The Prognostic Significance of Microvessel Density in Intrahepatic Cholangiocarcinoma

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Objective: *Intrahepatic cholangiocarcinoma (IHCC) is the second most common primary cancer of the liver. Tumor angiogenesis seem to play an important role in tumor growth and prognosis of cancer patients. The purpose of the present study was to determine the prognostic value of tumor microvessel density (MVD) in patients with IHCC.*
Material and Method: *Clinicopathological prognostic factors, recurrence rate, and survival in 22 patients with IHCC who underwent liver resection for IHCC were reviewed. Tumor MVD was estimated using immunohistochemical methods. Overall probabilities of recurrence and survival were estimated using Kaplan-Meier methods. Prognostic significance of MVD and other factors was tested using Cox proportional hazards regression.*
Results: *There was no significant association between any clinicopathologic factors (age, sex, tumor markers, and pathologic factors including MVD) and time-to-tumor recurrence. The only prognostic factor associated with survival was tumor stage. MVD was neither a significant survival predictor nor a predictor of tumor recurrence.*
Conclusion: *The only factor associated with poor prognosis in patients with IHCC in the present study was higher tumor stage. MVD was not a significant prognostic factor in patients with IHCC.*

Keywords: *Intrahepatic cholangiocarcinoma, IHCC, Tumor microvessel density, MVD, Tumor angiogenesis, Tumor neovascularization, Survival*

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Intrahepatic cholangiocarcinoma (IHCC), a malignancy arising from the intrahepatic bile ducts, is the second most common primary cancer of the liver after hepatocellular carcinoma. Overall, approximately 15% of all liver cancers are estimated to be IHCC[1-3]. The frequency of IHCC among all liver cancers ranges from 5% in men and 12% in women in Osaka, Japan, to 90% in men and 94% in women in Khon Kaen, Thailand[3,4]. The prognosis of IHCC is generally poor because the disease is usually advanced at the time of diagnosis.

Surgery is considered the most effective way to treat IHCC. Chu et al[5] found the median survival time after conservative therapy and hepatic resection to be 1.8 months and 12.2 months, respectively. However, the outcome of patients with advanced IHCC is extremely poor even after resection. The presence of lymph node metastasis in the resected specimen has been reported to be the worst prognostic factor in most previous studies[6-8].

Angiogenesis is involved in cancer formation, growth, and metastasis[9]. Moreover, highly vascularized carcinomas have a more aggressive clinical course than carcinomas with low vascularization[10]. Shirabe et al. found tumor angiogenesis to be a good prognostic factor in node-negative IHCC[11]. CD31 and CD34 have been used as the markers for the measurement of tumor angiogenesis[11-13]. Microvessel density (MVD) determination was modeled by using immunohistochemical staining of CD31 and CD34[12]. Several studies found that MVD is one of the prognosis

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factors of_cancer aggressiveness and survival[11,12,14]. The aim of the present study was to identify prognostic factors, including tumor angiogenesis, which might be significantly associated with cancer-related death and recurrence in Thai IHCC patients.

Material and Method

Patients

Between January 1995 and December 2004, 39 patients underwent liver resection for IHCC at the Department of Surgery, Ramathibodi Hospital, Bangkok, Thailand. Of these, 22 had leftover pathological specimens suitable for immuno-histochemical study. Information such as clinicopathologic data, survival, and recurrence patterns were reviewed and abstracted from clinical records.

Histologic examination

Resected specimens were cut along the largest diameter and fixed with 10% buffered formalin. Sections were made from the slice with the largest diameter and embedded in paraffin. The 5-mm thick sections were stained with hematoxylin and eosin. Pathologic information noted included tumor diameter, tumor grading, tumor foci (single or multiple), and status of the resection margin. Tumors were graded as well, moderately, and poorly differentiated adenocarcinoma according to their morphology[15].

Immunohistochemical staining with CD-31 and CD-34

Sections were deparaffinized with xylene and dehydrated in ethanol. The cut surface of the slice with the largest diameter was immunohistologically stained. Endothelial adhesion molecule antigens (CD-31 and CD-34) were treated with 0.3 mg/ml trypsin in phosphate-buffered saline at 37°C for 30 minutes. The sections were then exposed to 10% non-immunized serum in phosphate-buffered saline for 10 minutes and then treated at 4°C overnight with primary antibodies. The primary antibodies were monoclonal mouse anti-human CD-31 (Dako, Glostrup, Denmark) and CD-34 (Immunotech, France) at a dilution of 1: 20.

The staining for CD-31 was too faint to be counted (Fig. 1); therefore only CD-34 staining was used in the present study (Fig. 2).

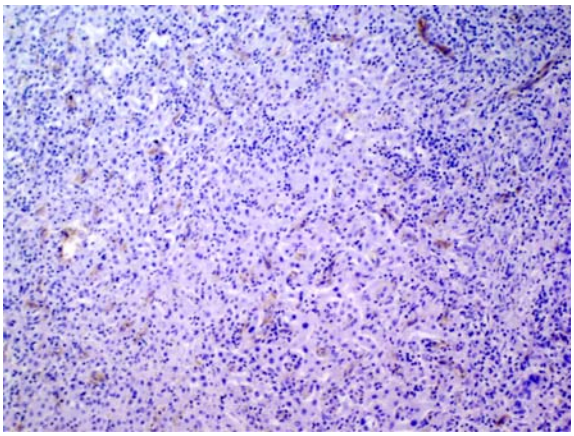

Fig. 1 Immunohistochemical staining for CD-31 in IHCC

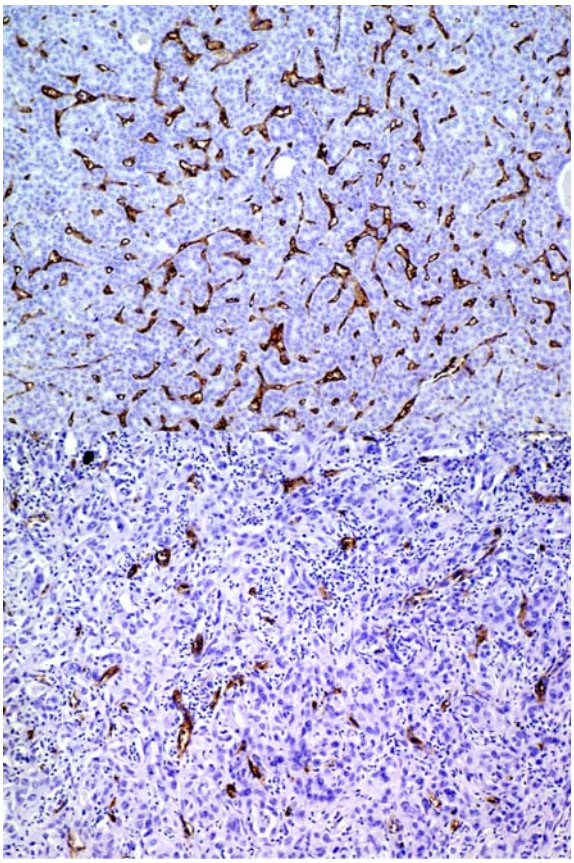

Fig. 2 Immunohistochemical staining for CD-34 in IHCC: top showed high microvessel density; bottom showed low microvessel density

Microvessel density

Microvessel count was performed according to method of Takahashi et al[16]. Briefly, the tumoral areas containing the highest number of capillaries and small vessels were identified by light microscopy at low power (X40 and X100 magnification). For each tumor, three areas considered to have the densest

neovascularization were identified. The representative areas were at the most invasive portion of the tumor near the normal hepatic parenchyma[17]. The representative microvessel count was the average of the vessel counts obtained at X200 magnification in the three areas[11]. Any brown-stained endothelial cell or endothelial cluster clearly separated from the adjacent microvessels, tumor cells, and connective tissue was considered a single, countable microvessel[17]. The microvessel count was divided by the area of observation (in mm^2), to obtain the microvessel density (MVD). Patients were divided into two groups based on MVD: the high MVD group (> 10.4 microvessels/mm^2) with 12 patients and the low MVD group (≤ 10.4 microvessels/mm^2) with 10 patients.

Statistical analysis

Continuous data were summarized as mean (SD) or median (range). Categorical data were summarized as counts and percentages. The survival and recurrence-free probability curves were estimated using the Kaplan-Meier method. Univariable and multivariable Cox (proportional hazards) regression were used to identify significant prognostic factors for cancer recurrence and survival as well as to estimate the hazard ratios and their 95% confidence intervals (CI). Statistical significance was defined as a p-value of 0.05 or less. All statistical analyses were performed using STATA v. 7 (Stata Corp, College Drive, TX, USA) software.

Results

Microvessel density

The mean microvessel density (SD) was 10.4 (5.2) microvessels/mm^2 (Table 1), the median microvessel density was 9.0 (range 4.4 to 24.4) microvessels/mm^2.

Survival

The survival probabilities of IHCC patients in the present study were 45% at 1 year, 16% at 3 years, and 5% at 5 years. The median survival time was 8.6 months (Fig. 3).

Recurrence

The recurrence-free probability of IHCC patients in the present study was 54% at 1 year, 18% at 3 years, and 9% at 5 years. The median time-to-recurrence was 13.8 months (Fig. 4). Postoperative recurrence was observed in 13 patients (59%). Of these, intrahepatic recurrence was observed in 10 (77%). The remaining three patients (23%) had extrahepatic recurrence, including lung metastasis in one patient and lymph node metastasis in two patients (one at aorto-caval node and the other at the lymph node near the 3rd part of the duodenum).

Prognostic factors

Significant risk factors for cancer mortality on univariable analysis were high tumor staging and tumor recurrence (Table 1). On multivariable analysis only tumor staging was significantly associated with cancer mortality. There were no significant risk factors for cancer recurrence.

Discussion

Angiogenesis is indispensable for cancer growth, invasion, and metastasis. There have been

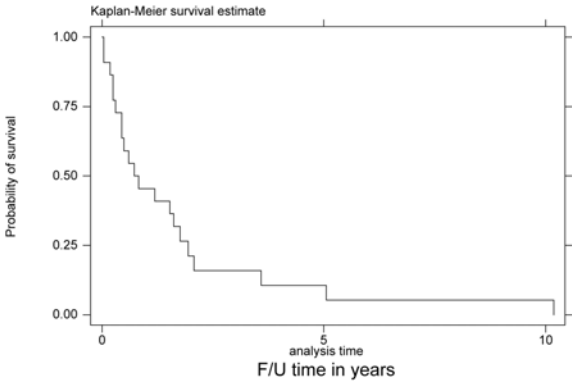

Fig. 3 Cancer survival was estimated using the Kaplan-Meier method. The 1, 3 and 5 years survival probabilities were 45, 16 and 5%, respectively

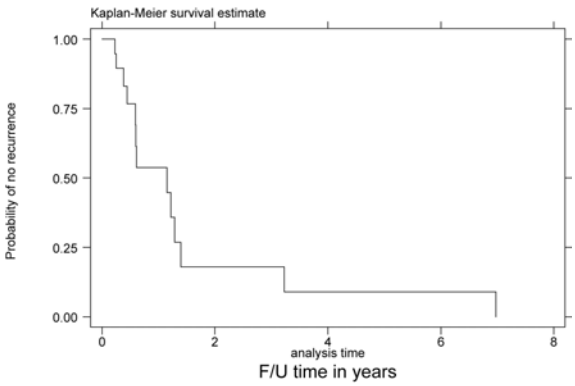

Fig. 4 Recurrence-free probability was estimated using the Kaplan-Meier method. The 1, 3 and 5 years recurrence-free probability were 54, 18 and 9%, respectively

Table 1. Prognostic factors for cancer mortality and recurrence

Factor	Summary (n = 22)	Hazard ratio for death (95% CI)	p-value	Hazard ratio for recurrence (95% CI)	p-value
Age (year)					
Mean (SD)	55.7 (11.9)	1.03 (0.99-1.08)	0.094	1.05 (0.99-1.11)	0.067
Sex (male)					
Number (%)	13 (59)	1.50 (0.58-3.88)	0.399	1.32 (0.39-4.44)	0.651
CEA (ng/ml)					
Median (range)	7.2 (1.1-387.5)	1.01 (0.99-1.01)	0.093	1.00 (0.99-1.01)	0.393
CA 19-9 (u/ml)					
Median (range)	120.9 (0-4097.4)	1.00 (0.99-1.00)	0.418	1.00 (0.99-1.00)	0.209
Tumor size (cm)					
Mean (SD)	6.11 (2.9)	0.92 (0.77-1.10)	0.370	0.89 (0.70-1.13)	0.338
Multiple tumor foci					
Number (%)	14 (64)	0.86 (0.35-2.13)	0.747	1.53 (0.45-5.17)	0.492
Staging: number (%)		1.74 (1.11-2.73)	0.016	1.01 (0.60-1.68)	0.970
I	0 (0)				
II	5 (22.7)				
IIIA	5 (22.7)				
IIIB	5 (22.7)				
IIIC	6 (27.2)				
IV	1 (4.5)				
Grading: number (%)		0.79 (0.36-1.74)	0.562	0.91 (0.35-2.36)	0.844
Well diff	5 (23)				
Moderate diff	13 (59)				
Poorly diff	4 (18)				
Positive margin					
Number (%)	10 (62.5) (n = 16)	0.56 (0.18-1.69)	0.304	0.46 (0.10-2.09)	0.316
Recurrence					
Number (%)	13 (59)	0.55 (0.01-0.27)	0.001	-	-
Average MVD					
Mean (SD)	10.4 (5.2)	0.98 (0.92-1.06)	0.721	0.99 (0.91-1.08)	0.900

95% CI = 95% confidence interval

several reports demonstrating the prognostic significance of angiogenesis in various kinds of cancers[18-24]. Rapid tumor growth and invasion into the surrounding organs, frequent presence of metastatic lymph nodes at diagnosis, and early recurrence after curative resection indicate that the disease has disseminated early by systemic spread and that the patient has a poor prognosis.

Angiogenesis and the development of metastasis are intrinsically connected. Experimental data have suggested that the establishment and growth of metastasis are influenced by soluble angiogenetic factors secreted from the originating solid tumor[25].

At present, CD-31 is considered the most sensitive and panspecific marker for endothelial cells. The extent of angiogenesis as estimated by CD-31 staining is closely linked to nodal metastasis[26] and prognosis[25,27]. The use of CD-34 has also been shown to yield a similar relationship between tumor angiogenesis and the prognosis of IHCC[28]. In the present study, the staining of CD-34 was used as the main marker of angiogenesis, as the staining of CD-31 was too weak to be useful.

In the present study, MVD was not significantly associated with IHCC-related death or recurrence. For each unit increase in the MVD, the hazard ratio for IHCC-related death was 0.98, with 95% CI from 0.92 to 1.06. The hazard ratio for cancer recurrence was 0.99 for each unit increase in the MVD, with 95% CI from 0.91 to 1.08. Factors that increased the hazard of death in the present study were high tumor staging and the absence of recurrence. The latter finding, seemingly paradoxical, can be explained by the fact that most patients who died early on did not

have a chance to develop recurrent cancer.

The 5-year survival for the present series of patients was only 5%. Most of the patients were classified as having stage III disease or worse (77%). Thus, the population of IHCC patients seen in the present institution generally has a poor prognosis.

One of the important steps in tumor progression is the formation of new blood vessels from preexisting vascular network. CD31 play a crucial role on leucocyte migration through vascular endothelial tntercellular junction. CD31 also exhibits signal transduction and up regulate intregrin function. It involves in thrombosis, angiogenesis, wound healing, and inflammation[12]. CD34 is a glycosylated transmembrane protein that is expressed on small vessel endothelial cells and tumors of epithelial origin[12]. CD31 and CD34 markers have been shown to have a strong correlation with the measurement of MVD[5,9,10]. The mean MVD in the present study was 10.4 microvessels per mm^2. This number was used to divide IHCC into two groups according to the study of Shirabe et al[11].

One possible biological reason for the lack of association between MVD and survival or recurrence is that IHCC patients in the present study had such a poor prognosis from the very start that the MVD could not provide additional prognostic information. This is in contrast to a previous study that found MVD to be a significant predictor of clinical outcomes in node-negative IHCC[11]. In addition, the average MVD for IHCC in the present study was relatively low, compared with that of other types of tumors such as esophageal, breast, and liver cell carcinoma[17,25,29] or with that of IHCC in other studies[11]. Given that in cancers with low MVD the relationship between MVD and clinical outcomes might be weak, a much larger sample of patients will be needed to clearly show such a relationship.

Conclusion

In the present study, microvessel density was not found to be a significant risk factor for death and tumor recurrence in Thai patients with intrahepatic cholangiocarcinoma. However, the average microvessel density obtained from the present study may be used as a reference value for future studies focusing on tumor angiogenesis, especially in Thai patients.

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ความสำคัญของการพยากรณ์โรคมะเร็งท่อน้ำดีในตับจากความหนาแน่นของหลอดเลือดจุลภาค

ไพสิฐ บุญศิริไพบูลย์, พัฒนา ศรมยุรา, จุมพล วิลาศรัศมี, ภานุวัฒน์ เลิศสิทธิชัย

วัตถุประสงค์: *มะเร็งท่อน้ำดีในตับเป็นมะเร็งตับปฐมภูมิที่พบได้บ่อยเป็นอันดับ 2 การเกิดหลอดเลือดใหม่ในก้อนเนื้องอกดูเหมือนจะมีความสำคัญ ในการเจริญเติบโตของเนื้องอกและพยากรณ์โรคในผู้ป่วยมะเร็งชนิดนี้ วัตถุประสงค์ของการศึกษานี้ คือ การหาความสำคัญของหลอดเลือดจุลภาคที่เกิดขึ้นจากเนื้องอกมะเร็งท่อน้ำดีในตับ*

วัสดุและวิธีการ: *การศึกษาทำโดยทบทวนผู้ป่วยมะเร็งท่อน้ำดีในตับ 22 ราย ที่ได้รับการผ่าตัดตับออก เกี่ยวกับลักษณะทางคลินิกและพยาธิวิทยาที่ใช้ในการพยากรณ์โรค ความหนาแน่นหลอดเลือดจุลภาคที่เกิดจากเนื้องอกได้รับการประมาณโดยวิธี immunohistochemical ความน่าจะเป็นของการกลับเป็นซ้ำ และอัตราการอยู่รอดของผู้ป่วย รวมถึงความสำคัญในการพยากรณ์โรคของหลอดเลือดจุลภาคที่เกิดจากเนื้องอกได้รับการประมาณ และวิเคราะห์โดยวิธีทางสถิติ*

ผลการศึกษา: *การศึกษานี้พบว่าความหนาแน่นหลอดเลือดจุลภาคที่เกิดจากเนื้องอกไม่มีความสำคัญในการกลับเป็นซ้ำของโรคและอัตราการอยู่รอดของผู้ป่วยมะเร็งท่อน้ำดีในตับ เช่นเดียวกับอายุ เพศ และสารส่อมะเร็ง ปัจจัยที่มีผลต่ออัตราการอยู่รอดของผู้ป่วยได้แก่ ระยะของโรค*

สรุป: *การศึกษานี้ไม่พบความสำคัญของความหนาแน่น หลอดเลือดจุลภาคที่เกิดจากเนื้องอกในการพยากรณ์โรคมะเร็งท่อน้ำดีในตับ*