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Clinical Studies

Expression pattern of angiogenic factors and prognosis after hepatic resection in hepatocellular carcinoma: importance of angiopoietin-2 and hypoxia-induced factor-1α

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Abstract: *Background:* Hepatocellular carcinoma (HCC) is a hypervascular tumor and angiogenesis plays an important role in its progression. Angiogenesis is regulated by a balance between pro and antiangiogenic molecules. The aim of this study was to investigate the expressions of angiogenic factors and elucidate their roles in angiogenesis in HCC. *Methods:* We investigated immunohistochemical expression of vascular endothelial growth factor (VEGF), angiopoietins (Ang-1 and Ang-2), hypoxia-induced factor-1α (HIF-1α) and thrombospondin-1 (TSP-1) in 60 specimens of surgically resected HCC. We investigated the relationship between their expressions and clinicopathological factors or prognosis. *Results:* Ang-2 staining had a significant correlation with the grade of differentiation of HCC cells ($P = 0.0082$). VEGF and Ang-2 expression correlated positively with microvessel density (MVD) ($P = 0.0061$ and 0.0374, respectively). MVD of well-differentiated HCC were significantly lower than those of moderately and poorly differentiated HCC. The disease-free survival time of patients with high Ang-2 and/or HIF-1α expression was significantly shorter than that of the low expression group ($P = 0.0278$ and 0.0374, respectively). *Conclusion:* Our study showed that the expression of VEGF and Ang-2 correlated with MVD. Strong Ang-2 expression and/or high nuclear expression of HIF-1α is a significant predictive factor for recurrence after curative resection in HCC patients.

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Key words: angiogenesis – angiopoietin – hepatocellular carcinoma – hypoxia-induced factor – vascular endothelial growth factor

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Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world, especially in Japan and other East Asian countries. HCC is characteristically a highly vascular tumor with a propensity for vascular invasion and intrahepatic metastasis. Hepatic resection is considered a curative treatment for HCC, but the prognosis after resection remains unsatisfactory because of the high incidence of recurrence (1). In various cancers, including HCC, tumor angiogen-

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Abbreviation: Ang, angiopoietin; ARNT, aryl hydrocarbon receptor nuclear translocator; HBV, hepatitis B virus; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; HIF, hypoxia-induced factor; MVD, microvessel density; PBS, phosphate-buffered saline; TSP-1, thrombospondin-1; VEGF, vascular endothelial growth factor.

esis plays an important role of invasiveness, progression and metastasis.

Angiogenesis, the formation of the new blood vessels, is an essential process in both the embryo and adult. Without a new blood supply, solid tumors cannot grow beyond a small diameter because of lack of oxygen and nutrition (2). With angiogenesis, the tumor grows rapidly and can metastasize to remote sites (3). This is a multistep process regulated by a balance between pro and antiangiogenic molecules produced by tumors and host component cells (4). To date, many factors to promote or inhibit angiogenesis have been identified, including growth factors, cytokine or protease (5). Among the factors, vascular endothelial growth factor (VEGF) is a vascular endothelium-specific growth factor and plays a central role in tumor angiogenesis. VEGF, which was initially identified as vascular permeability factor, promotes the growth, proliferation and migration of endothelial cells, and inhibits apoptosis of endothelial cells in pathological angiogenesis (6–8). Overexpression of VEGF mRNA and/or protein correlates with microvessel density (MVD), invasiveness and poor prognosis in various cancers (9, 10). In HCC, the relationship between VEGF and clinicopathological features remains controversial.

A new family of endothelial growth factor, angiopoietins (Ang), has been identified, which comprises ligands for the vascular endothelium-specific tyrosine kinase receptor Tie2 (11–13). Angiopoietin-1 (Ang-1), which is an agonist of Tie2 and induces its phosphorylation, serves as a survival factor for the endothelial cells and promotes recruitment of pericytes and smooth muscle cells. Therefore, Ang-1 is thought to help to maintain and stabilize vascular networks (14). Angiopoietin-2 (Ang-2) is a biological antagonist of Ang-1 and reduces vascular stability to block the stabilizing action of Ang-1. However, in the presence of VEGF, Ang-2 induces vascular sprouting and angiogenesis (15). Ang-2 was markedly expressed in organs that undergo vascular remodeling, such as the ovaries and placenta, and also in several malignancies including HCC. In addition, overexpression of Ang-2 protein and/or mRNA correlates with poor prognosis in some cancers (16–20). Several reports have investigated the correlation between expression of Ang-2 protein or mRNA and clinicopathological factors in HCC. The role of the Ang/Tie system in HCC remains unclear, especially the relationship between expression of Ang-2 protein and prognosis after hepatic resection.

We have also focused on hypoxia-induced factor-1α (HIF-1α) and thrombospondin-1 (TSP-1). HIF-1α and HIF-1β (also known as ARNT; the aryl hydrocarbon receptor nuclear translocator) are subunits of HIF-1, which is a heterodimer and transcriptional factor that plays a crucial role in O_2 homeostasis (21). Under normoxic conditions, HIF-1α is rapidly ubiqitinated and degraded in the ubiquitin-proteasome pathway. Under hypoxic conditions, HIF-1α is stabilized and several genes may be activated by HIF-1α (a transcriptionally regulator), such as VEGF, erythropoietin and glycolytic enzymes (22, 23). HIF-1α protein is expressed in various human malignancies (24) and HIF-1α protein overexpression is associated with chemotherapic resistance and poor prognosis in some cancers (25–27). However, the role of HIF-1α in HCC is less clear. TSP-1, a multifunctional matrix protein, is one of the five members of the thrombospondin gene family. TSP-1 has been recognized as an antiangiogenic factor and inhibits tumor growth to suppress angiogenesis (28–31). TSP-1 has also been reported to promote tumor growth, migration and angiogenesis (32, 33). The role of TSP-1 in tumor progression and angiogenesis is controversial in some cancers including HCC.

We previously reported that cooperation between Ang-2 and VEGF plays an important role in enhancing the formation of new blood vessels in hepatic metastases of colorectal cancer (34). To date, there are only a few studies of the relationship between the expression of VEGF, Ang-1, Ang-2, HIF-1α and TSP-1 and angiogenesis in HCC. Therefore, the aim of this study was to elucidate their role in angiogenesis in HCC. We investigated the expression of these angiogenic factors serially by immunohistochemistry and compared their expression with MVD and clinicopathological factors. In addition, we aimed to clarify the relationship between angiogenic factors and prognosis after curative resection.

Materials and methods

Patients and specimens

Surgical specimens were obtained from 60 patients with HCC who underwent hepatic resection in the Osaka University Hospital from 1992 to 1998. The clinicopathological characteristics of these patients were shown in Table 1. The 60 patients comprised 45 males and 15 females, with a median age of 63 years (range, 44–79). Hepatitis B virus (HBV) and hepatitis C virus (HCV) serology was positive in 12 and 40 patients, respectively. One case was coinfected with HBV and HCV in this series. HCC specimens were fixed in 10% buffered formalin, embedded in

Table 1. Clinicopathological features of 60 patients with hepatocellular carcinoma (HCC).

	No. of cases
Median age (range)	63 (44–79)
Sex	
Male	45 (75.0%)
Female	15 (25.0%)
Etiology of hepatitis*	
HBV	12 (20.0%)
HCV	40 (66.7%)
Non-HBV/non-HCV	9 (15.0%)
Cirrhosis	
Presence	37 (61.7%)
Absence	23 (38.3%)
Tumor size	
<3 cm	27 (45.0%)
≥ 3 cm	33 (55.0%)
Capsule formation	
Presence	44 (73.3%)
Absence	16 (26.7%)
Capsule invasion	
Presence	23 (52.3%)
Absence	21 (47.7%)
Septal formation	
Presence	34 (56.7%)
Absence	26 (43.3%)
Intrahepatic metastasis	
Presence	22 (36.7%)
Absence	38 (63.3%)
Portal vein tumor thrombus	
Presence	21 (35.0%)
Absence	39 (65.0%)
Histological grade†	
Well	7 (11.7%)
Mod.	26 (43.3%)
Poor	27 (45.0%)
TAE pretreated	
Yes	22 (36.7%)
No	38 (63.3%)

*One case co-infected HBV and HCV. †Well, well-differentiated HCC; mod., moderately differentiated HCC; poor, poorly differentiated HCC.

paraffin, and stained with hematoxylin–eosin for study of the pathological features of HCC in accordance with the classification proposed by the Liver Cancer Study Group of Japan (35).

Immunohistochemical staining

Formalin-fixed, paraffin-embedded 5 μm thick sections were deparaffinized in xylene and rehydorated through a graded series of ethanol. Immunohistochemical procedure was performed using Vectastain ABC peroxidase kit (Vector Labs, Burlingame, CA) as described previously (36). Briefly, the sections were treated with an antigen retrieval procedure in 0.01 M sodium citrate buffer (pH 6.0) for 40 min at 95 °C and were incubated in methanol containing 0.3% hydrogen peroxide at room temperature for 20 min to block endogenous peroxidase. After blocking endogenous biotin, the sections were incubated with normal protein block serum solution at room temperature for 20 min, to block nonspecific staining, and then incubated overnight at 4 °C with anti-VEGF (rabbit polyclonal IgG, diluted 1:100, Santa Cruz Biotechnology, Santa Cruz, CA), anti-angiopoietin-1(goat polyclonal IgG, diluted 1:50, Santa Cruz), anti angiopoietin-2 (goat polyclonal IgG, diluted 1:50, Santa Cruz), anti-HIF-1α (mouse monoclonal IgG2a, diluted 1:50, Novus Biologicals, Inc., Littleton, CO) and anti-thrombospondin-1 (mouse monoclonal IgG2a, diluted 1:50, Lab Vision, Fremont, CA) as primary antibodies. After washing three times for 5 min in phosphate-buffered saline (PBS), sections were incubated with a biotin-conjugated secondary antibody (goat anti-rabbit for VEGF, rabbit anti-goat for Ang-1 and Ang-2, horse anti-mouse for HIF-1α and TSP-1) at room temperature for 20 min and finally incubated with peroxidase-conjugated streptavidin at room temperature for 20 min. The peroxidase reaction was then developed with 3, 3′-diaminobenzidine tetrachloride (Wako Pure Chemical Industries, Ltd). Finally, the sections were counter stained with Meyer's hematoxylin. For negative controls, sections were treated the same way except they were incubated with nonimmunized rabbit IgG or Tris-buffered saline instead of the primary antibody.

Evaluation of immunostaining

Immunohistochemical staining was assessed by two investigators independently without knowledge of the corresponding clinicopathological data. In case of disagreement, two investigators rejoined evaluation and resolved disagreement together. Immunoreactivity of VEGF, Ang-1, Ang-2 or TSP-1 was located in cytoplasm. For each section, the intensity of immunohistochemical staining of VEGF, Ang-1, Ang-2 and TSP-1 was scored on a scale from 0 to 2, where 0 represented negative or faint staining; 1, moderate; and 2, strong staining. Vascular epithelium expressed moderate levels of Ang-1, Ang-2 and TSP-1, and the bile duct epithelium expressed moderate levels of VEGF. Accordingly, these levels of staining were used as an endogenous control within the sample, which was designated arbitrarily as intensity level 1, as described previously (36).

Immunoreactivity of HIF-1α was located in both nuclei and cytoplasm. Cells considered expression of HIF-1α when distinct nuclear staining was identified, as previously described (24). The immunohistochemical staining of HIF-1α was classified as follows, where 0 represented negative nuclear staining; 1, less than 1% nuclear staining

and/or with moderate cytoplasmic staining; and 2, more than 1% nuclear staining and/or with strong cytoplasmic staining.

MVD assay

MVD was assessed based on the method described previously (34). Briefly, after immunostaining with anti-CD34 antibody (mouse monoclonal IgG2a, diluted 1:50, Novocastra Laboratories, Newcastle, UK), five visual fields were randomly selected in the tumor and the average counts of vessel in five areas at high power magnificent (×200) were determined to the MVD of an individual tumor.

Statistical analysis

Statistical analysis was performed using the Statview J-4.5 program (Abacus Concepts Inc., Berkeley, CA). The χ^2-test, Fisher's exact probability test, Student's *t*-test, Mann–Whitney *U*-test or Kruskal–Wallis test were used to examine the association between VEGF, Ang-1, Ang-2, HIF-1α, TSP-1, MVD and clinicopathological parameters. The Kaplan–Meier method was used to calculate the probability of disease-free survival, comparisons was made using the log-rank test. A Cox proportional hazard model was used to assess the risk ratio with simultaneous contribution from several covariates. A level of $P<0.05$ was considered statistically significant.

Results

Immunohistochemical staining of VEGF, Ang-1, Ang-2, HIF-1α and TSP-1

Immunohistochemical results in cancerous tissue were summarized in Table 2. VEGF immunoreactivity was localized in the cytoplasm of cancer cells and hepatocyte. In some instances, VEGF staining at the boundary of the tumor was higher than in the center of tumor. VEGF signals were also present in hepatocytes of adjacent liver (Fig. 1A–C). VEGF expression was not detected in 11 cases (18.3%), whereas 12 cases (20.0%) of HCCs displayed marked VEGF expression, and 37 cases (61.7%) expressed moderate levels of VEGF protein (Table 2). Both Ang-1 and Ang-2 proteins was localized in the cytoplasm of cancer cells and hepatocytes in adjacent liver. Ang-1 was not detected in 19 cases (31.7%) of HCC, 34 cases (56.7%) showed moderate staining and seven cases (11.7%) showed strong staining (Fig. 1D–F). Ang-2 proteins were not observed in 18 cases (30.0%) of HCC, 29 cases (48.3%) showed moderate staining and 13 cases (21.7%) showed strong staining (Fig. 1G–I). The TSP-1 immunostaining was localized in the cytoplasm of cancer cells. TSP-1 expression was not detected in 34 cases (56.7%) of HCC, 17 cases (28.3%) showed moderate staining and nine cases (15.0%) showed strong staining in the cytoplasm of HCC cells (Fig. 1M–O). In noncancerous liver tissue, VEGF expression was not detected in 24 cases, moderately in 30 cases and strongly in six cases. Ang-1 expression was not detected in 24 cases, moderately in 35 cases and strongly in one case. Ang-2 expression was not detected in 28 cases, moderately in 31 cases and strongly in one case. Fourty-four cases showed no or faint staining and 16 cases showed moderately staining of TSP-1 in hepatocytes of adjacent liver. There was no case which showed strong TSP-1 expression. In each angiogenic factor, there was no correlation between the expression in HCC and in an adjacent liver tissue ($P>0.05$).

Table 2. Immunohistochemical analysis of VEGF, Ang-1, Ang-2, HIF-1α and TSP-1 expression

	Intensity		
	0	1	2
VEGF	11 (18.3%)	37 (61.7%)	12 (20.0%)
Ang-1	19 (31.7%)	34 (56.7%)	7 (11.7%)
Ang-2	18 (30.0%)	29 (48.3%)	13 (21.7%)
HIF-1α	28 (46.7%)	25 (41.7%)	7 (11.7%)
TSP-1	34 (56.7%)	17 (28.3%)	9 (15.0%)

Ang, angiopoietins; HIF, hypoxia-induced factor; TSP, thrombospondin; VEGF, vascular endothelial growth factor.

Immunohistochemical staining for HIF-1α revealed positive nuclear staining in seven cases (11.7%) of HCC. There was no nuclear staining and only negative or faint cytoplasmic staining of the hepatocytes in normal and adjacent liver. HIF-1α staining was not detected in 28 cases (46.7%) of HCC and less than 1% nuclear staining and/or with moderate cytoplasmic staining (score 1) was detected in 25 cases (41.7%) (Fig. 1J–L). In hepatocytes of adjacent liver, Hif-1α staining was not detected in 56 cases and moderately in four cases.

Relationship between expression of VEGF, Ang-1, Ang-2, HIF-1α or TSP-1 and clinicopathological characteristics

We examined the correlations between the intensity of VEGF, Ang-1, Ang-2, HIF-1α or TSP-1 expression and several clinicopathological features. Significant differences were noted for the histological grade of HCC cells. Ang-2 expression was not detected in six of seven cases of well-differentiated HCC and only one case displayed

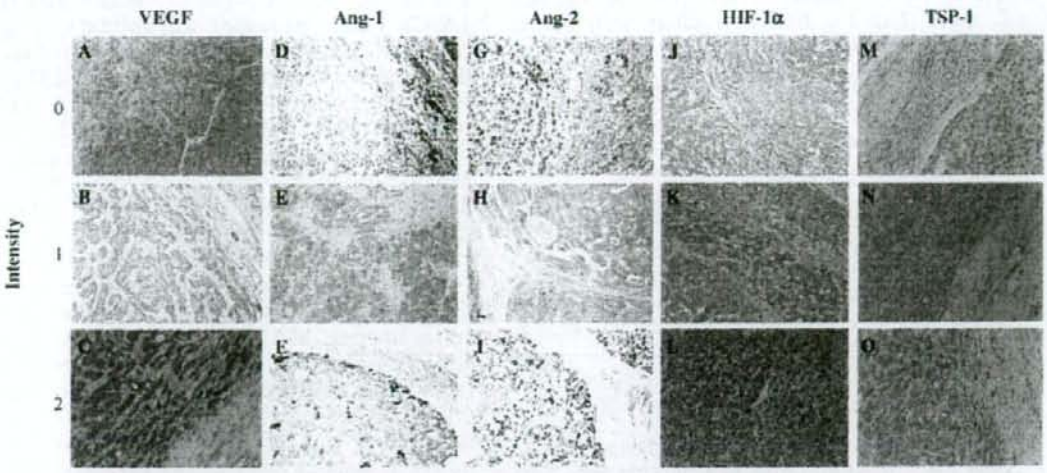

Fig. 1. Immunohistochemical staining of (A–C) vascular endothelial growth factor (VEGF), (D–F) angiopoietins-1 (Ang-1), (G–I) Ang-2, (J–L) hypoxia-induced factor-1α (HIF-1α) and (M–O) thrombospondin-1 (TSP-1). For VEGF, Ang-1, Ang-2 and TSP-1, staining was localized to the cytoplasm of hepatocellular carcinoma (HCC) cells. No or faint staining (score 0) of (A) VEGF, (D) Ang-1, (G) Ang-2 and (M) TSP-1. Moderate staining (socre 1) of (B) VEGF, (E) Ang-1, (H) Ang-2 and (N) TSP-1. Strong staining (score 2) of (C) VEGF, (F) Ang-1, (I) Ang-2 and (O) TSP-1. (L) Positive staining of HIF-1α was detected in nuclear and cytoplasm of HCC cells (score 2). (K) HIF-1α staining was found in cytoplasm and no nuclear staining (score 1). (J) No nuclear and cytoplasm was stained by HIF-1α (score 0). Magnification, ×100.

moderate staining. Strong Ang-2 staining was noted in 13 cases, five of which were of moderate histological grade and eight were poorly differentiated. There was significant correlation between the intensity score of Ang-2 expression in HCC cells and histological grade ($P = 0.0082$). There was no significant correlation between the intensity of Ang-2 expression in HCC cells and age, sex, tumor size, capsule formation, infiltration of capsule, portal vein invasion, intrahepatic metastasis or clinical stage of the HCC (Table 3). No correlation was observed between VEGF, Ang-1, TSP-1 or HIF-1α and clinicopathological characteristics.

Correlation between each angiogenic factors, clinicopathological factors and MVD

The mean value of MVD in 60 cases of HCC were 93.1 ± 43.8, which was significantly higher than those in adjacent liver tissues (data not shown). The mean value of MVD in well-differentiated HCC was 48.5 ± 24.9, which was significantly lower than that in moderately (104.6 ± 39.2) and poorly differentiated HCC (90.7 ± 39.9), ($P = 0.0012$ and 0.0143, respectively). There was significant correlation between MVD and septum formation ($P = 0.0096$). There was no correlation between MVD and the other variables (age, sex, hepatitis virus infection, cirrhosis, tumor size, capsule formation, capsule invasion, portal vein invasion, intrahepatic metastasis and TAE treatment). With respect to relationship between each angiogenic factor and MVD, VEGF and Ang-2 expression was significantly correlated with MVD ($P = 0.0061$ and 0.0374, respectively). Ang-1, TSP-1 and HIF-1α expressions had no significant correlation with MVD (Table 4).

Relationship between expression of VEGF, Ang-1, Ang-2, HIF-1α or TSP-1 and postoperative disease-free survival

For statistical analysis of cumulative survival, cases were divided into two groups, high expression group (intensity 2) and low expression group (intensity 0 and 1), with respect to VEGF, Ang-1, Ang-2, HIF-1α and TSP-1 levels, respectively. We calculated the probability of disease-free survival using the Kaplan–Meier method, and made comparisons using the log-rank test. As shown in Fig. 2, the disease-free survival time was significantly different between the high and low Ang-2 expression groups ($P = 0.0278$) and between the high and low HIF-1α expression groups ($P = 0.0374$). There was no significant correlation between VEGF, Ang-1 and TSP-1 expression and disease-free period. In clinicopathological factors including age, sex, tumor size, histological grade, portal vein invasion and intrahepatic metastasis, portal vein invasion and intrahepatic metastasis were significant prognosis factors in the univariate analysis. In the multi-variate analysis, high HIF-1α expression was a borderline independent factor for poor prognosis (hazard ratio 2.167

Table 3. Relationship with Ang-2 and HIF-1α expression and clinicopathological factors in cases with hepatocellular carcinoma (HCC)

	Ang-2 intensity				HIF-1α intensity			
	0	1	2	*P*-value*	0	1	2	*P*-value*
Median age (range)	67 (49–79)	61 (44–76)	59 (48–73)		63 (49–76)	65 (44–79)	59 (48–72)	
Sex								
Male	14	22	9	0.6387	21	19	5	0.9456
Female	4	7	4		7	6	2	
Etiology of hepatitis								
HBV	1	8	3		5	7	0	
HCV	13	18	9	NS	17	18	5	NS
Non-HBV/non-HCV	4	4	1		6	1	2	
Cirrhosis								
Presence	10	20	7	0.9455	19	14	4	0.4381
Absence	8	9	6		9	11	3	
Tumor size								
<3 cm	8	13	6	0.9349	10	15	2	0.4530
≥3 cm	10	16	7		18	10	5	
Capsule formation								
Presence	10	25	10	0.1554	18	21	5	0.2772
Absence	8	4	3		12	4	2	
Capsule invasion								
Presence	4	12	7	0.2308	11	7	5	0.9999
Absence	6	13	3		7	14	0	
Septum formation								
Presence	8	16	9	0.1995	13	16	4	0.2538
Absence	10	13	4		15	9	3	
Intrahepatic metastasis								
Presence	5	12	5	0.5144	11	7	4	0.9572
Absence	13	17	8		17	18	3	
Portal vein tumor thrombus								
Presence	5	10	6	0.3445	9	9	3	0.6420
Absence	13	19	7		19	16	4	
Histological grade†								
Well	6	1	0		4	3	0	
Mod.	8	13	5	0.0082	12	12	2	0.6813
Poor	4	15	8		12	10	5	
TAE Pretreated								
Yes	6	11	5	0.9350	10	7	5	0.7277
No	12	18	8		8	18	2	

*P-value was calculated using Mann-Whitney's *U*-test or Kruskal–Wallis test. †Well, well-differentiated HCC; mod., moderately differentiated HCC; poor, poorly differentiated HCC; NS, not significant.

Table 4. Relationship between each angiogenic factor and microvessel density (MVD) level in hepatocellular carcinoma (HCC)

	Intensity			
	0	1	2	*P*-value
VEGF	63.5 ± 31.1	94.7 ± 43.8	123.5 ± 38.7	0.0061*
Ang-1	77.1 ± 40.8	99.0 ± 45.4	115.6 ± 36.4	0.0828
Ang-2	72.1 ± 38.5	100.2 ± 43.7	110.1 ± 44.5	0.0374*
HIF-1α	88.3 ± 46.0	99.2 ± 42.2	101.7 ± 47.9	0.6281
TSP-1	98.9 ± 45.4	91.4 ± 45.1	82.8 ± 40.5	0.5258

Data are mean ± SD. *P*-value was calculated by Kruskal–Wallis test. *There was significantly difference between MVD and VEGF or Ang-2 expression.

$P = 0.0904$) as well as presence of portal vein tumor thrombus (Table 5). For overall survival, no significant relationship was noted for any five angiogenic factors examined (data not shown).

Discussion

In this study, we found that the increased expressions of VEGF and Ang-2 were correlated with increased MVD, and that expression of Ang-2 was correlated with the histological grade of HCC. In addition, MVD levels of well-differentiated HCC were clearly lower than those of moderately or poorly differentiated HCC. In the survival analysis, we showed that overexpression of Ang-2 and/or HIF-1α was a prognostic factor for recurrence after curative resection of HCC. There was no significant association between each angiogenic factor and overall survival rate. These results suggest that interaction of angiogenic factors increases tumor angiogenesis in HCC and that overexpression of angiogenic factors is related to early recurrence.

In our present study, we found strong expression of VEGF in hypervascular HCC. Among 12

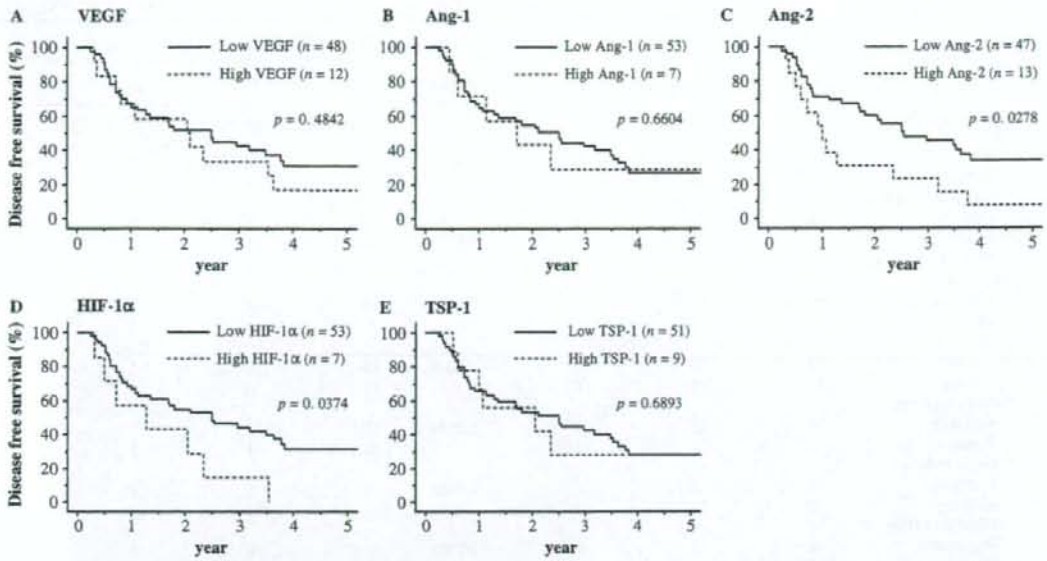

Fig. 2. Disease-free survival based on each angiogenic factor (vascular endothelial growth factor (VEGF), angiopoietins-1 (Ang-1), Ang2, hypoxia-induced factor-1α (HIF-1α) and thrombospondin-1 (TSP-1) (A–E) Disease free survival curves of hepatocellular carcinoma (HCC) patients with high expression (score 2) or low expression (score 0, 1) of each angiogenic factor and those were plotted by Kaplan–Meier method and their difference was evaluated by logrank test. (C) High Ang-2 group had significantly early recurrence than low Ang-2 group ($P = 0.0278$). (D) High HIF-1α group was significantly poorer prognosis than low HIF-1α group ($P = 0.0374$) (A, B, E). There was no relationship between VEGF, Ang-1 or TSP-1 and disease free survival time after hepatic resection.

Table 5. Prognostic factors for disease-free survival analyzed by the Cox proportional hazard model

	Univariate analysis			Multivariate analysis		
	Hazard ratio	95% CI	*P*-value	Hazard ratio	95% CI	*P*-value
Portal vein tumor thrombus						
Absence	ref.			ref.		
Presence	2.805	1.233–6.382	0.0139	2.297	0.861–6.126	0.0996
Intrahepatic metastasis						
Absence	ref.			ref.		
Presence	1.842	1.006–3.372	0.0477	1.328	0.672–2.625	0.4141
Ang-2						
Intensity 0, 1	ref.			ref.		
Intensity 2	2.098	1.068–4.121	0.0315	1.564	0.760–3.216	0.2245
HIF-1α						
Intensity 0,1	ref.			ref.		
Intensity 2	2.349	1.026–5.378	0.0434	2.167	0.885–5.305	0.0904

CI, confidence interval; ref., reference.

cases (20.0%) with strong VEGF expression, none were well-differentiated. However, there was no significant difference between expression of VEGF and clinicopathological factors, including histological grade. Several investigators reported and discussed relationship of VEGF to clinicopathological factors. Yamaguchi et al. reported that VEGF was highly expressed in well-differentiated HCC and the number of VEGF-positive cases gradually decreased with tumor size (37). On the other hand, VEGF-positive tumors had a significantly higher MVD and larger size than VEGF-negative tumors in a recent report (38). In another report (39, 40), VEGF expression in HCC cells did not correlate with any clinicopathological features, including MVD. In our cases, the adjacent liver histology of all cases excepting one was chronic hepatitis or cirrhosis. In chronic hepatitis or cirrhosis, lymphocyte infiltration or regeneration of sinusoid was observed. The expression of VEGF is regulated by a variety of hormones, growth factors and in-

flammatory cytokines secreted by infiltrating lymphocytes (37). Mitsuhashi et al. (41) reported that mRNA expression of VEGF was not correlated with clinicopathological factors or MVD and there was no difference between HCC cells and adjacent liver cells. Therefore, considering the complex and special nature of the HCC carcinogenic and angiogenic processes, we considered that VEGF was probably necessary, but not the sole contributor to angiogenesis of HCC.

We also studied angiopoietins in this series. By immunohistochemical staining, we found that almost two-third of HCC cases expressed Ang-1 and Ang-2, and Ang-2 expression was significantly correlated with histological differentiation and MVD. Sugimachi et al. (42) reported that Ang-1 and Ang-2 were expressed in 68% and 81% of HCCs, respectively, and Ang-2 expression was significantly higher in poorly differentiated HCC. Torimura et al. (43) also reported that expression of Ang-2 mRNA and protein was significantly associated with tumor differentiation and vascularity (assessed by angiography). Our results were consistent with these previous reports. Ang-1 expression was not correlated with MVD or any clinicopathological factor. The role of Ang-1 in tumor angiogenesis remains controversial. *In vitro*, Ang-1 overexpression did not promote angiogenesis or enhance tumor growth (44). In an *in vivo* xenograft model, tumors comprised of Ang-1-transfected cells were smaller than those comprised of Ang-2-transfected cells (45). On the other hand, several reports showed that Ang-1 expression was correlated with angiogenesis and tumor progression (46). Among our seven cases (11.7%) with strong Ang-1 expression, positive staining for Ang-2 and VEGF was found in all 7 and 6 cases, respectively. MVD levels of HCC was influenced by Ang-2/Ang-1 mRNA ratio (41), we therefore speculated that if Ang-2 protein was more highly expressed than Ang-1 protein in the HCC cells, angiogenesis would be stimulated. Furthermore, we found a correlation between expression of VEGF and Ang-2 in HCC cells ($P<0.05$, data not shown). *In vitro*, angiopoietins alone could not induce tube formation within a collagen substrate. In the presence of VEGF, Ang-2 promoted neovascularity with vessels of greater length than Ang-1 (15). There is a strong correlation between VEGF protein and Ang-2 mRNA expression in HCC (38). These findings suggest that VEGF and Ang-2 may play complementary and coordinated roles in HCC.

With respect to disease-free survival analysis, the 2-year disease-free survival rates of patients with high Ang-2 expression and low Ang-2 expression were 36% and 70%, respectively. There was no significant association between VEGF, Ang-1 expression and disease-free survival rates. This result was consistent with the finding, where patients with a high Ang-2/Ang-1 mRNA ratio had a worse prognosis than did patients with low Ang-2/Ang-1 ratio (41). Therefore, we propose that Ang-2 expression influenced not only tumor angiogenesis, but also progression and aggressive behavior of HCC.

We observed strong nuclear staining for HIF-1α in seven of 60 cases (11.7%) of HCC and no staining in the normal liver and adjacent liver tissues. Immunoreactivity of HIF-1α was detected in both nuclei and cytoplasm. As HIF-1a is transcriptional factor of angiogenic and glycolytic enzymes (22, 23), it can be assumed that nuclear HIF-1α is the active form. Therefore, we assessed the HIF-1α protein expression based on only nuclear staining, as previously described (24). Five cases with high HIF-1α expression received preoperative TAE or TACE. However, only two cases of 38 nontreated cases showed strong HIF-1α expression. HIF-1α expression was not common in HCC compared with other malignant tumors that expressed HIF-1α protein in 53% cases (24). However, HIF-1α expression was upregulated around the necrotic region in six of 23 cases in TAE-treated HCC (47), which is consistent with our present results. Furthermore, among our seven cases, strong and moderate VEGF expression was evident in five and two cases, respectively. HIF-1α protein expression showed a significant positive correlation with VEGF protein expression. Recent reports have shown that overexpression of HIF-1α protein was correlated with tumor progression and poor prognosis (25–27). Furthermore, Lee et al. (48) analyzed prediction of survival in HCC by gene expression profiling using oligo microarray containing 21,329 genes. They classified two groups of HCC patients by gene expression profiles and the clustering was highly associated with survival. Expression of HIF-1α mRNA was enhanced and egl nine homolog 2 (ENGL2) which is a negative regulator of HIF-1α, was reduced in the poor prognosis group. In our analysis of survival, patients with high HIF-1α expression had worse disease-free survival rates than the patients with low HIF-1α expression. In multi-variate analysis, high HIF-1α expression was a borderline independent factor of early recurrence. We speculate that HCCs do not usually express HIF-1α, however, once cancer cells acquire HIF-1α expression, they transform to more progressive and metastatic behavior.

There are few reports that compare TSP-1 protein expression and clinicopathological fac-

tors in HCC. The relative increase in TSP-1 mRNA level and the relative decrease in VEGF mRNA level compared with adjacent liver tissues may relate to angiogenesis in cholangiocellular carcinoma, but TSP-1 mRNA levels were not correlated with vascularity in HCC (49). In recently report (50), high TSP-1 expression was associated with tumor progression in HCC. However, in the present study, we found no correlation among TSP-1 protein expression and another angiogenic factors, MVD and prognosis. The difference between previous reports and our results may reflect methodological differences between the studies. However, among seven cases with strong TSP-1 expression, Ang-2 positivity was found in all except one case, but MVD levels were slightly less than those of the negative TSP-1 group. The role of TSP-1 in tumor angiogenesis remains to be controversial.

Our results and other recent reports suggested that Ang-2 is upregulated during the process of dedifferentiation and that it influences the angiogenesis and progression of HCC. VEGF was produced by both cancer cells and hepatocytes in the adjacent live and cooperated with Ang-2 induced angiogenesis in HCC. Expression of HIF-1α, which is a transcriptional factor for many genes including VEGF, is not common in HCC. However, if HCC cells acquire HIF-1α expression, angiogenesis may be induced through expression of VEGF, which is upregulated by HIF-1α. In addition, HIF-1α appears to regulate tumor progression and metastasis after curative resection in HCC, similar to its actions in other malignancies.

In conclusion, our study showed increased Ang-2 expression with dedifferentiation of HCC cells, and that the expression of VEGF and Ang-2 protein correlated with MVD in HCC. Strong Ang-2 expression and/or high nuclear expression of HIF-1α is a significant predictive factor for recurrence after curative resection in HCC patients. The number of cases with high Ang-2 and/or HIF-1α expression were small in our study, therefore further studies are essential to revealed the relationship with prognosis and expression of angiogenic factors in HCC.

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Clinical and Pathological Features of Allen's Type C Classification of Resected Combined Hepatocellular and Cholangiocarcinoma: A Comparative Study with Hepatocellular Carcinoma and Cholangiocellular Carcinoma

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The clinical features of Allen's type C of combined hepatocellular and cholangiocarcinoma (cHCC-CC) are not well known. In this study, we aim to define the clinicopathologic features of cHCC-CC and to evaluate the preoperative diagnosis and surgical treatment results in comparison with those of hepatocellular carcinoma (HCC) and cholangiocellular carcinoma (CCC). We retrospectively analyzed 13 patients with cHCC-CC, 509 patients with HCC, and 41 patients with CCC treated in our hospital within past two decades. Viral hepatitis B or C backgrounds were more prominent in HCC and cHCC-CC groups than in the CCC group. Elevated serum alpha-fetoprotein (AFP) levels were found in 60.3% of HCC patients and in 46.2% of cHCC-CC patients. Only one patient of cHCC-CC was correctly diagnosed before surgery. The postoperative survival rates between the cHCC-CC and HCC or the CCC group were not significantly different. Both intrahepatic and extrahepatic postoperative recurrences were frequent in cHCC-CC patients, and CCC component recurrences were more frequently seen. In conclusion, the preoperative diagnosis is difficult; liver masses similar to those of HCC, together with moderately elevated serum AFP and CA19-9 levels, are reliable indicators of cHCC-CC. Surgical resection of this tumor yields results intermediate between those of HCC and CCC in character. More cases are needed to further define the characteristics of this tumor. (J GASTROINTEST SURG 2006;10:987–998)

KEY WORDS: Primary liver cancer, combined hepatocellular and cholangiocarcinoma, diagnosis, prognosis, surgery, tumor recurrence

Primary liver cancer (PLC) is one of the most common cancers in Eastern Asia in regions such as Japan and China. There are three major PLC types in adults, classified according to the histopathologic components of the tumor. The most common PLC type is hepatocellular carcinoma (HCC) that originates from hepatocytes. The second most common type is cholangiocellular carcinoma (CCC), which originates from the intrahepatic bile duct epithelium. The least common type is combined hepatocellular and cholangiocarcinoma (cHCC-CC), representing 0.40% to 14.2% of PLC cancer cases.[1–6] To date, the origins of cHCC-CC remain unclear, although various possibilities have been suggested.[7,8] The World Health Organization defined cHCC-CC as a rare tumor comprised of two groups of malignant cells with the histological features of HCC or CCC. However, this general definition is not discriminating enough to define various histopathologic conditions when elements from these two tumor types occur together. To date, Allen's classification has been widely used in various reports.[2,3,9] This classification includes: type A, double cancer of HCC and CCC, with HCC and CCC present at different sites without contact; type B, HCC and CCC are present at adjacent sites and mingle with

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continued growth; and type C, HCC and CCC are mixed, growing within the same tumor. Type C is also named as mixed hepatocellular and cholangiocellular carcinoma (MHC) by the Liver Cancer Study Group of Japan.[10] Histologically, only type C displays the characteristic of genuine mixture of both HCC and CCC elements, and in many published studies of cHCC-CC, only Allen's type C was included and regarded as true cHCC-CC.[2,3,5,11,12] In the present study, we have focused solely on Allen's type C of cHCC-CC.

The clinical features, therapy modalities, and the prognosis of HCC and CCC have been extensively researched and reported on by various groups. However, cHCC-CC remains relatively uninvestigated, and there is little information to enable useful comparisons of this tumor type with HCC or CCC. Furthermore, results from the few cHCC-CC studies have shown great variability between research groups.[3]

In this study, we summarized the clinicopathological features, surgical management, and results of 13 histologically confirmed cases of resected cHCC-CC. In addition, we compared our data with 550 cases of resected HCC and CCC and defined the clinicopathologic features of cHCC-CC. We also evaluated the preoperative diagnosis and surgical treatment of this rare tumor.

MATERIAL AND METHODS

Patients

From 1982 to 2003, in the Department of Surgery, Osaka University Hospital, 13 patients with resected Allen's type C of cHCC-CC were included in this study. In our series, number of HCC patients is 509, number of CCC patients is 41 and number of cHCC-CC patients is 13. The percentage of cases showed among all 563 patients. HCC patients are 509 of all 563 patients (90.4%) and CCC patients are 41 of all 563 patients (7.3%). Typical hepatocellular differentiation of cHCC-CC was recognized by the following features: (1) trabecular pattern composed of tumor cells forming tissue strands of various thicknesses, separated by sinusoidlike blood spaces lined with a single layer of endothelial cells, (2) abundant eosinophilic cytoplasm and bile production, and (3) intracytoplasmic hyaline globules. The typical cholangiocellular differentiation was recognized by the following features: (1) a tubular and/or papillary structure covered by small cubical to low columnar cells resembling the biliary epithelium, (2) mucin production, and (3) accompanying abundant fibrous stromal. All 13 cases of cHCC-CC (Tables 1 and 2) were confirmed by pathological examination including immunohistochemistry of alpha fetoprotein (AFP) and cytokeratin (CK7) (Figs. 1 and 2). Preoperative diagnosis was based on preoperative laboratory investigations and imaging diagnosis.

Clinicopathologic Features

Preoperative investigation of patient data, including demographics, was collated from computer-based medical records. Hepatitis B or C infection status of each patient was determined by screening for hepatitis B virus surface antigen and hepatitis C antibody. Chronic hepatitis and liver cirrhosis status of each patient was confirmed by a liver biopsy of peripheral nontumor liver tissue. Levels of serum

Table 1. Clinical characteristics of patients with cHCC-CC

Patient's No.	Age (Yr)	Sex	HBsAg	HCVAb	AFP (ng/ml)	PIVKA-II (ng/ml)	CEA (ng/ml)	CA 19-9 (U/ml)
1	65	M	−	ND	8	ND	ND	ND
2	57	M	−	−	<5	4037	ND	ND
3	47	F	+	−	171	<62.5	1	14
4	36	M	+	−	5773	<62.5	ND	ND
5	61	M	−	−	117	359	27	50
6	52	M	+	−	<5	69	ND	1218
7	74	M	−	−	3297	9408	3	37
8	58	M	−	−	6	71	5	<5
9	60	M	−	−	<5	<40	<1	20
10	58	M	−	−	7	162	2	19
11	75	M	−	−	564	1698	2	892
12	47	M	+	−	<5	626	ND	ND
13	50	M	+	−	52	2733	ND	ND

M = male; F = female; HBsAg = hepatitis B surface antigen; HCVAb = hepatitis C virus antibody; AFP = alpha fetoprotein; PIVKA = protein in vitamin K absence; CEA = carcinoembryonic antigen; CA19-9 = carbohydrate antigen 19-9; ND = note done.

Table 2. Preoperative diagnosis, macroscopic findings, surgical treatment, and outcome of cHCC-CC

Case No.	Tumor size (mm)	Preoperative diagnosis	Macroscopic differentiation	Segment resected†	Additional surgery	Recurrent time (mo)	Recurrence (organs)	Recurrent component	Survival (mo)
1	12.5	Liver abscess	Infiltrative	7, 8	Right pulmonary lobectomy	1	Skin	CCC?	13 (dead)
2	5.0	HCC	Simple nodular	6, 7	—	24	LN, bone, sacrum	ND	61 (dead)
3‡	5.6	HCC	Infiltrative	6, 7, 5*	Hilar LN dissection	15	Liver	CCC	135 (alive)
4	4.5	HCC	Infiltrative	6, 7, 8	—	2	Pleura	CCC?	2 (dead)
5	9.0	CCC	Infiltrative	2, 3, 4	Hilar LN dissection	2	bone, lung	CCC	7 (dead)
6	6.9	HCC	Infiltrative	5, 8, 4*	—	8	Liver	CCC	21 (dead)
7	6.8	HCC	Confluent multiple nodular	6, 6*, 8*	—	6	Liver, bone	HCC	28 (dead)
8	4.3	HCC	Simple nodular type with extra nodular growth	8	—	56	Liver	CCC	57 (alive)
9	11.5	HCC	Infiltrative	7, 8*	Portal vein thrombi extraction	—	—	—	36 (alive)
10	3.0	HCC	Confluent multiple nodular	6, 5	—	18	Lung, liver	NA	19 (alive)
11	9.4	cHCC-CC	Infiltrative	2, 3, 4, 5, 8	—	—	—	—	14 (dead)§
12	3.0	HCC	Simple nodular	6, 7	—	—	—	—	9 (alive)
13	4.2	HCC	Confluent multiple nodular multinodular	2, 3, 5, 8	—	—	—	—	10 (alive)

CCC? = clinically suspected CCC; NA = data not available.
*Partial resection of Couinaud's segment.
†Segments are based on Couinaud's classification.
‡Re-resection was performed after recurrence.
§Patient died of carcinoma of the prostate.

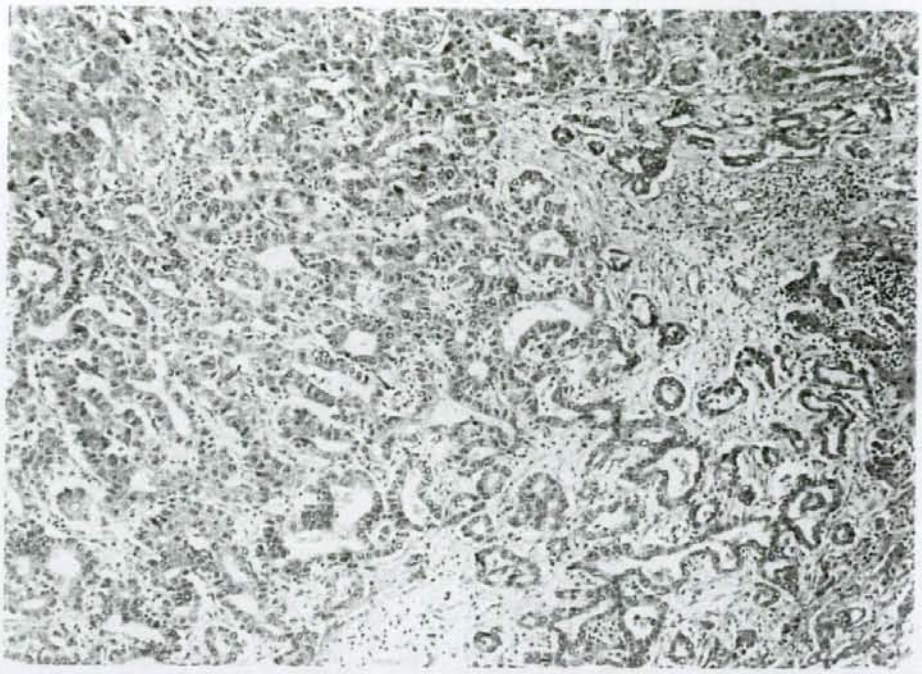

Fig. 1. A representative specimen of histological features of cHCC-CC, Allen's type C. HCC-like area (left), and (right) a CC-like area (magnification ×100).

tumor markers AFP, carcinoembryonic antigen (CEA), and CA19-9 were collected when available. Data were also collected about surgical procedures, including surgical time, bleeding, and additional lymph node dissection. Macroscopic and microscopic features such as lesion diameter, capsule formation, bile duct invasion, vascular involvement, and lymph node metastasis were also collected from the computer-based medical records. Macroscopic classification of cHCC-CC was carried out using criteria of HCC classification by the Liver Cancer Study Group of Japan,[13] including simple nodular type, infiltrative type, confluent multiple nodular type, and simple nodular type with extra nodular growth. Tumor staging was performed according to the Union International Contrele Cancer TNM staging system. Surgical procedures were grouped according to the classification by the Liver Cancer Study Group of Japan,[13] including the following categories: Hr0, resection of less than one Couinaud's segment; HrS, resection of Couinaud's segment; Hr1, anterior, posterior, or lateral segmentectomy; Hr2, right or left lobectomy or central bisegmentectomy; and Hr3, right or left trisegmentectomy. Survival time was calculated from the date of operation to the date of death due to tumor recurrence, or from the last follow-up date.

Immunohistochemical Staining

Formalin-fixed, paraffin-embedded specimens including tumors and peripheral tissue were selected for analysis. Sections measuring 4 μm in thickness were deparaffinized in xylene and rehydrated and stained with hematoxylin-eosin solution for histopathologic examination. After deparaffinization in xylene and rehydration in a graded series of ethanol, immunohistochemistry was performed using a Vectastain ABC peroxidase kit (Vector Labs, Burlingame, CA). Briefly, the sections were treated with an antigen retrieval procedure in 0.01 mmol/L sodium citrate buffer (pH 6.0) for 40 minutes at 95° C and were incubated in methanol containing 0.3% hydrogen peroxide at room temperature for 20 minutes to block endogenous peroxidase. The sections were incubated with normal protein block serum solution (room temperature, 20 minutes) to block nonspecific staining, and then incubated overnight at 4° C with anti-AFP (mouse monoclonal IgG, diluted 1:400; Sigma-Aldrich, Inc., St Louis,

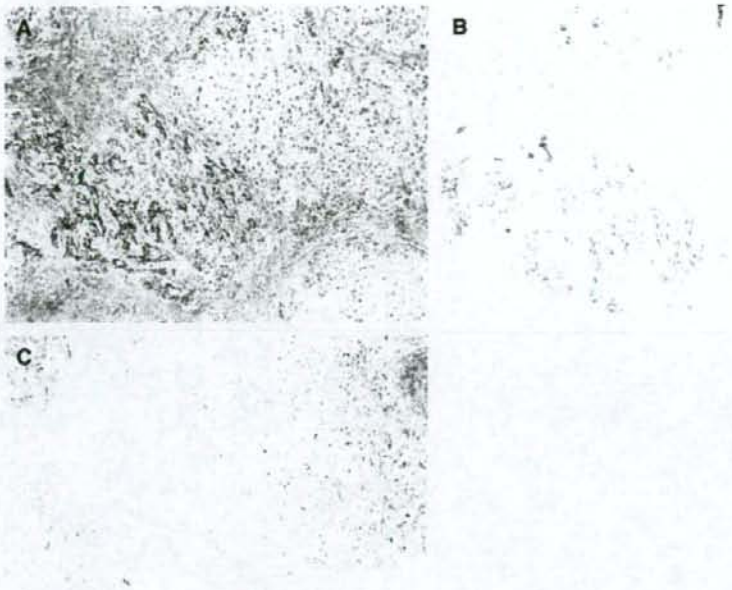

Fig. 2. (**A**) Hematoxylin-eosin stain of one specimen of cHCC-CC. (**B**) In CCC-like area (right), a strong reaction for CK7 is seen. However, in HCC-like area (left), negative reaction is seen. (**C**) In HCC-like area (right), a weak positive reaction for AFP is seen. However, in CCC-like area (left), a negative reaction is seen (magnification ×100).

MO), anti-CK7 (mouse monoclonal IgG, diluted 1:200; Sigma-Aldrich, Inc., St Louis, MO). Sections were washed three times for 5 minutes in phosphate-buffered saline, incubated with a biotin-conjugated secondary antibody (horse antimouse; room temperature, 20 minutes), and finally, incubated with peroxidase conjugated streptavidin (room temperature, 20 minutes). Peroxidase reaction was developed with 3, 3′-diaminobenzidine tetrachloride (Wako Pure Chemical Industries, Ltd, Osaka, Japan). Sections were counterstained with Meyer's hematoxylin. For negative controls, sections were incubated with Tris-buffered saline instead of the primary antibody.

Statistical Analysis

Unless otherwise indicated, numerical data are presented as mean ± SD. Differences in proportions of categorical data were tested by chi-square test. Unless otherwise indicated, differences in mean values of numerical data were tested using a two-tailed Student's *t* test. Survival (mean, medium survival days, and 1-, 3-, and 5-year cumulative survival rates) was assessed using the Kaplan-Meier method, and comparisons were made using the log-rank test. Statistical software used for all assessments was SPSS 11.5 (SPSS, Inc., Chicago, IL).

RESULTS

Patient Demographics and Laboratory Investigations of HCC, CCC, and cHCC-CC

Of the 563 cases of PLC resected, 2.3%, 90.4%, and 7.3% were cHCC-CC, HCC, and CCC, respectively. On average, the age of the patients in each of these three groups was late 50s or early 60s. The sex ratio of cHCC-CC and HCC showed an apparent male predominance, whereas in the CCC group, the male predominance was far less prominent. A positive viral hepatitis B or C status was prominent in the HCC group and noticeable in cHCC-CC group, but not in the CCC group. Serum AFP levels were more frequently elevated and tended to be higher in the HCC group than in the cHCC-CC group. However, the difference was not statistically significant. Serum CEA and CA19-9 levels were more frequently elevated and tended to be higher in the CCC group than in the cHCC-CC group, but the difference was not statistically significant (Table 3).

Pathological Examinations and pTNM Stages of HCC, CCC, and cHCC-CC

The postoperative pathological examination of the peripheral nontumor liver tissue showed that the majority of cHCC-CC and HCC patients had liver cirrhosis or chronic hepatitis (Table 4). However, in the cHCC-CC group, the incidence of chronic hepatitis was more frequent than liver cirrhosis, whereas in the HCC group, the opposite scenario was noted. Tumor size was largest in the cHCC-CC group, followed by the CCC group, then the HCC group. However, differences in tumor size were not statistically significant. The incidence of microscopic vascular invasion (portal vein or hepatic vein) in cHCC-CC was almost the same as the other two groups. The cHCC-CC and HCC groups both had a low lymph node metastatic rate, whereas the CCC group had more than a 50% possibility of developing lymph node metastasis. This difference in the rate of lymph node metastasis was statistically significant. As for tumor's capsule, there were significantly more "no capsule formation" and "partial formation" in the cHCC-CC group than in the HCC group. Concerning the pTNM stages, we found that in the cHCC-CC and HCC groups, more than half the patients were in the early or middle stages (pTNM 1 or 2 stage), whereas significantly more patients in CCC group were in the advanced stages (pTNM 3 or 4 stage).

Surgical Procedure for HCC, CCC, and cHCC-CC

We combined Hr0 and HrS as one category because Hr0 was seldom performed in the cHCC-CC and CCC groups. Operation times and blood loss are summarized in Table 5 for the hepatic resections performed. We noticed that significantly more Hr2 or Hr3 were performed in the cHCC-CC group (33.4%) and the CCC group (39%) than in the HCC group (21%). There were no perioperative deaths in the cHCC-CC and CCC groups; six (1.2%) patients died in the perioperative stage in the HCC group. In addition to a hepatectomy, patient 1 had local invasion of the right diaphragm and the inferior lobe of the right lung by the tumor of Couinaud's segments 7 and 8. A right pulmonary lobotomy was also performed at the same time as the hepatectomy patient 1. Patient 9 was found to have cancerous thrombi in

Table 3. Demographic and laboratory findings in cHCC-CC, HCC, and CCC patients

Items	Groups of patients			
	cHCC-CC (n = 13)	HCC (n = 509)	CCC (n = 41)	*P* value*
Case number (%)	13 (2.3%)	509 (90.4%)	41 (7.3%)	
Age (yr), (range)	57 ± 10.9 (36–75)	60 ± 9 (29–84)	61.5 ± 9.7 (33–80)	
Sex ratio (M/F)	12/1	4.9/1	1.2/1	2–3 $P = 0.000$
				1–3 $P = 0.019$
Viral hepatitis marker				
HBsAg (+)	38.5% (5/13)	20.3% (101/498)	5% (2/40)	2–3 $P = 0.019$
				1–3 $P = 0.007$
HCVAb (+)	0% (0/12)	61.4% (227/370)	14.3% (5/35)	1–2 $P = 0.000$
				1–3 $P = 0.000$
				2–3 $P = 0.000$
Serum tumor markers				
AFP level (ng/ml) (range)	769 ± 1752 (0–5773)	899 ± 44380 (0–500500)	NA	
<20	53.8% (7/13)	39.7% (201/506)	NA	
20–10000	46.2% (6/13)	51.8% (262/506)	NA	
>10000	0% (0/13)	8.5% (43/506)	NA	
CEA (ng/ml) (range)	5.7 ± 9.5 (0–27)	3 ± 2 (0–12)	12.0 ± 39 (0–245)	2–3 $P = 0.005$
CEA ≤10	85.7 (6/7)	98% (151/154)	90.2 (37/41)	2–3 $P = 0.037$
CEA >10	14.3 (1/7)	2.0% (3/154)	9.8% (4/41)	
			21126 ± 68594	2–3 $P = 0.000$
CA19-9 (ng/ml) (range)	254 ± 462 (5–1218)	30 ± 104 (0–1160)	(5–330000)	
CA19-9≤10	88.9% (8/9)	66.7% (84/126)	89.2% (33/37)	2–3 $P = 0.007$
CA19-9>10	11.1% (1/9)	33.3% (42/126)	10.8% (4/37)	

M = male; F = female; HBsAg = hepatitis B surface antigen; HCVAb = hepatitis C virus anitbody; AFP = alpha-fetoprotein; CEA = carcinoembryonic antigen; CA19-9 = carbohydrate antigen 19-9; NA = data not available.

*1–2 means comparison of cHCC-CC and HCC; 1–3 means comparison of cHCC-CC and CCC; 2–3 means comparison of cHCC-CC and CCC.

Table 4. The pathological features and pTNM stage in cHCC-CC, HCC, and CCC patients

Items	Groups of patients			*P* value*
	cHCC-CC (n = 13)	HCC (n = 509)	CCC (n = 41)	
Liver cirrhosis	23.1% (3/31)	59% (292/495)	NA	1–2 $P = 0.010$
Chronic hepatitis	61.5% (8/13)	34.9% (173/495)	NA	1–2 $P = 0.048$
Hepatitis of cirrhosis	84.6% (11/13)	94.0% (465/495)	NA	
Tumor size, cm (range)	6.5 ± 3.2 (3–12.5)	4.8 ± 3.9 (0.5–2.4)	5.3 ± 3.3 (0.5–16)	
Microscopic vascular invasion	23.1% (3/13)	31.6% (161/509)	36.6% (15/41)	
Bile duct invasion	0%	3.7% (19/509)	NA	
Lymph node metastasis	7.7% (1/13)	1.2% (6/509)	53.7% (22/41)	1–3 $P = 0.004$ 2–3 $P = 0.000$
Capsule formation				
No formation	46.2% (6/13)	20.3% (103/509)	NA	
Partial formation	38.5% (5/13)	9.6% (49/509)	NA	1–2 $P = 0.000$
Complete formation	15.4% (2/13)	70% (356/509)	NA	
pTNM stages				
1 or 2	61.5% (8/13)	58.5% (298/509)	24.4% (10/41)	1–3 $P = 0.013$
3 or 4	38.5% (5/13)	41.5% (211/509)	75.6% (31/41)	2–3 $P = 0.000$

NA = data not available.
*1–2 means comparison of cHCC-CC and HCC; 1–3 means comparison of cHCC-CC and CCC; 2–3 means comparison of HCC-CC and CCC.

both the right branch and trunk of the portal vein. Thrombi extraction was performed. During hepatectomy, patients 3 and 5 were suspected of having liver hilar lymph node metastasis. Additional hilar lymph node dissections were performed, but lymph node metastasis was found only in patient 5.

Survival of HCC, CCC, and cHCC-CC

The mean, median survival time and cumulative 1-, 3-, and 5-year survival of patients in the cHCC-CC, HCC, and CCC groups after hepatic resection are listed in Table 6 and illustrated in Fig. 3. Mean survival time and 1-, 3-, and 5-year cumulative survival rates were highest in the HCC group, followed by the cHCC-CC group, and were lowest in the CCC group. Patients in the cHCC-CC and HCC groups had similar median survival rates. Cumulative survival rates for 1, 3, and 5 years were significantly better in the HCC group than in the CCC group. There was no significant difference in postoperative survival rates between patients from the HCC group and the cHCC-CC group, or from the CCC group and the cHCC-CC group.

Recurrence of cHCC-CC

Up to the date of our investigations, 9 of the 13 cases experienced tumor recurrences (Table 2). This included four cases of extrahepatic recurrences,

Table 5. Surgical procedures in cHCC-CC, HCC, and CCC patients

Items	Group of patients			*P* value*
	cHCC-CC (n = 13)	HCC (n = 509)	CCC (n = 41)	
Hepatic resection				
Hr0 or Hrs	6.7% (1/13)	38.7% (197/509)	7.3% (3/41)	
Hr1	60% (8/13)	40.7% (204/509)	7.3% (3/41)	1–3 $P = 0.000^{\dagger}$
Hr2	26.7% (3/13)	17.5% (89/509)	53.7% (22/41)	2–3 $P = 0.000^{\dagger}$
Hr3	6.7% (1/13)	3.7% (19/509)	31.7% (13/41)	1–2 $P = 0.000^{\ddagger}$
Operation time (min)	397 ± 224	303 ± 147	548 ± 72	2–3 $P = 0.000^{\ddagger}$ 1–3 $P = 0.000^{\ddagger}$
Blood loss (ml)	2722 ± 3310	2196 ± 4085	2211 ± 1464	

*1–2 means comparison of HCC-CC and HCC; 1–3 means comparison of HCC-CC and CCC; 2–3 means comparison of HCC-CC and CCC.
†*P* value was calculated for the difference of the ratio of Hr0, Hr1, Hr2 to Hr3, Hr4 between groups.
‡One-way ANOVA test.

Table 6. Mean median survival time and cumulative survival rates in cHCC-CC, HCC, and CCC patients

Groups	Mean survival	Median survival (days)	1-year survival rate	3-year survival rate	5-year survival rate
cHCC-CC (n = 13)	1710.8	1801	84.6%	50.1%	50.1%
HCC (n = 504)	2453.9	1769	85.3%	66.3%	50.3%
CCC (n = 41)	1034.6	402	51.2	30.0%	25.8%

three cases of remnant liver recurrence, and two cases of both intrahepatic and extrahepatic recurrences. The recurrent sites included five cases of liver, two cases of lumbar bone, two cases of lung, one case of lymph node, one case of bone, one case of pleura, one case of skin, and one case of sacrum. The recurrent components included four cases of CCC, one case of HCC, two cases of clinically suspected CCC components, and two cases of unclear components.

DISCUSSION

cHCC-CC was first described by Wells in 1903.[14] However, the first comprehensive description and classification of this tumor type was reported in 1949.[6] Although various studies on the classification, origin, clinicopathological features, and therapy of cHCC-CC have been published (Table 7), several aspects of these data were inconsistent and often showed disparities. For example, in assessments of the clinicopathological features of cHCC-CC, (including sex ratio, background chronic liver disease, and hilar lymph node involvement), inconsistent and disparate results have been recorded.[2–5,11] Indeed, some studies from Asian regions have reported that, as with HCC, cHCC-CC was commonly seen in patients with backgrounds of liver cirrhosis or viral hepatitis.[2,11] However, this observation was not noted in other studies from Western regions.[3]

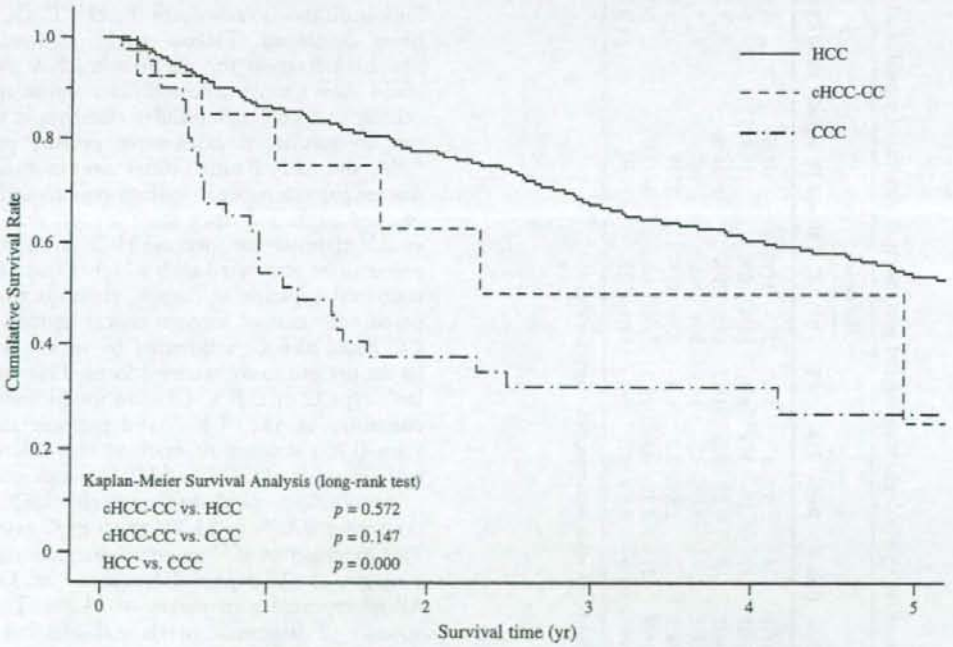

Fig. 3. Comparison of cumulative survival rates among cHCC-CC, HCC, and CCC patients. The HCC group had a significantly better postoperative survival rate than the CCC group. The postoperative survival rates between the cHCC-CC group and the HCC group or the cHCC-CC group and the CCC group were not significantly different.

Table 7. The clinico-pathologic features and postresection survival of a published series of cHCC-CC

Author	Time	Case No	Ratio	Type	M/F	Hepatitis B	Hepatitis C	Mean AFP (ng/ml)	Mean CEA (U/ml)	Mean CA 19-9 (U/ml)	LN	Postoperative survival Medium	1 year	3 years	5 years
Vano[2]	2003	26	2.4%	C	23/3	27%	38%	75.5†	3.6†	NA	0.76%	23	73%*	34.6	23.1
Liu[16]	2003	12	2%	NS	8/4	58%	0%	19.5	NA	NA	NA	17	58%*	35%	0%
Lee[1]	2002	17	0.4%	NS	15/2	45%	50%	16.8	19.8	172.3	23.5%	NA	NA	NA	NA
Jarnagin[3]	2002	27	3.6%	C	14/13	NA	NA	187	5	NA	NA	31*	78%*	38%*	24%
Sasaki[11]	2001	7	2.3%	C	6/1	57%	75%	32787	2.5	9.5	28.5%	13	NA	NA	NA
Ng[8]	1998	21	2.3%	ABC	18/3	75%	0%	4812.9	NA	NA	22%	7	NA	NA	NA
Nakamura[5]	1996	6	2.8%	C	5/1	50%	0%	350	3.7	58.4	0%	52.5	100%	60%	60%

LN = hepatic hilar lymph mode metastasis; NA = data not available.
*Estimated data.
†Medium value.

In some reports, cHCC-CC had an apparent male predominance,[2,5] whereas other reports did not show this.[3,4] Additionally, problems relating to the recurrence pattern, the necessity of additional hilar lymph node dissection, and the postoperative prognosis are yet to be fully clarified.

It would appear that the biggest obstacle to this research lies in the rarity of this tumor type. The largest published study included 36 cases of all cHCC-CC types, and was done by Maeda and colleagues in 1995.[9] Statistically, the results of small-sized study reports are more easily influenced by "randomized noise" and may not be able to show clearly the distinct features of this disease entity. Another problem is the unavailability of universally accepted diagnosis and classification criteria. This can result in inconsistent diagnosis criteria and selection of cases in these published studies. For example, some published reports regarding only type C of cHCC-CC showed a mixture of two lines of differentiations throughout and included what appeared to be true combined tumors[2,3]; others either included all three types of cHCC-CC[4,8,15] or did not state the criteria for diagnosis, classifications used, or cHCC-CC types included.[1,16] However, pathological diagnosis techniques for cHCC-CC are still being developed. Tickoo et al.[17] showed that in situ hybridization for albumin mRNA techniques could allow a more precise differentiation of hepatocellular and cholangiocellular elements in comparison to results of cytokeratin profile, polyclonal CEA, and AFP. Besides, differences in etiology and disease pattern between eastern regions and western regions might influence these aspects deeply. Fong et al.[18] claimed that, because HCC of Asian regions seems to be associated with a higher rate of concurrent viral hepatitis or chronic cirrhosis when compared with that of western cancer centers, cHCC-CC could also be influenced by such differences.[3] In the present study, we have focused just on the Allen's type C of cHCC-CC and used immunohistochemistry of AFP, CK-7, and periodic acid-Schiff stain (PAS) staining to examine the differentiation and diagnosis of CCC and HCC components.

According to published research, cHCC-CC accounts for 0.40% to 14.2% of all PLC cases (Table 7). Goodman et al.[19] reported frequency of 2.4%, Jarnagin et al.[3] reported frequency of 3.6%, and Allen[6] reported a frequency of 14.2%. The inconsistency of diagnosis criteria and selection of cases in published studies may have resulted in these variations. Besides, some ratios were calculated only from resected clinical samples[2,4] and some were calculated from both resected clinical samples and autopsied or biopsied samples.[9] Differences in

regional/temporal variances and etiology may also play a role. In addition to the cHCC-CC to HCC ratio, the HCC to CCC ratio is also interesting. In Japan, Sharp and colleagues[20] reported a ratio of 6.4 in a population-based follow-up of the extended life span study. Our data showed that the percentages of HCC, CCC, and cHCC-CC in PLC were 90.4%, 7.3%, and 2.3%, respectively.

The clinical pathological features differed from each other in many of the published studies. In our study (Table 1), a prominent male predominance (93.3%) and noticeable viral hepatitis rate (38.5%) were found in cHCC-CC group. These made cHCC-CC, Allen's type C, more similar to HCC than CCC. As for tumor marker, Nakamura reported serum AFP and CA19-9 levels were increased in three of five patients and four of five patients, respectively.[5] Our data showed that elevated AFP and CA19-9 levels were detectable in almost half of cHCC-CC patients. The tumor size in the present studies was generally larger in the cHCC-CC group in comparison to the HCC and CCC groups. This finding was in line with data from several other research groups.[2,3,16] We speculate that the relatively small portion of viral hepatitis rate in cHCC-CC group (30% in cHCC-CC vs. 81.4% in HCC) may result in the major part of patients not being aware of the possibility of liver cancer. This hypothesis is supported by the fact that in our present study, the patients with negative viral hepatitis status had larger tumors than patients positive for viral hepatitis (7.68 vs. 4.84, $P = 0.067$).

The preoperative diagnosis of cHCC-CC appeared to be challenging. Taguchi et al.[4] reported a study of 23 cases of cHCC-CC, among which none could be correctly diagnosed before hepatectomy procedures. In the present study, although preoperative angiography/CT scan were routinely carried out before surgery (data not shown), only one case of a patient with both elevated serum AFP and CA19-9 could be correctly diagnosed. The other 12 cases were misdiagnosed either as HCC (10 cases), CCC (one case), or liver abscess (one case). It seems that imaging diagnosis alone could not differentiate cHCC-CC from HCC. Strategies for improving the preoperative diagnosis of cHCC-CC have been developed. Nakamura et al.[5] hypothesized that a hypervascular tumor with high CEA and CA19-9 levels or a hypovascular tumor with a high level of AFP may indicate a preoperative diagnosis of MHC. In our studies, liver mass similar to that of HCC in CT scans plus moderately elevated serum AFP and CA19-9 were reliable indicators of cHCC-CC. Primary colon or rectum cancer with liver metastasis can also present both elevated serum AFP and CA19-9. However, these possibilities can be easily excluded if a primary lesion is not found.

Surgical procedures in the present study showed that more Hr2 or Hr3 were performed in the cHCC-CC group (33.4%) and the CCC group (85.4%) than in the HCC group (21%). The operation time was longest in the CCC group, followed by the cHCC-CC group, and then the HCC group. The increase in surgical time in the CCC group was largely due to the additional hilar lymph node dissections for CCC patients. Blood loss in the cHCC-CC group was larger than that of HCC and CCC. However, the difference between the groups was not significant due to the small size of the cHCC-CC group. These facts implied that patients in the cHCC-CC group, as a whole, probably underwent more invasive surgical procedures than the HCC group. The necessities of additional hilar lymph node dissection for cHCC-CC are still under debate. Sasaki et al.[11] reported that two in three cases of the multinodular type of cHCC-CC had hilar lymph node metastasis and indicated that additional hilar lymph node dissection should be necessary for this type of cHCC-CC. However, many series did not show a high lymph node metastasis rate or did not mention it.[2,3,16] Nakamura et al.[5] suggested that additional hilar lymph node dissection was unnecessary because of the negative finding of lymph node metastasis at the time of surgery. In the present study, only two cases were suspected to have metastatic carcinoma in hilar lymph node during surgery, so additional hilar lymph node dissection was performed. The results showed one positive and one negative. The low incidence of metastatic hilar lymph nodes in our study (7.7%) does not support the necessity of additional hilar lymph node dissection for cHCC-CC patients, especially in cases of accompanying liver cirrhosis, which inevitably leads to further surgical invasion.

In many studies, the survival of cHCC-CC after surgery was worse than that reported for HCC. Yano et al.[2] reported that cHCC-CC patients had a significantly poorer rate of postoperative survival than patients with either HCC or CCC. Jarnagin et al.[3] also reported a worse survival of cHCC-CC patients in comparison to HCC or CCC patients. However, these differences in survival were not significant. In the present study, the mean and the 1-, 3-, and 5-year survival rates were highest in the HCC group, followed by the cHCC-CC group, and then the CCC group. The HCC group had a significantly better postoperative survival rate than the CCC group. Although cHCC-CC had an intermediate survival rate between that of HCC and CCC in the graph of postoperative survival (Fig. 3 and Table 6),