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# The prognostic role of Angiogenesis, Lymphangiogenesis and Vasculogenic mimicry in Hepatocellular Carcinomas treated with Transplantation

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## ABSTRACT

**Objective:** Angiogenesis plays a decisive role in the carcinogenesis and progression of hepatocellular carcinoma (HCC), a hypervascular tumor. This study aimed to quantitatively assess angiogenesis and evaluate other vascularization patterns, such as lymphangiogenesis and vasculogenic mimicry (VM), using morphometric and immunohistochemical methods. Additionally, we investigated the prognostic relevance of vascularization in the postoperative outcomes of HCC patients treated with liver transplantation.

**Material and Methods:** Sixty HCC cases treated with orthotopic liver transplantation were included. Intratumoral angiogenesis was assessed using anti-CD34 and anti-CD105 antibodies, while lymphangiogenesis was evaluated with the anti-D2-40 antibody, all via immunohistochemistry on tissue microarray slides. Vascularization was quantified using image analysis software, measuring vessel area and the number and area of objects per mm². Morphological and immunohistochemical data were analyzed statistically to assess correlations with prognostic factors and survival outcomes.

**Results:** Both tubular and patterned matrix types of VM were observed in HCC cases. However, no statistically significant association was found between the presence of VM and survival or prognostic indicators. A statistically significant relationship was identified between overall survival and the number of CD34-positive vessels per unit area, which was significantly lower in patients with poorer outcomes ($p = 0.006$). No significant correlation was found between CD34 vessel count and clinicopathologic parameters. CD105 expression was lower than CD34, with no statistically significant association observed between their expressions or with clinical outcomes.

**Conclusion:** Our findings support the existence of distinct and independent vascularization patterns in HCC. An increased level of vascularization, as detected by CD34 staining, was associated with better prognosis. The image analysis method employed proved effective but should be validated further using both tissue microarray and routine histological slides.

**Keywords:** Hepatocellular carcinoma, angiogenesis, lymphangiogenesis, vasculogenic mimicry

## INTRODUCTION

Hepatocellular carcinoma (HCC) is a highly vascular solid tumor in which angiogenesis plays a crucial role in tumor development, carcinogenesis, progression, and metastasis (1,2). Despite the prominence of vascularization as a morphologic feature of HCC, antiangiogenic therapies have generally proven ineffective. It has been hypothesized that these therapies target only specific molecular pathways of vascularization while overlooking others.

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In HCC, tumor vessel development occurs through various molecular mechanisms. The primary process involves sprouting of new blood vessels from pre-existing ones, a phenomenon measured histologically as microvessel density (MVD) using immunohistochemical markers (2,3). MVD serves as a key parameter reflecting tumor angiogenesis, and its prognostic value has been investigated across numerous tumor types (4). However, variability in vessel counting methods and subjective assessment may explain the inconsistent findings regarding its prognostic utility (3,5). In this study, we employed image analysis software to quantify vascular structures objectively and reduce observer bias.

A secondary tumor-feeding mechanism in HCC, termed vasculogenic mimicry (VM), involves the formation of vascular-like channels lacking endothelial cells and surrounded by tumor cells. This phenomenon, originally described in melanomas and prostate carcinomas, has also been observed in HCC (6,7). Tumor cells exhibit remarkable plasticity, acquiring endothelial-like properties to form these channels (7–10). VM facilitates tumor perfusion and metastatic spread without relying on angiogenic factors (11,12). Two distinct VM patterns have been described: the tubular type, consisting of vessel-like channels lined by tumor cells; and the patterned matrix type, in which laminin- and collagen-rich extracellular matrix forms loops enclosing clusters of tumor cells (2,13).

Tumor-associated lymphangiogenesis represents another neovascularization pathway in HCC (6). Lymphatic vessels may be located intratumorally or peritumorally and, like tumor vasculature, differ structurally from their normal counterparts (14). These newly formed lymphatics are often fragile, lacking supportive structures, and may facilitate tumor cell dissemination via the lymphatic system (15). However, the prognostic implications of lymphangiogenesis in HCC remain unclear (5).

The aim of this study was to evaluate the roles of angiogenesis, vasculogenic mimicry, and lymphangiogenesis in the biological behavior of HCCs treated with hepatic transplantation. To achieve this, tumor vascularization was assessed through immunohistochemistry and quantitatively analyzed using image analysis techniques.

# MATERIALS AND METHODS

### Patients

Sixty patients who underwent orthotopic liver transplantation (OLT) for hepatocellular carcinoma (HCC) at Dokuz Eylul University Hospital were included in this study. Demographic data, underlying liver disease, tumor size and number, tumor stage, recurrence or metastasis status, vital status at last follow-up, cause of death, and disease-specific survival were obtained through review of patient archives, medical records, and correspondence with attending physicians. Pathological slides were reviewed by two pathologists. The decision to perform OLT was based on the Milan criteria. Seven patients received transcatheter arterial chemoembolization prior to transplantation.

### Pathologic Examination

Liver explants were routinely fixed in buffered formalin and sliced into 0.5 cm-thick coronal sections for macroscopic evaluation. In some cases, tumors were identified preoperatively by imaging, while in others, tumor nodules were detected incidentally during gross examination. Tumor characteristics, including size, number, and evidence of gross vascular invasion, were recorded in pathology reports. Routine hematoxylin-eosin (H&E)-stained sections were reviewed by two pathologists (GGN and OS) for diagnosis. Tumors were graded and staged according to the AJCC 8th Edition pTNM system. H&E sections were also assessed for the presence of vasculogenic mimicry (VM). All pathological data were revised and confirmed from archived reports.

### Immunohistochemistry

Four-micrometer-thick sections were cut from formalin-fixed, paraffin-embedded tissue blocks and mounted on lysine-coated slides. Whole tissue sections were stained with anti-laminin (Neomarkers, Rabbit Polyclonal Antibody; RB-082-A, dilution 1:50). Representative areas from each tumor were selected to construct tissue microarray (TMA) blocks using 3.0 mm punch cores (Tissue-Tek® Quick-Ray™ 8018).

To quantify angiogenesis, two antibodies were used: anti-CD34 (Dako, Monoclonal Mouse Anti-Human; IR632, Clone QBEnd 10, prediluted) and anti-CD105 (Novocastra, Lyophilized Mouse Monoclonal Antibody, dilution 1:50).

For lymphangiogenesis, anti-D2-40 (Invitrogen, Monoclonal Mouse, Clone D2-40, dilution 1:50), a specific marker for lymphatic endothelium, was used on 4 µm TMA sections. All immunohistochemical staining was performed using the Ventana Benchmark XT automated slide staining system.

### Evaluation and Quantification of Tumor Vascularization

Microvessel density (MVD) was assessed independently by two pathologists who were blinded to clinical and pathological data. Staining intensity and endothelial area were quantitatively analyzed on TMA sections using the 3D Histech HistoQuant image analysis system. Slides stained with CD34, CD105, and D2-40 were scanned using the 3D Histech Mirax scanner. The resulting images were imported into HistoQuant software, calibrated to a defined measurement area. "Hot spot" regions were identified at 4x magnification. Vascular structures were selected using the “color selection” tool, and the “area/density (intensity) measurement” function was applied to quantify values (**Figure 1**). Data were recorded and transferred to Microsoft Excel for subsequent statistical analysis.

**Parameters assessed using the HistoQuant software (3DHistech) included:**

**Number of detected objects (NO):** Total count of positively stained vascular structures.

**Summed area (MA):** Total stained vessel wall surface area in mm².

**Number of objects per mm² (NO/FA):** Vascular object count per selected field area.

**Area of selected field (FA):** The defined area in mm² within which vascular structures were quantified.

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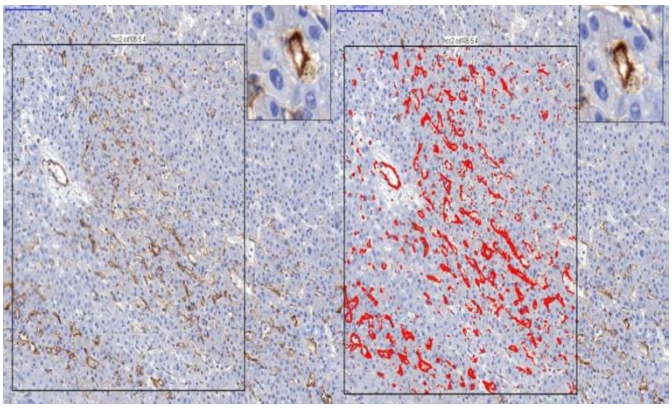


**Figure 1:** Marking the relevant area to be measured in the software program by us and selecting the area to be measured by the program.

### Evaluation of Vasculogenic Mimicry

Each case was examined for the presence of vasculogenic mimicry (VM). Two types of VM, as described in the literature, were evaluated: (1) tubular type and (2) patterned matrix type (13). Tubular-type VM was identified when vascular-like channels containing erythrocytes in the lumen—lacking endothelial cells and surrounded by tumor cells—were observed on hematoxylin-eosin (H&E) sections. These findings were confirmed by negative staining for CD34.

Patterned matrix-type VM was assessed on immunohistochemically stained sections using anti-laminin antibody. This type was identified by the presence of laminin-positive connective tissue loops encircling clusters of 3 to 15 tumor cells (13). Laminin-stained sections were reviewed by two pathologists (GG and OS).

### Statistical Analysis

Morphological and immunohistochemical findings were analyzed statistically to determine associations with prognostic factors and overall survival. Power analysis was performed using a publicly available sample size calculator, assuming an alpha level of 0.05 and 80% power for detecting differences in dichotomous variables (presence/absence) between two independent groups. The sample size was deemed adequate to identify statistically significant prognostic differences.

All statistical analyses were performed using SPSS version 15.0. Spearman correlation was used for non-parametric data. The Mann–Whitney U test was applied for comparisons between unpaired groups. Survival analyses were conducted using Kaplan–Meier survival estimates.

## RESULTS

### Clinical Data

The mean age of the patients was 51 years (range: 37–64). Seven patients (12%) were female, and 53 (88%) were male. Fifteen patients (25%) underwent transplantation due to cirrhosis and were incidentally diagnosed with HCC upon pathological examination.

Regarding cirrhosis severity, 17 patients (28.3%) were classified as CHILD A, 25 (41.7%) as CHILD B, and 18 (30%) as CHILD C. MELD scores were <9 in 15 patients (25%), 10–19 in 44 patients (73.4%), 20–29 in two patients (3.3%), and 30–39 in two patients (3.3%).

The etiology of cirrhosis was unknown in two patients (3.3%) with negative viral serology and no history of alcohol use. Among cases with known etiology, 27 (45%) were HBV-positive, 10 (16.7%) were HCV-positive, one (1.7%) had HBV + HCV coinfection, and 16 (26.7%) had HBV + HDV coinfection. Alcohol use alone was reported in three cases (5%), and one HBV-positive patient (1.7%) reported additional alcohol use.

A majority of patients (49; 81.8%) did not receive any treatment for the tumor prior to transplantation. Treatment history was unknown in three patients (5%). Seven patients (11.6%) underwent transcatheter arterial chemoembolization (TACE) before transplantation, and one patient (1.6%) underwent pre-transplant surgical resection.

The mean follow-up duration was 47.9 months (range: 0.4–107 months). During follow-up, seven patients (11.6%) experienced recurrence, and one of these patients remains alive with disease. At the time of analysis, 39 patients (65%) were alive and disease-free, while 20 (33.3%) had died. The mean overall survival was 75.05 ± 5.74 months.

A statistically significant reduction in recurrence was observed among patients meeting the Milan and UCSF criteria ($p < 0.01$). However, no statistically significant associations were identified between recurrence and other clinical parameters.

### Pathologic data

Twenty-eight patients (46.7%) had a single tumor nodule, while 32 patients (53.3%) had multiple nodules. Among those with multifocal tumors, the number of nodules ranged from 2 to 10, with an average of 3.7 nodules per patient. Tumor size ranged from 10 to 110 mm, with a mean size of 32.57 mm. Nine patients had large HCCs (>5 cm), and 19 patients had small HCCs (≤2 cm). No patient had evidence of extrahepatic metastasis at the time of surgery. The detailed histopathological findings are presented in **Table 1**.

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**Table 1:** The histopathological findings of the cases

| | Number of cases (n) |
|---|---|
| Focality | |
| One tumor nodule | 28 (46,7%) |
| >1 tumor nodule | 32 (53,3%) |
| Tumor size | |
| ≤2 cm | 19 (31,7 %) |
| 3-5 cm | 32 (53,3 %) |
| >5 cm | 9 (15%) |
| Tumor grade | |
| 1 | 4 (6,7%) |
| 2 | 38 (63,3%) |
| 3 | 16 (26,7%) |
| 4 | 2 (3,3%) |
| pT stage | |
| T1 | 25 (41,7%) |
| T2 | 31 (51,7%) |
| T3 | 3 (5%) |
| T4 | 1 (1,7%) |
| Vascular invasion | |
| Absent | 46 (76,7%) |
| Present | 14 (23,3%) |
| Macroscopic | 2 (3,3%) |
| Microscopic | 12 (20%) |

**Blood vessels (MVD)**

All cases showed varying degrees of CD34 positivity. A statistically significant relationship was found between the number of CD34-positive vessels per unit area and overall survival. Interestingly, the number of vessels per square millimeter (NO/FA) was significantly lower in patients with poorer outcomes (p = 0.006). Patients with a lower density of CD34-positive vessels had shorter survival times.

CD105 expression in tumor vessels was generally less intense than that of CD34. However, no statistically significant correlations were observed between CD105 expression and CD34 expression, or between CD105 expression and overall survival. Furthermore, there were no significant associations between the number of vessels per unit area (as assessed by CD34 or CD105) and clinicopathologic features, including tumor size, number of nodules, pT stage, tumor grade, vascular invasion, patient age, CHILD score, MELD score, or recurrence.

**Vasculogenic Mimicry(VM)**

Both types of vasculogenic mimicry were identified in our HCC cases. Tubular-type VM—defined as channels filled with erythrocytes, lacking endothelial lining, and surrounded by tumor cells—was observed in 16 cases (26.6%) (Figure 2). Patterned matrix-type VM—characterized by laminin-positive loops encircling clusters of 3–15 tumor cells—was present in 11 cases (18.3%) (Figure 3).

The relationships between VM subtypes and clinicopathological parameters are detailed in **Tables 2** and **3**. No statistically significant association was found between the presence of either VM subtype and overall survival or other prognostic factors.

**Table 2:** Relationship between tubular type VM and clinicopathological parameters

| | Tubular type VM positive (n=16) | Tubular type VM negative (n=44) |
|---|---|---|
| Latest Status | | |
| Alive | 9 (%56,3) | 31 (%70,4) |
| Dead | 7 (%43,7) | 13 (%29,6) |
| Recurrence | 3 (%18,75) | 4 (%9) |
| Pathologic T stage | | |
| T1 | 4 (%25) | 21 (%47,7) |
| T2 | 9 (%56,3) | 22 (%50) |
| T3 | 2 (%12,5) | 1 (%2,3) |
| T4 | 1 (%6,3) | 0 (%0) |
| Grade | | |
| 1 | 1 (%6,3) | 3 (%6,8) |
| 2 | 8 (%50) | 30 (%68,2) |
| 3 | 6 (%37,5) | 10 (%22,7) |
| 4 | 1 (%6,3) | 1 (%2,3) |
| Vascular invasion (vi) | | |
| Present | 4 (%25) | 10 (%22,7) |
| None | 12 (%75) | 34 (%77,3) |
| Laminin | | |
| Patterned matrix type VM negative | 1 (%6,3) | 10 (%22,7) |
| Patterned matrix type VM positive | 15 (%93,7) | 34 (%77,3) |
| CD34 (Mean ±SD) | | |
| MA | 3,9 ±2,1 | 2,6 ±1,6 |
| NO/FA | 494,2 ±616,7 | 612,9 ±1107,0 |
| CD105 (Mean ±SD) | | |
| MA | 2,5 ±2,0 | 2,9 ±2,6 |
| NO/FA | 824,0 ±728,5 | 696,0 ±835,8 |
| D2-40 (Mean ±SD) | | |
| MA | 2,4 ±3,3 | 1,8 ±2,8 |
| NO/FA | 171,3 ±339,4 | 148,3 ±318,2 |

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**Table 3:** Relationship between patterned type VM and clinicopathological parameters

| | Patterned type VM positive (n=11) | Patterned type VM negative (n=49) |
|---|---|---|
| Latest Status | | |
| Alive | 7 (%63,6) | 33 (%67,3) |
| Dead | 4 (%36,4) | 16 (%32,7) |
| Recurrence | 1 (%9) | 6 (%12,2) |
| Pathologic T stage | | |
| T1 | 5 (%45,5) | 20 (%41) |
| T2 | 5 (%45,5) | 26 (%53) |
| T3 | 1 (%9) | 2 (%4) |
| T4 | 0 (%0) | 1 (%2) |
| Grade | | |
| 1 | 0 (%0) | 4 (%8,2) |
| 2 | 7 (%63,6) | 31 (%63,3) |
| 3 | 3 (%27,3) | 13 (%26,5) |
| 4 | 1 (%9,1) | 1 (%2) |
| Vascular invasion (vi) | | |
| Present | 3 (%27,3) | 11 (%22,5) |
| None | 8 (%72,7) | 38 (%77,5) |
| Tubular type VM | | |
| positive | 1 (%9,1) | 15 (%30,6) |
| negative | 10 (%90,9) | 34 (%69,4) |
| CD34 (Mean ±SD) | | |
| MA | 2,4 ±1,1 | 3,1 ±1,9 |
| NO/FA | 396,4 ±149,7 | 622,8 ±1098,1 |
| CD105 (Mean ±SD) | | |
| MA | 2,7 ±2,9 | 2,8 ±2,4 |
| NO/FA | 1042,3 ±1091,1 | 660,0 ±721,3 |
| D2-40 (Mean ±SD) | | |
| MA | 1,6 ±2,6 | 2,1 ±3,01 |
| NO/FA | 140,8 ±354,3 | 157,5 ±317,21 |

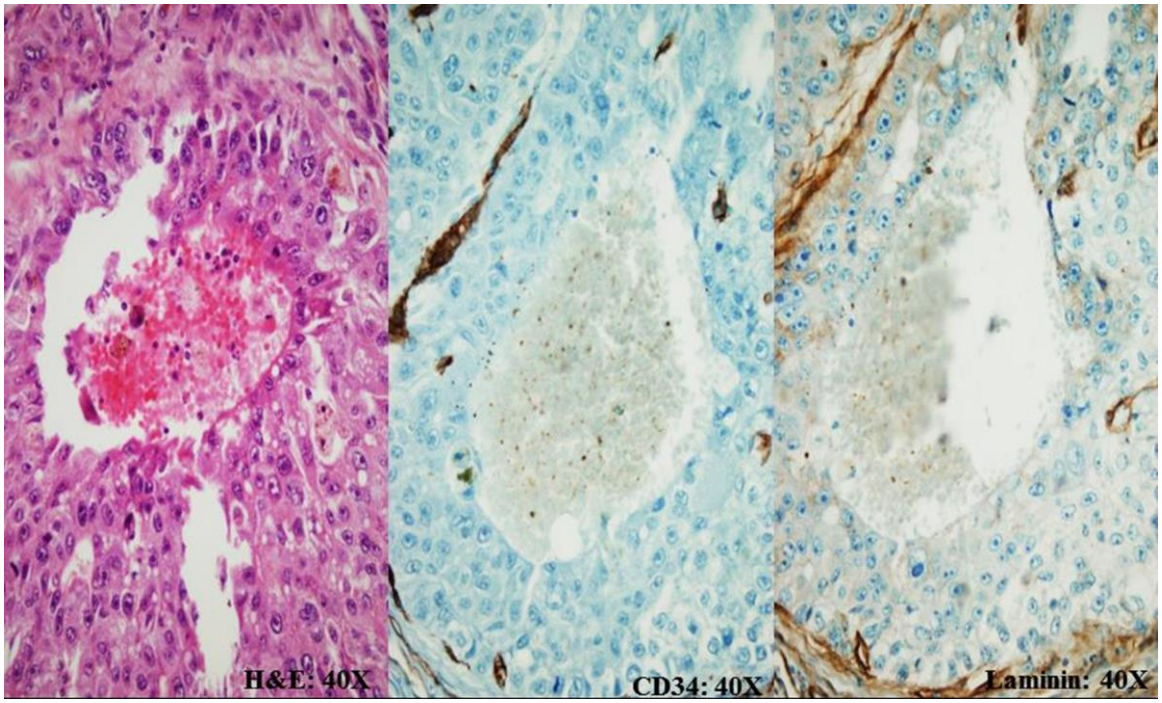



**Figure 2:** Tubular type VM: HCC with channels filled with erythrocytes, lacking endothelial cells and surrounded by tumour cells (H&E and CD34 immunohistochemistry)

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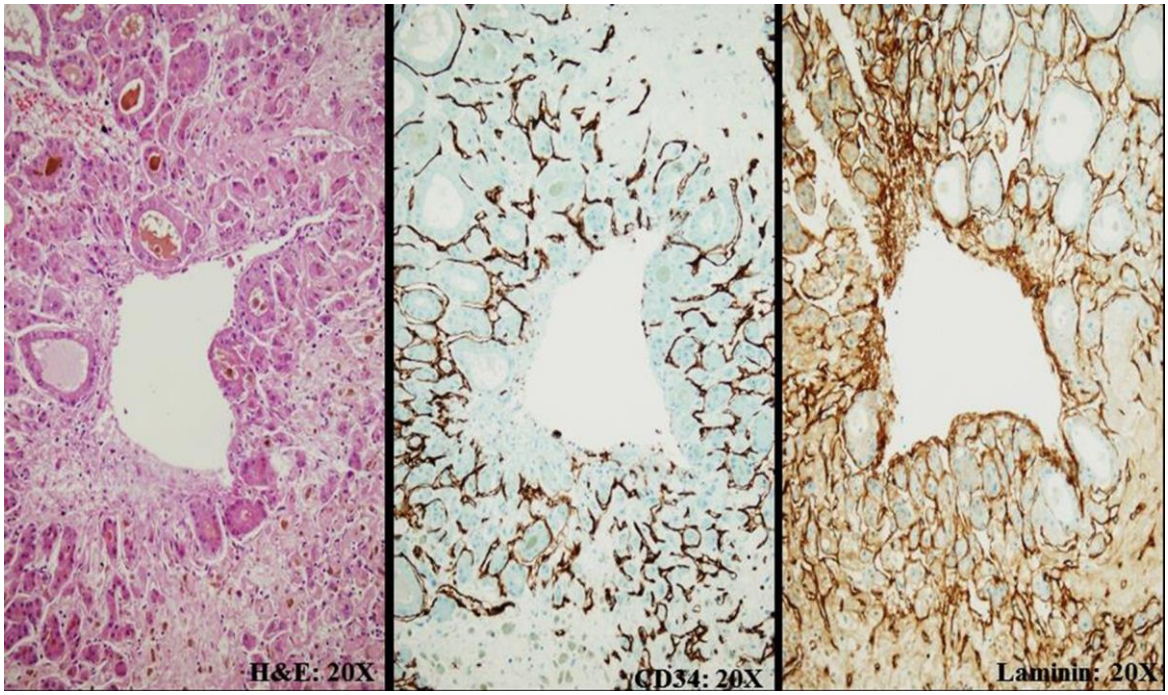



**Figure 3: Patterned type VM:** HCC showing VM expression, by laminin immunostain which did not express the endothelial cell marker CD34, indicating that the laminin-positive loops are not hepatic sinusoids.

**Lymphatics**

Using D2-40 immunohistochemistry, lymphatic vessels were identified in 25 tumors, while 35 tumors showed no D2-40 positivity. Lymphatics were present both within the tumor parenchyma and in the fibrous capsule surrounding the tumor nodules. However, lymphatic vessel counts did not show any statistically significant association with survival or other prognostic factors.

## DISCUSSION

The relationship between the number of intratumoral vessels and tumor biological behavior and patient survival has been investigated in various tumor types. A high intratumoral microvessel density (MVD) has been identified as an independent adverse prognostic factor in breast, lung, and prostate cancers, malignant melanomas, and central nervous system tumors [16].

Hepatocellular carcinoma (HCC) is a hypervascular tumor. For many years, treatment strategies such as ligation and embolization of feeding arteries have been employed to halt tumor growth. However, these approaches have not consistently been effective due to the tumor's angiogenic potential and its heterogeneity [4]. Although molecular mechanisms regulating angiogenesis have become new therapeutic targets in HCC, anti-angiogenic therapies alone remain insufficient in advanced stages of the disease [2,3,8]. Despite limited evidence, studies in the literature suggest that tumor angiogenesis is an important prognostic factor in HCC and involves multiple molecular mechanisms [5]. While sprouting of new vessels from pre-existing ones is the predominant angiogenic pathway, other mechanisms—such as the migration of bone marrow-derived endothelial progenitor cells, lymphangiogenesis, and vasculogenic mimicry (VM)—also contribute to the complex vascular organization of HCC [4].

The limited efficacy of anti-angiogenic treatments may be due to their inability to target all these distinct angiogenic pathways [3].

Although some studies have investigated the prognostic role of angiogenesis in HCC, data on the contribution of specific mechanisms, including VM and lymphangiogenesis, remain insufficient. In our study, we utilized tissue microarray (TMA) sections and image analysis to evaluate the prognostic value of vessel-related parameters and VM in transplant-treated HCC cases.

The first study to evaluate CD105 and VEGF expression using TMA in HCC was conducted by Yao et al., who found a statistically significant correlation between vessel counts in TMA and conventional sections [17]. Conversely, a study in breast cancer using TMA and image analysis found no such correlation, possibly due to selective sampling of tumor tissue rather than stroma-rich regions during TMA construction [18]. Given the relatively lower stromal content in HCC compared to other solid tumors and the limited number of TMA-based studies, we chose the TMA method for our analysis.

Among vascular markers, the anti-CD34 antibody has been frequently reported to provide more prognostically relevant information than others in angiogenesis studies [4,19,20]. However, conflicting reports exist, with some studies failing to find a prognostic association between CD34 and clinicopathological parameters [21,22].

Some literature suggests that MVD assessed by anti-CD34 staining is a significant prognostic factor for disease-free survival post-resection in HCC [20].

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In our cohort, there was no significant association between anti-CD34 vessel density (number per mm²) and clinicopathological features. Nevertheless, higher CD34-positive vessel counts were significantly associated with longer overall survival (p = 0.006).

Our study focused solely on transplant-treated patients, which limited the number of recurrence cases. Previous research has reported that a high MVD score is a poor prognostic factor for disease-free survival following resection in HCC tumors <5 cm [3,4]. In contrast, we observed no significant correlation between tumor size and CD34-assessed vascular parameters.

Yamamoto et al. reported an inverse correlation between MVD (measured using Factor VIII) and recurrence in 84 resected HCC cases, but found no association with CD34 [23]. Murakami et al. demonstrated in 136 HCC cases that low CD34-assessed MVD was an adverse prognostic factor for overall survival [24]. In line with Murakami et al., our study found that patients with lower CD34-positive vessel counts had poorer outcomes (p = 0.006).

These contradictory findings in the literature may reflect the unique complexity of liver vasculature and HCC angiogenesis. In addition to conventional tumor-associated growth factors, liver sinusoidal endothelial cells, Kupffer cells, and hepatic stellate cells contribute to angiogenesis and regeneration, supporting HCC progression [6]. El-Assal et al. showed that MVD correlates with tumor size and is heterogeneously distributed, being more concentrated in peritumoral regions [21]. Murakami et al. observed that VEGF expression, a key angiogenic factor, decreases with increasing tumor grade, with poorly differentiated HCCs showing lower MVD. They also noted a negative correlation between tumor size and MVD [24]. Similarly, Huang et al. identified the peritumoral vascular encapsulation of tumor clusters (VETC) as a recurrence risk factor in HCC [25].

In our study, sampling was performed near the tumor periphery, yet no correlation between tumor size and vessel number was detected. These findings suggest that high MVD may be associated with lower tumor stage and histological grade, supporting the hypothesis that MVD could be a favorable prognostic indicator. Moreover, MVD may have predictive value for anti-angiogenic treatment efficacy, as it directly reflects intratumoral angiogenesis [2].

The prognostic significance of anti-CD105 expression in HCC remains underexplored. In a study by Ho et al. involving 86 resected HCCs, anti-CD105 stained only microvessels, with no significant prognostic advantage over CD34 [22]. Some reports, however, suggest CD105 may be superior to CD34 as a vascular marker [3,4,17,26,27]. CD105 is known to be involved in neovascular formation and may indicate abnormal vasculature in various tumor types [19,28]. Conversely, Yu et al. reported CD105 expression not only in tumor vessels but also in sinusoidal endothelium of cirrhotic liver tissue, suggesting its limited specificity and potential unsuitability as a therapeutic target in cirrhotic HCC [29]. Despite this, Kasprzak et al. and Jenk KS et al. have advocated for CD105 as a viable anti-angiogenic target in HCC [30,31].

In our study, CD105 expression was lower than CD34 expression, and no significant correlation was found between their values. In sections containing cirrhotic parenchyma, staining was limited to tumor vasculature; however, TMA sections containing cirrhotic regions were scarce. No significant association was observed between CD105-positive vessel counts and clinicopathologic parameters.

VM represents a non-endothelial pattern of tumor blood supply. Although its mechanism is not fully understood, it has attracted growing interest. VM has been linked to more aggressive tumor phenotypes, metastasis, and poor survival outcomes [9–11,13]. Both tubular and patterned matrix VM types have been associated with cancer progression and poor prognosis in many studies [10]. Nevertheless, the literature remains divided.

Although VM has been identified in various malignancies, its presence and prognostic relevance in HCC are still debated. Some studies associate patterned matrix VM with larger tumor size, higher grade, vascular invasion, and advanced pTNM stage, while others link it only to early recurrence [9,10,13]. In our series, tubular VM was observed in 16 cases, and patterned matrix VM in 11 out of 60. No statistically significant relationship was found between either VM type and clinicopathological parameters. Additionally, VM was not associated with CD34 or CD105 vascular measurements. It is noteworthy that our cohort had an uneven distribution of early- and late-stage cases, with few cases displaying adverse prognostic features, which may have limited the power of statistical analyses. Given these inconsistencies, larger studies are needed to clarify the prognostic role of VM in HCC [13].

There is increasing interest in tumor-associated lymphangiogenesis [6,32–34], though studies on its prognostic value in HCC remain scarce. Thelen et al. analyzed D2-40-stained conventional sections of 60 HCCs and found that while normal liver parenchyma lacked lymphatic vessels, lymphangiogenesis occurred in HCC, particularly within the tumor parenchyma and septa. Their findings suggest that tumor-associated lymphangiogenesis contributes to neovascularization and may serve as an independent prognostic factor for disease-free survival [5].

In our study, similar D2-40-positive lymphatic vessels were observed in both tumor parenchyma and stroma. No staining was seen in 25 cases. There was no statistically significant association between D2-40-positive lymphatic vessel count per mm² and clinicopathologic features. Additionally, no relationship was found between lymphatic vessel parameters and CD34/CD105 expression or the presence of VM.

## CONCLUSIONS

In conclusion, our study suggests that vascularization patterns in hepatocellular carcinoma (HCC) operate independently and through distinct mechanisms. However, when evaluated individually, increased vascularization could not be definitively associated with poor prognosis or adverse tumor biology in this patient cohort. On the contrary, elevated vascularity—as indicated by CD34 expression—was associated with improved prognosis. This finding may be influenced by the predominance of early-stage tumors in the study cohort and the limited number of high-stage cases available for comparison.

While the image analysis method employed in this study appears effective in identifying vascular structures, it may not be optimal. Future research should consider validating this approach against established stereological techniques to enhance reliability. Nonetheless, image analysis provides the advantage of quantitative data generation, contributing to increased objectivity and reproducibility.

The use of tissue microarrays (TMA) may have also impacted data reliability, particularly in the evaluation of lymphangiogenesis. Although TMAs are valuable tools for high-throughput screening and comparative analyses, they may fall short in representing tumor heterogeneity. This limitation should be acknowledged when interpreting the results.

Our findings demonstrate the presence of both tubular-type and matrix-type vasculogenic mimicry (VM) in HCC. However, to better understand the biological implications of these morphological patterns, it is crucial to include more advanced and less differentiated tumors in future studies. A larger cohort with a balanced distribution across tumor stages will be essential to assess the prognostic significance of vascular features more comprehensively and accurately.

**Acknowledgement**

None

**Conflicts of Interest**

None of the authors have any conflict of interest or financial ties to disclose.

**Ethical Considerations**

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and/or with the Helsinki Declaration of 1964 and later versions.

**Authors' Contribution**

Conceptualization: FO, SA
Data curation: -
Formal analysis: -
Funding Acquisition: -
Investigation: -
Methodology: -
Statistics: -
Project administration: -
Supervision: -
Validation: -
Visualization: -
Writing–original draft: -
Writing–review & editing:: -

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