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RAPID COMMUNICATION

Relationship between microvessel count and post-hepatectomy survival in patients with hepatocellular carcinoma

Atsushi Nanashima, Toshiyuki Nakayama, Yorihisa Sumida, Takafumi Abo, Hiroaki Takeshita, Kenichirou Shibata, Shigekazu Hidaka, Terumitsu Sawai, Toru Yasutake, Takeshi Nagayasu

Atsushi Nanashima, Yorihisa Sumida, Takafumi Abo, Hiroaki Takeshita, Kenichirou Shibata, Shigekazu Hidaka, Terumitsu Sawai, Toru Yasutake, Takeshi Nagayasu, Division of Surgical Oncology, Department of Translational Medical Sciences, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 8528501, Japan

Toshiyuki Nakayama, Department of Molecular Pathology, Atomic Disease Institute, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan

Author contributions: Nanashima A and Nakayama T contributed equally to this work; Nanashima A, Sumida Y, Abo T, Takeshita H, Hidaka S, Sawai T, Yasutake T, and Nagayasu T designed research; Nakayama T and Shibata K performed research; Abo T contributed analyzed data; And Nanashima A wrote the paper.

Correspondence to: Atsushi Nanashima, MD, Division of Surgical Oncology, Department of Translational Medical Sciences, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 8528501, Japan. a-nanasm@alpha.med.nagasaki-u.ac.jp
Telephone: +81-95-819-7304 Fax: +81-95-819-7306

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Abstract

AIM: To elucidate the relationship between the microvessel count (MVC) by CD34 analyzed by immunohistochemical method and prognosis in hepatocellular carcinoma (HCC) patients who underwent hepatectomy based on our preliminary study.

METHODS: We examined relationships between MVC and clinicopathological factors in 128 HCC patients. The modified Japan Integrated Staging score (mJIS) was applied to examine subsets of HCC patients.

RESULTS: Median MVC was 178/mm^2, which was used as a cut-off value. MVC was not significantly associated with any clinicopathologic factors or postoperative recurrent rate. Lower MVC was associated with poor disease-free and overall survivals by univariate analysis (P = 0.039 and P = 0.087, respectively) and lower MVC represented an independent poor prognostic factor in disease-free survival by Cox's multivariate analysis (risk ratio, 1.64; P = 0.024), in addition to tumor size, vascular invasion, macroscopic finding and hepatic dysfunction. Significant differences in disease-free and overall survivals by MVC were observed in HCC patients with mJIS 2 (P = 0.046 and P = 0.0014, respectively), but not in those with other scores.

CONCLUSION: Tumor MVC appears to offer a useful prognostic marker of HCC patient survival, particularly in HCC patients with mJIS 2.

Key words: Hepatocellular carcinoma; Hepatic resection; Microvessel count; CD34; Modified Japan integrated staging score

Peer reviewer: Qin Su, Professor, Department of Pathology, Cancer Hospital and Cancer Institute, Chinese Academy of Medical Sciences and Peking Medical College, PO Box 2258, Beijing 100021, China

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INTRODUCTION

Hepatic resection is a useful option for radical treatment of hepatocellular carcinoma (HCC). However, recurrence rate after resection remains high[1,2]. Patient survival thus remains unsatisfactory due to high recurrence rate even at this stage. Clinicopathological factors in HCC are related to tumor recurrence[3] and tumor biological characteristics provide useful information regarding the activity of HCC. According to previous reports, candidates for tumor biological factors and molecular markers include abnormal expression of p53[4], nm23 (a tumor suppressor gene)[5], tumor angiogenesis[6], proliferative activity[7], growth factors[8], DNA ploidy[9] and other molecular markers[10]. Some of

these markers are related to prognosis in HCC patients. Combination of conventional clinicopathological factors and prognostic factors of tumor biology may improve prediction of prognosis after hepatectomy for HCC and may contribute to a new staging classification.

Tumor angiogenesis may be important to support tumor growth[11], and HCC is a hypervascular tumor expressing several angiogenic factors[12]. Levels of angiogenic factors such as vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (b-FGF) are increased in HCC and might affect patient survival[12,13]. Recent studies have also shown that microvessel density (MVD) in HCC or non-cancerous liver surrounding tumor correlates with tumor aggressiveness and prognosis[6,14-16]. We have previously provided a preliminary demonstration of the comprehensive analysis of various biological factors, revealing that microvessel count (MVC) using CD34 antibody was independently associated with poor prognosis in HCC patients undergoing hepatic resection by multivariate analysis[17]. However, contrary to other reports, poor patient prognosis was related to lower MVCs. We hypothesize that hypovascularity in HCC represents a factor associated with treatment-resistance such as chemoembolization, which causes poor prognosis.

The present study examined the relationship between MVC in HCC using immunohistochemical stains and conventional clinicopathological factors and prognosis in a larger number of HCC patients with longer follow-up period to clarify our hypothesis. Furthermore, we examined this relationship in subsets of patients after applying the modified Japan Integrated Staging score (mJIS).

MATERIALS AND METHODS

Patients

HCC specimens from 128 patients (104 men, 24 women) were obtained during surgery on patients admitted to the Division of Surgical Oncology at Nagasaki University Graduate School of Biomedical Sciences (NUGSBS) between 1990 and 2005. Mean age for patients at the time of surgery was 62.9 ± 8.2 years (range, 28-78 yr). Prior to surgery for HCC, 36 patients (28.1%) were treated using either chemoembolization (n = 30) or local ablation (n = 6), including alcohol injection in 2 patients and radiofrequency ablation (RFA) in 4 patients. After surgery, 3 patients (2.3%) received adjuvant 5-fluorouracil chemotherapy by intra-arterial injection through a subcutaneously implanted reservoir. Child-Pugh classification was B in 11 patients (8.6%) and A in 117 patients. The liver damage grade by the Liver Cancer Study Group (LCSG) of Japan in 2000 was B in 26 patients and A in 102 (Table 1)[18]. The operative procedures included lobectomy or extended lobectomy (n = 54), segmentectomy or subsegmentectomy (n = 43) and partial resection (n = 31). Radical hepatectomy was performed to remove hepatic tumor without leaving any residual tumor. All hepatic tumors were completely resected without macroscopic exposure of the amputated section to the remaining liver. The present series included no in-hospital deaths and the only causes of death were cancer-related. Minimum follow-up period after hepatic resection of HCC was 24 mo.

Table 1 Definition and criteria of Child-Pugh classification and liver damage grade

	A	B	C
Child-Pugh classification			
Encephalopathy	None	Mild	Coma
Ascites	None	Responsive	Unresponsive
Serum bilirubin (mg/dL)	< 2.0	2.0-3.0	> 3.0
Serum albumin (g/dL)	> 3.5	2.8-3.5	< 2.8
Prothrombin activity (%)	> 70	40-70	< 40
Liver damage grade[18]			
Ascites	None	Responsive	Unresponsive
Serum bilirubin (mg/dL)	< 2.0	2.0-3.0	> 3.0
Serum albumin (g/dL)	> 3.5	3.0-3.5	< 3.0
ICG R15 (%)	< 15	15-40	> 40
Prothrombin activity (%)	> 80	50-80	< 50

ICG R15: Indocyanine green retention rate at 15 min.

We used the classification system of the General Rules for the Clinical and Pathological Study of Primary Liver Cancer[19]. This system provides a clinicopathological evaluation of HCC. Macroscopic classification as described by Classification of Primary Liver Cancer[19] was also applied in the study. All study protocols were approved by the Human Ethics Review Board of our institution. Informed consent for data collection was obtained from each patient during this period. Anesthetic and patient data were retrieved from the NUGSBS database.

Immunohistochemical staining

Resected specimens were fixed in 10% formalin and embedded in paraffin. Thin sections (4 μm) were deparaffinized twice using xylene and rehydrated in a series of ethanol solutions (100%, 90% and 80%). Sections were placed in 0.01 mol/L trisodium citrate dehydrate buffer (pH 6.0) and treated in a microwave oven for 10 min at 500 W. For CD34 staining[17,20], tissue sections were digested with 0.2% trypsin in 0.01 mol/L phosphate-buffered saline (PBS) for 20 min at 37℃. In the next step, tissues were immersed in 3% H_2O_2 with distilled water for 10 min to inactivate endogenous peroxidases. After blocking non-specific binding by normal goat serum, sections were incubated overnight at 4℃ with mouse anti-monoclonal CD34 antibody (1:25; QB-END/10, Novocastra Laboratories, Newcastle, United Kingdom) as the primary antibody. This was followed by reaction with biotinylated anti-immunoglobulin and reagent using labeled streptavidin-biotin (LSAB) kit peroxidase (Dako, Carpinteria, CA). The peroxidase reaction was visualized with 0.01% H_2O_2 and 3,3'-diaminobenzidine under light microscopy (× 200). For MVCs using CD34 staining, average count was determined in the 5 most-vascular areas in the HCC examined at 200 × magnification[17,20]. Two pathologists blindly assessed each slide.

Table 2 Definition and criteria of TNM stage for HCC according to the Liver Cancer Study Group of Japan[18]

Criteria for TNM categories	
(1) Number of tumors: 1	
(2) Tumor size: ≤ 2 cm	
(3) No vascular or bile duct invasion	
T category	T1: Fulfilling all three criteria
	T2: Fulfilling two criteria
	T3: Fulfilling one criterion
	T4: Fulfilling none of the criteria
N category	N0: Absence of lymph node metastasis
	N1: Presence of lymph node metastasis
M category	M0: Absence of distant metastasis
	M1: Presence of distant metastasis
Stage Ⅰ	T1 N0 M0
Stage Ⅱ	T2 N0 M0
Stage Ⅲ	T3 N0 M0
Stage Ⅳ-A	T4 N0 M0 or T1-T4, N1M0
Stage Ⅳ-B	T1-4, N0 or N1, M1

Table 3 Definition and criteria for JIS and mJIS

	Score			
	0	1	2	3
Original JIS score[21]				
Japanese TNM stage	Ⅰ	Ⅱ	Ⅲ	Ⅳ
Child-Pugh Classification	A	B	C	
Modified JIS score[22]				
Japanese TNM stage	Ⅰ	Ⅱ	Ⅲ	Ⅳ
Liver damage grade	A	B	C	

TNM: tumor-node-metastasis.

Staging criteria for the mJIS

We used the pathological tumor-node-metastasis (pTNM) classification system as defined by the Liver Cancer Study Group (LCSG) of Japan in 2000[18]. T category was determined based on 3 factors: number, size, and vascular or bile duct invasion. N category was determined as the presence of lymph node metastasis, while M category represented the presence of distant metastases. TNM staging comprises 4 stages based on the combination of T, N, and M categories (Table 2). The original Japan Integrated Staging score proposed by Kudo *et al*[21] comprised the sum of scores for the two variables of Japanese TNM classification and Child-Pugh classification. In the mJIS proposed by our institute[18,22], Child-Pugh classification was replaced by the score for liver damage grade as defined by the LCSG of Japan (Table 3).

Statistical analysis

Continuous data are expressed as mean ± standard deviation. Data from different groups were compared using one-way analysis of variance (ANOVA) and examined by Student's *t*-test or Dunnett's multiple comparison test. For univariate analysis, categorical data were analyzed using Fisher's exact test. Disease-free and overall survival rates were calculated according to the Kaplan-Meier method, and differences between groups were tested for significance using the log-rank test. Multivariate analysis was performed using proportional hazards regression modeling. A two-tailed value of $P < 0.05$ was considered statistically significant. All statistical analyses were performed using SAS software (Statistical Analysis System, Cary, NC).

RESULTS

Among the 128 patients in the present study, disease-free 1-, 3-and 5-year survival rates were 63%, 39% and 29%, respectively, and median disease-free survival was 3.5 years. Overall 1-, 3-and 5-year survival rates were 89%, 65% and 48%, respectively, and median overall survival was 5.9 years. Of 94 patients (75.0%) who displayed tumor recurrence after hepatectomy, 85 (90.4%) received chemoembolization ($n = 81$) or alcohol injection ($n = 4$).

Median MVC within the tumor area was 178/mm^2, and this value was applied as a cut-off value. Table 4 shows the relationship between MVC and clinicopathological features in 128 HCC patients. However, MVC was not significantly associated with any clinicopathological factors, TNM stage or postoperative recurrence.

Figure 1 shows disease-free and overall survival after hepatectomy compared to MVC. Disease-free survival rate was significantly lower in patients with lower MVC than in patients with higher MVC ($P = 0.039$). Overall survival rates tended to be lower in patients with lower MVC than in patients with higher MVC, but this difference was not significant. Table 5 shows the results of multivariate analysis for disease-free and overall survival after hepatectomy for various factors identified as displaying significant associations on univariate analysis. Multiple tumors, vascular involvement of the tumor, liver damage grade B and lower MVC represented independent risk factors for poor disease-free survival after hepatectomy ($P = 0.0004$, 0.003, 0.007 and 0.024, respectively). Multiple tumors, vascular involvement of the tumor and macroscopic findings were identified as independent risk factors for poor overall survival after hepatectomy ($P = 0.025$, 0.014 and 0.017, respectively), whereas MVC was not associated with overall survival ($P = 0.266$).

Figure 2 shows disease-free survival by comparing MVC for each mJIS score. In HCC with mJIS 0 or 1, MVC was not associated with disease-free survival. Lower MVC was significantly associated with poor disease-free survival in HCC with mJIS 2. Lower MVC tended to be associated with poor disease-free survival in HCC with mJIS ≥ 3, but this result was not significant. Figure 3 shows overall survival by comparing MVC at each mJIS score. In HCC with mJIS 0, 1 or ≥ 3, MVC was not associated with overall survival. Lower MVC was significantly associated with poor disease-free survival in HCC with mJIS 2.

DISCUSSION

Previous studies have investigated prognostic factors in HCC for patients who underwent radical hepatectomy

Table 4 Relationship between microvessel density and clinicopathological factors in HCC

	Microvessel count > 178/mm²/≤ 178/mm²	P
Pretreatment		
No (*n* = 98)	53/45	1.0
Yes (*n* = 30)	16/14	
Liver damage		
A (*n* = 102)	54/48	0.969
B (*n* = 26)	15/11	
Background liver		
Normal liver (*n* = 7)	3/4	0.459
Chronic hepatitis (*n* = 75)	38/37	
Cirrhosis (*n* =46)	28/18	
Viral status		
Hepatitis virus B (*n* = 50)	32/18	0.152
Hepatitis virus C (*n* = 61)	31/30	
Both hepatitis virus B and C (*n* = 4)	2/2	
Non-B, non-C (*n* = 13)	4/9	
Number of tumors		
Solitary (*n* = 92)	50/42	1.0
Multiple (*n* =36)	19/17	
Tumor size		
< 3 cm (*n* = 31)	15/16	0.371
3-5 cm (*n* = 49)	24/25	
> 5 cm (*n* = 48)	30/18	
Macroscopic finding[1]		
SN (*n* = 37)	16/21	0.207
SNEG (*n* = 39)	25/14	
CMN (*n* = 52)	28/24	
Vascular involvement		
No (*n* = 83)	42/41	0.476
Yes (*n* = 45)	27/18	
Histopathological differentiation		
Well (*n* = 19)	12/7	0.437
Moderately (*n* = 97)	55/42	
Poorly (*n* = 6)	2/4	
Japan tumor-node-metastasis stage[2]		
Ⅰ (*n* = 14)	5/9	0.542
Ⅱ (*n* = 51)	27/25	
Ⅲ (*n* = 44)	25/19	
Ⅵ-A (*n* = 19)	12/7	
Recurrence		
No (*n* = 32)	16/16	0.386
Yes (*n* = 96)	53/43	

[1]Macroscopic classification by Liver Cancer Study Group of Japan[19]; [2]The General Rules for the Clinical and Pathological Study of Primary Liver Cancer[18]. SN: Single nodular; SNEG: Single nodular with extranodular growth; CMN: Confluent multinodular group.

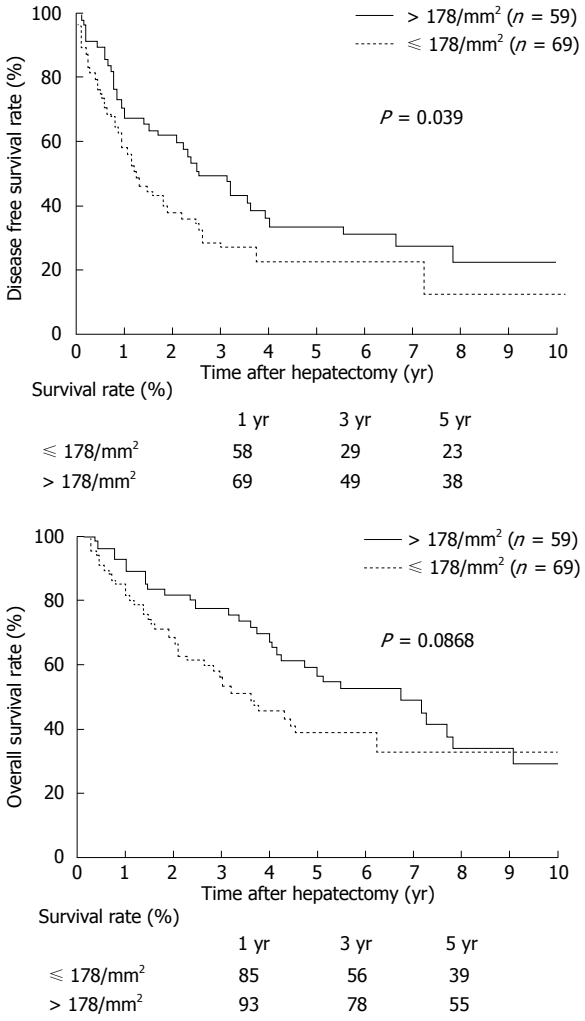

Figure 1 Relationship between microvessel count (MVC) and disease-free and overall survival in patients with hepatocellular carcinoma (HCC) who underwent hepatic resection.

and in whom tumor stage had been determined[23,24]. The International Union against Cancer (UICC) and Japanese TNM classification systems for predicting patient prognosis in HCC did not provide good reflection of patient survival in HCC patients[25,26]. Combined staging systems with hepatic function have recently been applied[21,22,27,28], and among these, the JIS score system was applied in Japan[21]. Child-Pugh classification was used in this system, but liver damage grade by the LCSG of Japan offers a better reflection of patient survival[18,29]. The mJIS score system thus includes liver damage grade and has been applied in a few reports[22]. The present study also applied mJIS score. In the next step, a staging system for HCC may need additional useful factors comprising tumor biological or molecular markers. The present study identified angiogenic factors of MVC as potentially useful. In the last decade, microvessel density using CD34 as a prognostic parameter in HCC patients has been reported and appears consistently very useful[6,16,30,31]. We also performed a preliminary study of the significance of MVC for survival in HCC patients and, unlike other reports, revealed hypovascularity (i.e., lower MVC) as a poor prognostic parameter[17].

MVC was not associated with any clinicopathological factors or recurrence rate after hepatectomy in either the present study or our pilot study[17]. In contrast to our results, El-Assal *et al*[30] and other investigators[6,14,15,30-32] have reported that microvessel density in HCC is increased in larger tumors, tumors with poor differentiation and cirrhotic patients, while higher microvessel density is associated with intra-hepatic recurrence. In the present study, however, MVC did not correlate with co-existing cirrhosis or with various etiological factors related to chronic hepatitis such as viral status. Conversely, Sun *et al*[33] found no relationship between MVC and either clinicopathological factors or patient prognosis. The relationship between tumor vascularity and clinicopathological features thus remains controversial. Increased micro-angiogenesis is definitely

Table 5 Multivariate analysis by Cox's proportional hazard test of prognostic factors influencing disease-free survival and overall survival in HCC after hepatectomy

	Disease-free survival			Overall survival		
	Risk ratio	95% CI	*P*	Risk ratio	95% CI	*P*
Number ⩾ 2 lesions	2.28	1.44-3.60	0.0004	1.87	1.08-3.24	0.025
Vessel involvement positive	2.04	1.28-3.24	0.003	2.07	1.16-3.68	0.014
Macroscopic finding SNEG or CMN	1.43	0.89-2.29	0.135	2.33	1.17-4.66	0.017
Surgical margin positive	1.75	0.93-3.33	0.086	1.59	0.65-3.91	0.310
Blood loss > 1500 mL	1.36	0.81-2.29	0.135	1.50	0.82-2.74	0.189
Liver damage grade B	1.99	1.20-3.31	0.007	1.44	0.77-2.69	0.255
PIVKA-Ⅱ > 400 mAU/mL	1.30	0.81-2.13	0.274	1.15	0.65-2.04	0.636
Microvessel count ⩽ 178/mm^2	1.64	1.06-2.50	0.024	1.35	0.71-2.04	0.266

Macroscopic classification by Liver Cancer Study Group of Japan[19]. SN: Single nodular; SNEG: Single nodular with extranodular growth; CMN: Confluent multinodular group; CI: Confidence interval.

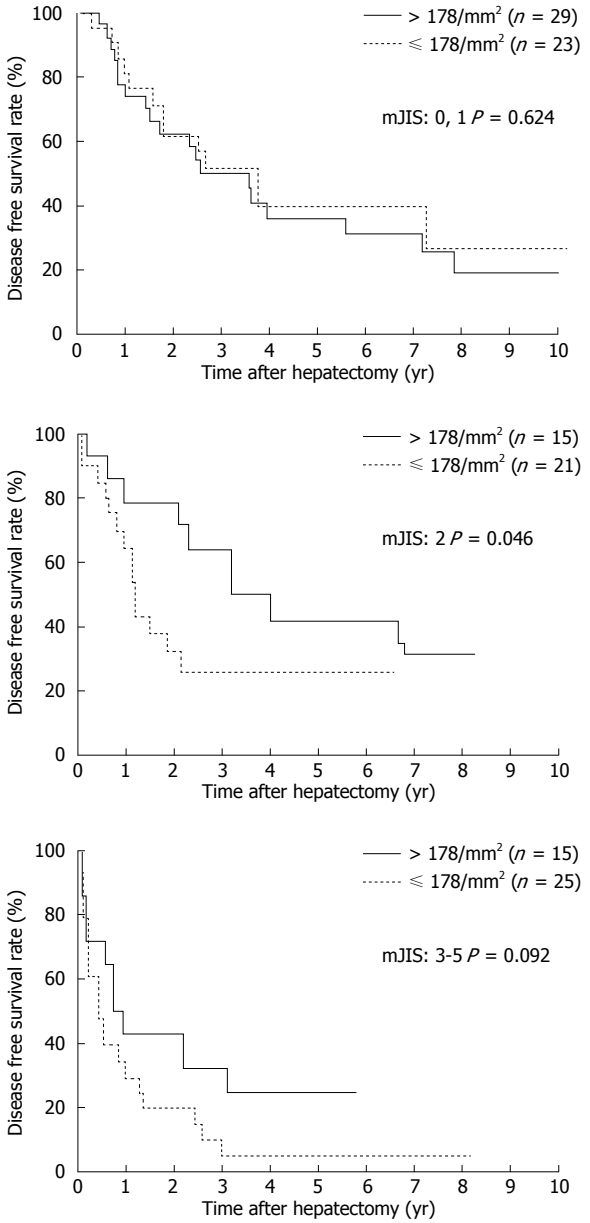

Figure 2 Relationship between MVC and disease-free survival using the modified Japan Integrated Staging score (mJIS) in HCC patients who underwent hepatic resection.

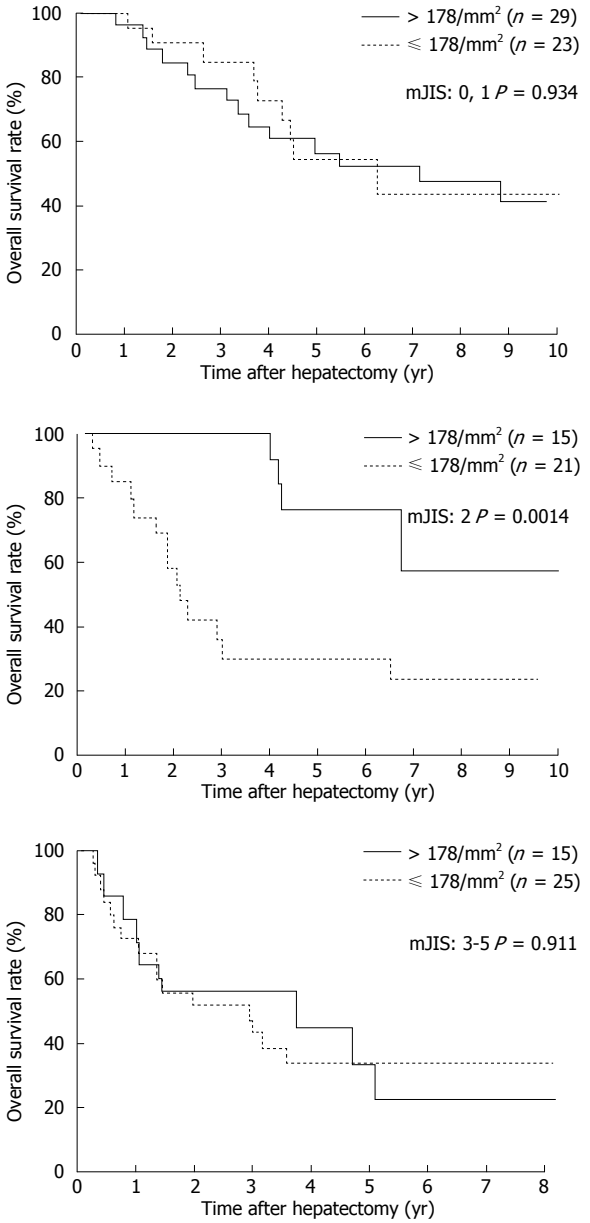

Figure 3 Relationship between MVC and overall survival using the mJIS in HCC patients who underwent hepatic resection.

associated with carcinogenesis of HCC and development to advanced tumor[12,32]. At the stage of smaller-sized HCC, tumor vascularity is already rich on computed

tomography or enhanced ultrasonography[34,35]. Considering the mechanisms of HCC characterized above, increased microvessel density of the tumor is a logical result. When tumor demonstrates a large tumor size or poor histologic differentiation, tumor vascularity may be decreased. Previous reports also showed that HCC tumor vascularity decreased with the progression of histopathological grade[36,37]. Our results showed that lower MVC tended to be higher in poorly differentiated HCC, but this finding was not significant. A larger sample of poorly differentiated HCCs is needed to clarify this issue, as only 6 cases were included in the present series.

With respect to disease-free and overall survival after hepatectomy[1,2,6,24], the results from our series were favorable. Counts $\leqslant 178/mm^2$ for HCC were associated with worsened prognosis in patients undergoing hepatectomy, particularly in disease-free survival by univariate and multivariate analysis in the present study. Compared to other independent risk factors, the odds ratio was lower for MVC than for other factors such as a number of tumors, vascular involvement, macroscopic findings or liver damage grade. The role of MVC for tumor relapse or progression might not be particularly strong. This result contradicts findings in other reports described above[6,14,16,30,31]. As explained above, HCC naturally acquires rich tumor vascularity in the early stages and most clinically treated HCCs represent tumor with radiological enhancement[34,35,38]. Hypervascularity is observed in the majority of HCC[36]. As tumor vascularity decreases in HCC with deterioration of histological differentiation and/or aggressive invasiveness[36,37], the malignant potential of hypovascular HCC could increase relative to that of hypervascular HCC, thus leading to poorer prognosis. In cases of hypervascular HCC, initial or recurrent tumor can be observed in earlier stages under various imaging modalities, and treatments combined with chemoembolization or chemotherapy via tumor microvessels may well prove effective[39]. We speculate that hypovascular HCC is difficult to detect by conventional imaging and to treat by chemoembolization or other therapy, and treatment selection is therefore limited. Previous studies showed that tumor vascularity correlates with the response to chemotherapy, which in turn correlates with survival[40-42]. Furthermore, tumor hypervascularity, which is observed in most HCC, correlated with good response to chemoembolization, whereas hypovascularity of early or sclerosing HCC did not correlate with good response to chemoembolization[40,41]. Thus, a better response to arterial chemoembolization is associated with prolonged survival[40,41]. In our series, most patients with postoperative recurrence of HCC received arterial chemoembolization and, therefore, the prognosis of these patients could have been influenced by tumor vascularity and response to chemoembolization. Although we could not provide such evidence in recurrent tumors in our follow-up study, it is possible that the vascularity of primary HCC could influence the biological characteristics of recurrent HCC. For these reasons, the survival of patients with hypovascular HCC logically would be worse after recurrence. Our results concerning MVC are thus biologically quite feasible. Anti-angiogenic therapy is a promising treatment for HCC[43]; however, the response of hypovascular HCC to such treatment remains problematic.

In addition to tumor-associated factors, hepatic functional reserve and liver function after surgery influence postoperative prognosis[3,6,44]. In the present series, we applied mJIS score to examine subsets of HCC patients. Poon *et al*[6] also reported that the significance of MVC differed between subsets of HCC patients. Our results showed no marked differences in disease-free and overall survival according to MVC in the early stage of mJIS 0 or the advanced stage of mJIS 3-5. A significant difference in MVC was only observed for mJIS 2. Poon *et al*[6] found microvessel density by CD34 as the only significant factor predictive of disease-free survival in patients with HCC (5 cm, but no significant prognostic influence was seen for larger HCC. In early-stage HCC, tumor can be sufficiently cured by hepatic resection even in the presence of malignant potential. Conversely, in severely advanced HCC, other significant prognostic factors might exert greater influence on tumor aggressiveness and survival, such as number of tumors, vascular involvement, macroscopic findings or liver damage grade, as indicated in the present study. For mJIS 2, patient survival with hepatic resection or ablation therapy was not particularly satisfactory compared to mJIS 0 or 1 according to our recent report[45]. In this stage of mJIS 2, treatment indications need to be defined according to the appropriate prognostic factors. In cases where lower vessel count was observed at initial resection, liver transplantation following recurrence may be a good option. Our results showed that MVC did not correlate with other clinicopathological parameters and is an independent risk factor for prognosis. Thus, according to the present results, MVC would represent a useful parameter to decide treatment modality.

In conclusion, we have demonstrated that lower MVC by CD34 in HCC offers an independent predictor of disease-free and overall survival in patients with HCC, particularly in HCC with mJIS 2. As a tumor biological factor, MVC representing tumor angiogenesis offer a new candidate prognostic factor in HCC to predict tumor recurrence and patient survival in combination with traditional pathological factors. Furthermore, this marker can be applied as a predictive marker to select molecular targeting treatments in future.

COMMENTS

Background

Tumor biological characteristics provide useful information on the activity of hepatocellular carcinoma (HCC). The combination of conventional clinicopathological factors and prognostic factors related to tumor biology may improve prediction of prognosis of patients with HCC. HCC is a hypervascular tumor that expresses various tumor angiogenic factors. Microvessel density (MVD) in HCC may correlate with tumor aggressiveness and prognosis.

Research frontiers

Tumor angiogenesis may be important for tumor growth. High levels of

angiogenic factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (b-FGF) have been described in HCC, and they correlate with patient survival. Microvessel counts (MVC) in HCC or non-tumorous liver tissue that surrounds HCC correlate with tumor aggressiveness and prognosis. On the other hand, it should be difficult to chemoembolize hypovascular HCC. Therefore, we hypothesized that low MVCs correlates with poor prognosis.

Innovations and breakthroughs

Our results presented new findings that were different than studies published previously by other investigators. However, we found that a low MVC is an independent prognostic factor for tumor relapse. This was particularly significant in HCC patients with mJIS 2.

Applications

MVC could be a potentially useful marker of prognosis in HCC by predicting tumor recurrence and patient survival, in addition to traditional pathological factors. Furthermore, MVC could help in clinical decision making with respect to the selection of treatment modality.

Peer review

Our study identified a novel prognostic factor that can be used to predict tumor recurrence and survival of patients with HCC. This marker can be potentially used to select the most appropriate surgical treatment modality, such as a liver transplantation particularly in patients with mJIS 2.

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