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Original contribution

The prognostic significance of vasohibin 1–associated angiogenesis in patients with hepatocellular carcinoma ☆

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Summary Vasohibin 1, an endothelium-derived negative feedback regulator of angiogenesis, is induced by fibroblast growth factor 2 (FGF-2) and vascular endothelial growth factor A (VEGF-A). In this study, we retrospectively evaluated immunoreactivity of FGF-2 and VEGF-A as well as microvessel density (MVD) determined by expression of vasohibin 1 and CD34 (MVD-CD34) and correlated the findings with clinical outcomes of 181 patients with hepatocellular carcinoma (HCC). Double immunostaining of an endothelial marker CD34 and vasohibin 1 with Ki-67 was also performed to assess angiogenic activity of endothelial cells in HCC. The ratio of Ki-67–positive endothelial cells in vasohibin 1–positive vessels (22%) was significantly higher than that of CD34-positive vessels (9%, $P < .001$), suggesting the correlation between vasohibin 1 and neovascularization in endothelial cells of HCC. MVD-CD34 decreased, but the ratio of MVD determined by expression of vasohibin 1 to MVD-CD34 (vasohibin 1/CD34) increased significantly according to histologic grade. Vasohibin 1 was significantly correlated with the status of FGF-2 ($P = .007$) but not with that of VEGF-A ($P = .055$). The 10-year overall survival and the 2-year disease-free survival rates of the low vasohibin 1/CD34 group (vasohibin 1/CD34 $\leq$ 0.459) were significantly higher than those of the high vasohibin 1/CD34 group (vasohibin 1/CD34 > 0.459) (survival, 48% versus 38% and 52% versus 35%; $P < .001$ and $P < .05$, respectively). In addition, vasohibin 1/CD34 in HCC patients was an independent marker of poor prognosis, as determined by multivariate analysis (risk ratio, 1.973; 95% confidence interval, 1.049-3.711; $P = .035$). Vasohibin 1/CD34 could identify the proliferative vessels and could be a useful biomarker for predicting the clinical outcome of HCC patients.

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1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most common malignancy worldwide and is the third most common cause of cancer-related deaths [1]. HCC is a highly vascularized tumor, which makes it pivotal to study its angiogenesis. The process of angiogenesis in HCC is generally characterized by sinusoidal capillarization and unpaired arteries [2]. Sinusoidal capillarization, which can be identified more sensitively by the endothelial marker CD34 than CD31 or others [3], is a relatively complex process and is regulated by a balance between angiogenesis stimulators and inhibitors [4]. Angiogenesis, also known as neovascularization, is a key event in numerous pathologic and physiologic conditions [5]. Homeostasis is strictly controlled by numerous negative feedback systems [6]. Little is known, however, about their role in angiogenesis associated with HCC. Vasohibin 1 is one of the first established negative feedback regulators of angiogenesis [7]. This factor was first identified in a study of gene expression induced by vascular endothelial growth factor (VEGF) [8]. Vasohibin 1 possesses antiangiogenic properties for endothelial cells (ECs) and was subsequently demonstrated to be specifically expressed in ECs [7]. Vasohibin 1 inhibits angiogenesis in an autocrine manner in response to angiogenic stimulators such as fibroblast growth factor 2 (FGF-2) and VEGF-A [7]. The subsequent analysis also revealed that vasohibin 1 is preferentially expressed in ECs of newly formed blood vessels behind the sprouting front where angiogenesis terminates [9]. In addition, vasohibin 1 (−/−) mice were reported to contain numerous immature vessels in the area where angiogenesis was supposed to be terminated behind the sprouting front [9]. The status of vasohibin 1 has, therefore, been studied in several human malignancies to explore the status of angiogenesis in pathology specimens [10-12]. Recently, poor prognostic value of vasohibin 1 was reported in prostate cancer [13]. In HCC, Wang et al [14] reported that vasohibin 1 expressed in the cytoplasm of HCC was one of the important factors influencing the clinical outcome of the patients. However, its details have not been described in the report.

Therefore, in this study, we first evaluated the potential validity of vasohibin 1 as an immunohistochemical marker of angiogenesis using double immunostaining with Ki-67 and vasohibin 1 or CD34 in archival materials of HCC. We then examined the status of vasohibin 1 in ECs and compared the findings with the results of CD34-labeled microvessel density (MVD) and the clinical outcome of the patients. We also conducted immunohistochemical analyses of FGF-2 and VEGF-A to further explore the details of angiogenesis in HCC.

2. Materials and methods

2.1. Patients

We recruited the surgical pathology materials of 181 consecutive Japanese patients with HCC undergoing curative surgical resection from 1999 to 2010 at Tohoku University Hospital, Sendai, Japan. The protocol of this study was approved by the Ethics Committee of Tohoku University School of Medicine, Sendai, Japan. Informed consent was obtained from each patient examined in this study. The median follow-up time was 36 months. None of the patients had received irradiation or chemotherapy before surgery. The relevant clinicopathologic information, including age, etiology, stage, and histologic grade, is summarized in Table 1. Staging was based on *TNM Classification of Malignant Tumours, Seventh Edition* by the International Union Against Cancer [15]. Cellular differentiation grades were classified according to the Edmondson-Steiner classification system (G1-G4) [16]. Those who developed a recurrence were treated with repeated hepatic resection, transcatheter arterial embolization, or radiofrequency ablation.

2.2. Immunohistochemistry

The specimens had been fixed in 10% formalin for 48 to 72 hours at room temperature and embedded in paraffin. Three-μm-thick sections were cut for both hematoxylin and eosin (HE) and immunohistochemical staining. Immunohistochemical staining for FGF-2 (Santa Cruz Biotechnology, Inc, Santa Cruz, CA), VEGF-A (Laboratory Vision, Fremont, CA), CD34 (Nichirei Bioscience, Tokyo, Japan), vasohibin 1, and Ki-67 (Dako, Copenhagen, Denmark) was performed as summarized in Table 2. After antigen retrieval, sections were

Table 1 Clinicopathologic characteristics of patients.

Age (range)	64 y (28-84)
Sex (M/F)	138:43
Etiology (%)	
HCV	86 (47.5%)
HBV	53 (29.3%)
HCV + HBV	4 (2.2%)
Others	5 (2.8%)
None	33 (18.2%)
TNM stage [a]	
Stage I	88 (48.6%)
Stage II	69 (38.1%)
Stage III	21 (11.6%)
IIIA	16
IIIB	4
IIIC	1
Stage IV	3 (1.7%)
IVA	1
IVB	2
Histologic grade	
G1	14 (7.7%)
G2	131 (72.4%)
G3	32 (17.7%)
G4	4 (2.2%)

NOTE. Etiologies of "Others" include alcohol, nonalcoholic steatohepatitis, and primary biliary cirrhosis.

[a] According to *TNM Classification of Malignant Tumours, 7th ed* [15].