD-CD105 showed significant correlation with TNM stage, with lower MVD observed in tumors with more advanced stage (stage I & II vs stage III, p = 0.011).

**Survival analysis.** Survival analysis was performed in the 105 patients by the Kaplan-Meier test (Fig. 5). The expression intensity of VEGF was significantly correlated with disease-free and overall survival (p = 0.008 and p = 0.015, respectively). When median scores of MVD were used as cut-off points, the patients with higher score of MVD-CD105 had a significantly poorer prognosis than those with lower score in either disease-free or overall survival analysis (p = 0.002 and p = 0.009, respectively), whereas the similar prognostic significance of MVD-CD34 was not observed in overall survival analysis (p = 0.052) but was observed in disease-free survival analysis (p = 0.022). No prognostic significance of MVD-MA was found in either disease-free or overall survival analysis (p =
Table 1. Correlations of immunohistochemical expression of MVD-MA, MVD-CD105, and MVD-CD34 with clinicopathological parameters in 105 patients with hepatocellular carcinoma.

<table>
<thead>
<tr>
<th>Clinical parameters</th>
<th>No. of subjects</th>
<th>MVD-CD105 (score/0.708 mm^2)</th>
<th>MVD-CD34 (score/0.708 mm^2)</th>
<th>MVD-MA (score/0.565 mm^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mean SD p</td>
<td>mean SD p</td>
<td>mean SD p</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>male</td>
<td>89</td>
<td>139.64 54.09 0.550</td>
<td>241.89 100.73 0.908</td>
<td>57.42 36.40 0.900</td>
</tr>
<tr>
<td>female</td>
<td>16</td>
<td>148.63 61.21</td>
<td>238.62 121.86</td>
<td>56.13 45.08</td>
</tr>
<tr>
<td>Age (yr)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>≤60 years</td>
<td>85</td>
<td>141.33 59.21 0.903</td>
<td>254.70 116.68 0.526</td>
<td>58.61 38.27 0.437</td>
</tr>
<tr>
<td>&gt;60 years</td>
<td>20</td>
<td>139.65 60.79</td>
<td>238.26 100.76</td>
<td>51.30 34.94</td>
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<tr>
<td>HBsAg</td>
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<tr>
<td>positive</td>
<td>85</td>
<td>136.91 51.87 0.115</td>
<td>237.28 100.11 0.405</td>
<td>56.26 36.83 0.592</td>
</tr>
<tr>
<td>negative</td>
<td>20</td>
<td>158.45 65.37</td>
<td>258.85 118.43</td>
<td>61.30 41.53</td>
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<td>Histopathological parameters</td>
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<td>Tumor diameter</td>
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<tr>
<td>≤5cm</td>
<td>39</td>
<td>163.21 45.48 0.001*</td>
<td>262.59 104.13 0.107</td>
<td>66.87 33.36 0.043*</td>
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<tr>
<td>&gt;5cm</td>
<td>66</td>
<td>127.89 56.23</td>
<td>228.86 101.98</td>
<td>51.52 39.03</td>
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<tr>
<td>Tumor encapsulation</td>
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<tr>
<td>present</td>
<td>23</td>
<td>134.70 51.77 0.536</td>
<td>206.22 93.65 0.065</td>
<td>59.52 34.48 0.741</td>
</tr>
<tr>
<td>absent</td>
<td>82</td>
<td>142.78 56.00</td>
<td>251.26 104.61</td>
<td>56.57 38.62</td>
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<td>Venous invasion</td>
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<tr>
<td>present</td>
<td>47</td>
<td>119.83 49.38 0.000*</td>
<td>210.55 86.62 0.000*</td>
<td>49.83 38.84 0.070</td>
</tr>
<tr>
<td>absent</td>
<td>58</td>
<td>158.17 53.70</td>
<td>266.38 110.01</td>
<td>63.21 35.80</td>
</tr>
<tr>
<td>Microsatellite lesions</td>
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<tr>
<td>present</td>
<td>43</td>
<td>133.21 44.84 0.228</td>
<td>224.33 79.27 0.161</td>
<td>53.00 34.51 0.341</td>
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<tr>
<td>absent</td>
<td>62</td>
<td>146.42 60.85</td>
<td>253.23 116.70</td>
<td>60.15 39.63</td>
</tr>
<tr>
<td>Adjacent non-tumorous liver</td>
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<tr>
<td>normal</td>
<td>33</td>
<td>141.12 60.18 0.989</td>
<td>240.91 98.02 0.974</td>
<td>56.64 43.51 0.915</td>
</tr>
<tr>
<td>cirrhosis</td>
<td>72</td>
<td>140.96 52.92</td>
<td>241.61 106.70</td>
<td>57.49 34.90</td>
</tr>
<tr>
<td>TNM stage</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I &amp; II</td>
<td>59</td>
<td>152.95 60.08 0.011*</td>
<td>253.44 113.36 0.178</td>
<td>63.14 37.82 0.068</td>
</tr>
<tr>
<td>III A&amp; IIIB</td>
<td>46</td>
<td>125.70 43.75</td>
<td>225.93 88.31</td>
<td>49.63 36.34</td>
</tr>
</tbody>
</table>

HBsAg, hepatitis B surface antigen; TNM, tumor-node-metastasis.
* p <0.05.

Fig. 5 (page 45). Plots of life-tables of survival analysis by the Kaplan-Meier test show: The expression intensity of VEGF was significantly correlated with both the disease-free and overall survival (p = 0.008 and p = 0.015, respectively) and patients with high score of MVD-105 had a significantly poorer prognosis than those with low score in either disease-free or overall survival analysis (p = 0.002 and p = 0.009, respectively). Similar prognostic significance of MVD-CD34 was not observed in overall survival analysis (p = 0.052), but was observed in disease-free survival analysis (p = 0.022). No prognostic significance of MVD-MA was found in either disease-free or overall survival analysis (p = 0.277 and p = 0.712, respectively). Furthermore, higher scores of MVD-CD105 indicated poorer prognosis of patients with large tumors (≥5 cm maximum diameter), whereas this was not the case with small tumors (<5 cm diameter).
Fig 5. See legend on page 44.
0.277 and \( p = 0.712 \), respectively). Moreover, when the patients were divided into 2 groups using a tumor diameter of 5 cm as the cut-off point, a significantly poorer prognosis with higher score of MVD-CD105 was found in the larger tumor size group (\( p = 0.007 \) for disease-free survival and \( p = 0.012 \) for overall survival, respectively), whereas this was not the case in the smaller tumor size group (\( p = 0.222 \) for disease-free survival and \( p = 0.184 \) for overall survival, respectively).

Discussion

Although it is well accepted that tumor growth is dependent on angiogenesis, whether MVD truly reflects the angiogenic activity of tumors, especially the vascular solid tumors, remains controversial. For counting MVD, most researchers have used several normal endothelial cell (EC) markers, such as CD31, CD34, and von Willebrand factor (vWF). But many researchers have found that it difficult to identify native or newborn vessels by using these EC markers, since they significantly occur on ECs of normal tissues [20].

Endoglin (CD105), a cell membrane glycoprotein, is up-regulated in endothelial cells of de novo blood vessels of various tumors compared to those of normal tissues [7, 20-22]. Therefore, CD105 has been considered to be a more selective marker for MVD and a better prognostic marker in many tumors such as breast, prostate, colon, and lung tumors [20, 23-25].

It is of particular interest to evaluate the clinicopathological significance of MVD in HCC because HCC is one of the most vascular solid tumors. Few studies have assessed the clinicopathological significance of MVD in HCC and fewer yet have identified CD105 expression in HCC. In this study, MVDs in HCC were assessed by use of anti-CD105 and anti-CD34 antibodies. Correlation with clinicopathological parameters demonstrated significantly lower MVD scores in larger and more advanced stage tumors with CD105 as the endothelial marker. Survival analysis showed that higher MVD-CD105 indicated poorer prognosis. It is noteworthy that the clinicopathological significance of MVD-CD34 was only found in disease-free survival, with a lower significance than that of MVD-CD105. The findings of the clinicopathological correlation with MVD-CD105, similar to Ho's report [19], were apparently contrary to those reported in other solid tumors [23-25], in which higher rather than lower MVD scores were associated with more aggressive tumors. However, most studies of breast cancer indicated that angiogenesis seems to be a good prognostic marker only in early-stage group [26], which had lower MVD scores. In this study, higher score of MVD-CD105 with poorer prognosis was found only in the larger tumor group, which also had lower MVD scores. Hence, angiogenesis may be a good prognostic marker only in the tumor stage with relative lower score of MVD.

VEGF is a member of a family of structurally related proteins that act as ligands for VEGF receptors. VEGF has effects on the development of new blood vessels (angiogenesis) and the survival of immature blood vessels (vascular maintenance). The role of VEGF in tumor angiogenesis has been extensively studied and has been shown to be a key mediator of angiogenesis in cancer, in which it is up-regulated by oncogene expression and a variety of growth factors. In this study, VEGF expression in HCC was assessed by tissue microarray and compared with the expression of CD105 and CD34 simultaneously. A significantly poorer prognosis with higher level of VEGF expression was clearly found. Moreover, VEGF expression intensity was positively correlated with MVD-CD105, but not with MVD-CD34. This indicates that MVD-CD105 is a better marker of angiogenesis in HCC than MVD-CD34.

The tissue microarray technique is a sophisticated and cost-effective technique of evaluating many immunohistochemical markers in tumors. However, the use of tissue microarray has been reported to be inappropriate in some tumors [25]. To our knowledge, this is the first study that evaluated the clinicopathological significance of the expression of CD105 and VEGF in HCC using the tissue microarray technique. Our results showed that the vessel counts in tissue microarrays significantly correlated with those in concurrent “large” paraffin sections. Vessel counts in tissue microarrays did not indicate the prognosis while the higher vessel counts in “large” paraffin sections
correlated with poorer prognosis. The expression intensity of VEGF analyzed in tissue microarray was significantly correlated with overall survival. Similar to Charpin's report [25], this likely stems from the fact that in current tissue microarrays, the stromal areas are not large enough for appropriate evaluation of tumor angiogenesis, since the tissue microarray blocks commonly included carcinoma-tous rather than stromal areas. Therefore, the use of tissue microarrays does not appear to be appropriate in analyzing angiogenesis in HCC. On the other hand, differing from Tanigawa’s report, we saw 3 patterns of vessels (ie, branching vessels, sprouting vessels, and sinusoidal vessels). The significance of counting these different patterns of vessels needs further studies.

In conclusion, this study demonstrates the superiority of CD105 over CD34 as a marker of angiogenesis in HCC, which is consistent with studies of breast cancer [27] and lung cancer [23]. Moreover, although the tissue microarray technique was very relevant for evaluating immunocytochemical markers within tumor cells, it was unsuitable for evaluating angiogenesis in HCC.

Acknowledgments

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