Expression of tight junction protein claudin-5 in tumor vessels and sinusoidal endothelium in patients with hepatocellular carcinoma.

Expression of tight junction protein claudin-5 in tumor vessels and sinusoidal endothelium in patients with hepatocellular carcinoma

Takanori Sakaguchi, M.D., Shohachi Suzuki, M.D., Hiroyuki Higashi, M.D., Keisuke Inaba, M.D., Satoshi Nakamura, M.D., Satoshi Baba*, M.D., Tomoyuki Kato*, Hiroyuki Konno M.D.,

Second Department of Surgery, *Faculty of Pathology, Hamamatsu University School of Medicine, Japan;

Subject Categoly: Oncology

Running Title: CL-5 in HCC tumor vessels and surrounding sinusoids

Abbreviations: HCC, hepatocellular carcinoma; CL, claudin; SEC, sinusoidal endothelial cell; MVD, microvessel density; BBB, blood-brain barrier; MCP-1, monocyte chemoattractant protein 1

Key words: hepatocellular carcinoma, tight junction, claudin, sinusoid, angiogenesis, microvessel density

Address correspondence to: Takanori Sakaguchi

Second Department of Surgery

Hamamatsu University School of Medicine

1-20-1 Handayama, Hamamatsu, 431-3192, Japan

Phone: 81-53-435-2279, Fax: 81-53-435-2273

E-mail: saka1119@hama-med.ac.jp

-Abstract-

Background: An increase of leaky vasculature is important for growth and metastasis of hepatocellular carcinoma (HCC). The paracellular permeability-regulating proteins in tumor vessels and adjacent sinusoids have not been studied in HCC patients.

Methods: Expression of an endothelial tight junction protein claudin-5 (CL-5) and a standard endothelial marker CD34 were immunohistochemically examined in resected specimens from 51 HCC cases. The relationship between hepatitic or fibrotic grade and CL-5 expression pattern in sinusoidal endothelial cells (SECs) was evaluated in the tumor-adjacent tissues. Microvessel density (MVD) highlighted by CD34 or CL-5 was examined in tumor tissues.

Results: In the normal liver, a ubiquitous CL-5 expression was seen in SECs, the arteries and portal veins but not in the central veins. Sinusoidal CL-5 expression was downregulated according to the increase of hepatitic or fibrotic grade. Poor differentiation and vasculobiliary invasion were significantly associated with a lower CL-5-MVD but not CD34-MVD. By multivariate analysis, vasculobiliary invasion and lower CL-5-MVD were independent factors associated with a lower postoperative overall survival rate.

Conclusions: Attenuated CL-5 expression in SECs may be related to SEC dysfunction in injured liver. Downregulated CL-5 expression in tumor vessels may serve as a potential marker for poor prognosis in HCC.

Keywords: hepatocellular carcinoma, tight junction, claudin, sinusoid, angiogenesis, microvessel density

-Introduction-

Hepatocellular carcinoma (HCC) is a typical hypervascular neoplasm[1]. For such a hypervascular solid tumor, growth and metastasis are supposed to be dependent on angiogenesis[2]. Tumor microvessel density (MVD), reflecting angiogenesis in tumor areas, was reported to be associated with a worse postoperative survival in HCC patients[3]. However, another report showed no significant correlation between MVD in HCCs and postoperative prognosis[4]. Not only quantity but also character differs between normal and tumor angiogenesis, in terms of morphology and hyperpermeable state[2]. It is well recognized that tumor-adjacent sinusoidal endothelial cells (SECs) in the cirrhotic liver are morphologically different from normal SECs[5].

The tight junction, an important interellular adhesion machinery, serves two main functions in cell layers: organization of the paracellular transport of solutes and ions maintaining concentration gradients driving transcellular transport; and restriction of membrane lipids and proteins to maintain cellular polarity[6]. Tight junctional control depends on the claudin (CL) family of tight junction proteins[6]. Among them, claudin-5 (CL-5) is important for maintenance of the tight junctional integrity between the vascular endothelium[6]. Nitta et al. reported that the blood-brain barrier (BBB) is leaky in CL-5-deficient mice, suggesting that CL-5 is crucial for endothelial tight junction[7].

Many investigators have showed abnormal regulation of claudin family proteins in cancer tissues[8]. Our previous report showed that CL-1 downregulation in HCC cells was correlated with dedifferentiation, portal invasion, and poor postoperative survival[9]. However, there has been no report investigating the expression status of claudins in tumor vessels and the adjacent SECs. This study,

therefore, aims to elucidate the relationship between: (1) hepatitic or fibrotic activity and sinusoidal CL-5 expression; and (2) MVD highlighted by a standard endothelial marker CD34[10] (CD34-MVD) or by CL-5 (CL-5-MVD) in HCC tissues and various clinicopathological parameters in HCC patients undergoing hepatectomy.

-Patients & Methods-

Patients

Fifty-one patients who underwent a hepatectomy for HCC in our institute between 1994 and 2003 were enrolled in this study. The patients included 43 men and 8 women, who ranged in age from 40 to 80 years with a median age of 64 years. A surgical resection of the HCC was done for therapeutic purposes. Each patient provided HCC tissues and nontumorous liver tissues for pathological examination. The cause of liver disease was the hepatitis B virus in 16 patients, the hepatitis C virus in 32, and cryptogenic in 3. Informed consent was obtained from all patients. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

Histopathology

Surgically removed specimens were fixed with 10% buffered formalin and embedded in paraffin, and 4-μm-thick sections were cut and stained with standard hematoxylin-eosin. The tumor cell differentiation was assessed according to criteria of the Japan Liver Cancer Study Group[11]. For HCCs showing histological diversity of tumor differentiation, the predominant pattern was taken into consideration when assigning a grade.

The specimens were evaluated for inflammation and fibrosis with reference to a semiquantitative standardized scoring system[12] by two pathologists in a blind fashion. The histologic grade of inflammation was scored by evaluating inflammatory activity as follows: grade 0, mild portal inflammation only, no activity; 1, minimal activity and scant piecemeal necrosis; 2, mild activity with piecemeal necrosis; 3, moderate activity with obvious periportal necrosis involving most portal triads; and 4,

severe activity with bridging necrosis. The histologic grade of fibrosis was scored as follows: grade 0, no fibrosis; 1, portal fibrous expansion; 2, mild to moderate portal fibrosis; 3, periportal fibrosis with bridging; and 4, cirrhosis.

Antibodies and immunohistochemistry

A mouse monoclonal antibody against CD34 (QBEnd 10; Dako A/S, Glostrup, Denmark) and mouse monoclonal antibody against human CL-5 (Zymed, San Francisco, CA) were used to perform immunohistochemical staining with ChemMate Envision system (Dako A/S, Glostrup, Denmark).

After dewaxing and quenching the endogenous peroxidase with H_2O_2/methanol, the sections were processed to retrieve antigens using a microwave method. In brief, the slides were immersed in 10 mM citric acid (pH 7.0) and heated in a microwave oven for 40 minutes (500 W output; Iwatani, Tokyo, Japan). They were then cooled to room temperature for 4 hours.

The sections were treated with normal serum for 1 hour to reduce background staining, then incubated with primary anti-CD34 antibody diluted 1:50 or anti-CL-5 antibody diluted 1:200 with 1% bovine serum albumin (BSA) / phosphate-buffered saline (PBS) in a humidified chamber at room temperature for 8 hours. They were then washed 3 times with cold 10 mM phosphate buffer (pH 7.2). Biotinated anti-mouse immunoglobulin G in ChemMate Envision system was used as the secondary antibody. The binding products were visualized with 3, 3'-diaminobenzidine tetrahydrochroride solution, and the nuclei were lightly counterstained with hematoxylin. We confirmed the specificity of CD34 and CL-5 staining by the negative staining in a control study. Controls were obtained by

replacing the first antibody with 1% BSA/PBS or by incubation with isotypic non-specific immunoglobulins.

Evaluation of immunostaining

In nontumorous tissues, the sinusoidal CL-5 expression pattern was evaluated as follows: 1, ubiquitous CL-5 expression; 2, weaker CL-5 expression when compared with those in the arteries or portal veins; and 3, no CL-5 expression. Typical patterns are shown in Figure 1A-C.

In cancer tissues, MVD was assessed under X 200 fold magnification. When HCCs showed histological diversity on tumor differentiation, the expression of CD34 and CL-5 was assessed in the area of the predominant grade. Only the vessels showing positive staining were selected for counting. Large vessels with thick muscular walls or with a lumen greater than 50 μm in diameter were excluded. Five areas showing the highest density of staining were selected for counting. Average counts were recorded and expressed as the absolute number of vessels per 0.74 mm^2 of the sections according to another report[3]. In the case of the large pseudoglandular type or large trabecular type, a microvessel of about 40 μm was counted as one vessel according to the modified method of Tanigawa et al[3]. Both CD34-highlighted MVD and CL-5-highlighted MVD were evaluated in the nearly same area using sequential sections.

Patients' follow up and prognosis

After the operation, patients were followed up regularly every month. When intrahepatic recurrence was indicated, we performed a repeat hepatectomy for patients

with satisfactory liver function, if feasible. Either transarterial chemoembolization (TACE), percutaneous ethanol injection therapy (PEIT), or radiofrequency ablation under laparotomy was selected as the treatment for patients excluded from surgical indication. The follow-up period of these patients ranged from 4 to 166 months (median 53 months).

Satistical analysis

Statistical differences were analyzed using the chi-square test with Fisher's exact test and the unpaired Student's *t*-test or analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test. Analyses of overall survival and disease-free survival were conducted with the Kaplan-Meire method and the differences in survivals between the groups were compared using the log-rank test. Clinicopathological variables as potential prognostic determinants were dichotomized and analyzed for their effect on overall and disease-free survival. Based on the results of a univariate analysis, a few prognostic factors were assessed by a subsequent multivariate analysis using Cox's proportional hazards model. Statistical analyses were done using Stat View (version 5.0, Abacus Concepts, Inc, Berkeley, CA). A p value < 0.05 was considered significant.

-Results-

Pathological features of HCC and underlying liver disease

Histological examination revealed the underlying disease of the nontumorous liver to be cirrhosis or bridging fibrosis in 23 cases and chronic hepatitis in 26 cases, while it revealed a normal liver in 2 cases. According to the hepatitic activity grades, two specimens were classified into grade 0, 3 into grade 1, 24 into grade 2, 18 into grade 3, and 4 into grade 4. Regarding to the fibrotic grades, two were classified into grade 1, 9 into grade 1, 17 into grade 2, 18 into grade 3, and 5 into grade 4. The indocyanine green (ICG) retention rate 15 min after administration (ICG-R15) was not significantly different between patients showing hepatitic activity grade 0-2 and those showing grade 3 or 4 (Table 1). ICG-R15 was significantly higher in patients showing fibrotic grade 3 or 4 than in those showing grade 0-2.

Fourteen HCCs were classfied as well differentiated, 17 as moderately differentiated, and 20 as poorly differentiated or undifferentiated.

Expression patterns of claudin-5 and CD34 in nontumorous sinusoidal endothelial cells. The correlation between CL-5 expression pattern in SECs and ICG-R15

CL-5 was expressed in large vessels like the arteries and portal veins but not in the central veins (Fig. 1A - C). In the liver without fibrosis and inflammation, CL-5 was ubiquitously expressed in every SEC (pattern 1 as shown in Fig. 1A). However, sinusoidal CL-5 expression was diminished in livers showing mild to moderate hepatitis or fibrosis (pattern 2 as shown in Fig. 1B). Furthermore, in cirrhotic liver, little CL-5 expression was seen in SECs (pattern 3 as shown in Fig. 1C). Increased hepatitic activity grade and fibrotic grade significantly correlated with loss of CL-5 expression in

SECs (Table 1). ICG-R15 in patients showing pattern 1, 2, and 3 of SEC CL-5 expression were 9.7 ± 4.7 %, 16.4 ± 6.8 %, and 20.7 ± 8.5 %, respectively. ICG-R15 was significantly higher in patients showing SEC CL-5 expression pattern 3 than in those showing grade SEC CL-5 expression pattern 1 (p=0.0008).

Unlike CL-5, CD34 was expressed in the endothelium of large vessels, such as arteries, portal veins and central veins and in the periportal SECs, regardless of hepatitic activity grades or fibrotic stages (Fig. 1D-F). Midzonal and pericentral SECs did not express CD34.

Expression of CD34 and CL-5 in tumor vessels

Figure 2A shows HE-stained sections of well differentiated HCC tissues. CD34 was ubiquitously expressed in tumor vessels in well differentiated HCCs (Fig. 2B). CL-5 was ubiquitously expressed in most CD34-positive tumor vessels in well differentiated HCCs (Fig. 2C). Figure 2D shows HE-stained sections of poorly differentiated HCC tissues. CD34-stained tumor vessels were very scarce in poorly differentiated HCCs (Fig. 2E). Of note, in poorly differentiated HCCs, CD34-positive tumor vessels did not necessarily show CL-5 staining (Fig. 2F, compare with Fig 2E). Figure 3 shows a typical expression pattern of CD34 (A) and CL-5(B) in the portal tumor thrombus derived from a poorly differentiated HCC. Tumor vessels with positive CD34 staining in the portal tumor thrombus frequently lacked CL-5 staining.

MVD highlighted by CD34 or CL-5 in HCCs was investigated as a quantitative approach. As shown in Figure 4, CL-5-highlighted MVD (CL-5-MVD), but not CD34-highlighted MVD (CD34-MVD), negatively correlated with the maximum size

of the tumor. CL-5-MVD well correlated with CD34-MVD.

The relationship between various pathological parameters and CL-5- or CD34-MVDs is shown in Table 2. CD34-MVD was significantly decreased in poorly differentiated HCCs compared to well and moderately differentiated HCCs. Other parameters, such as tumor size and the presence of vasculobiliary invasion or microsatellite lesions did not influence CD34-MVD.

Regarding CL-5-MVD, large HCCs (equal to or larger than 30mm) had a significantly lower CL-5-MVD compared with small HCCs (<30mm). Poorly differentiated HCCs showed a significantly lower CL-5-MVD than well and moderately differentiated HCCs. CL-5-MVD was significantly lower in HCCs with vasculobiliary invasion than those without vasculobiliary invasion. There was no significant difference in CL-5-MVD between HCCs with and without microsatellite lesions.

Patient overall and disease-free survival

Disease-free and overall postoperative survival rates in HCC patients undergoing hepatectomy were investigated for various clinicopathological parameters, including CD34- or CL-5-MVD. The cut-off value of CD34- and CL-5- MVD was set at 80 and 47 according to the median. By univariate analysis, poor differentiation, multiple nodules, and the presence of vasculobiliary invasion were significantly associated with a lower disease-free survival rate (Table 3). Vasculobiliary invasion, microsatellite lesions and lower CL-5-MVD significantly affected overall survival (Table 3). The overall survival rate was higher in HCC patients showing higher CD34-MVD (≥80) than those showing lower CD34-MVD (<80), however without any significant difference.

In the subsequent multivariate analysis, multiple nodules (Hazard ratio (HR) 2.915, 95% confidence interval (CI) 1.473-5.747, p=0.0021) and vasculobiliary invasion (HR 2.517, 95% CI 1.093-5.795, p=0.0301) were independent predictive factors for poor disease-free survival. Vasculobiliary invasion (HR 6.466, 95% CI 1.978-21.135, p=0.0020) and lower CL-5-MVD (HR 2.747, 95% CI 1.071-7.042, p=0.0355) were independent factors associated with a lower overall survival rate (Fig. 5).

-Discussion-

There have been many reports investigating the expression of claudins, major tight junction proteins, in various cancer tissues[8, 13-15]. However, investigators have focused on the expression in cancer cells. Our previous report demonstrated that the downregulation of CL-1 expression in HCC cells correlates with dedifferentiation, portal invasion, and poor prognosis after hepatectomy[9]. Cheung and colleagues[16] showed that CL-10 upregulation in HCC tissues is associated with recurrence of HCC after hepatectomy, but it remains unclear what kinds of cells are responsible for the upregulation. No information has been available on the expression status of claudins in tumor vessels. Therefore, we focused on tight junction protein expression in tumor vessels in HCC tissues as well as in adjacent SECs. CL-5 expression was immunohistochemically evaluated because of its specificity in vascular endothelial cells[6, 7].

Angiogenesis is essential for the development of malignant tumors and favors progression and metastasis[2], especially in HCC[1]. However, angiogenesis, usually estimated by MVD, does not necessarily correlate with postoperative prognosis in HCC patients. Tanigawa et al. demonstrated that increased CD34-MVD as well as dedifferentiation is an independent negative prognostic factor for postoperative survival[3]. On the other hand, Sun et al demonstrated that CD34-MVD does not correlate with disease-free or overall survival after hepatectomy for HCC[4]. It is well recognized that the maximum diameter of HCC is associated with poor postoperative survival[4]. El-Assal et al[1]. demonstrated that small HCCs (≤2cm) had a significantly lower CD34-MVD than middle-sized HCCs (2-5cm), whereas CD34-MVD of large HCCs (>5cm) was lower than that of middle-sized HCC.

Therefore, intratumoral CD34-MVD may not be a reliable prognostic factor for HCC patients.

Tumor vessels have specific characteristics such as a tortuous form, irregular shape, and hyperpermeable state[2]. We have speculated that disruption of the integrity of interepithelial tight junctions may be one of the causes of hyperpermeability. To test this hypothesis, we examined the expression status of CL-5, an important protein in epithelial tight junctions[6, 7], in HCC tissues. Furthermore, SECs change their phenotype, so-called "sinusoidal capillarization", with the development of cirrhosis[5]. Since we speculate that such phenotypic change affects the status of tight junctions in SECs, the CL-5 expression pattern in the tumor-adjacent liver tissues was also evaluated in the present study.

CD34, a standard epithelial marker[10], is expressed in the periportal SECs as well as large vessels such as the arteries, portal veins, and central veins, irrespective of hepatitic or fibrotic grade (Fig 1D-F). These results are identical to the observations of Tanigawa and colleagues[3]. Ohmori and associates also reported that CD34 was sporadically expressed in the periportal SECs in the hepatitis C-associated chronic hepatitic livers, using biopsy specimens[17]. Moreover, they demonstrated that higher CD34-positive SEC count was correlated with higher stage of hepatic fibrosis as well as HCC development within 5 years of the liver biopsy[17]. Taken together, many investigators suggest that CD34 expression in the periportal SECs is a marker for sinusoidal capillarization[18]. In our results, CD34 expression was seen in the periportal SECs of the normal liver as well. We speculate that the discrepancy of CD34 expression in SECs from periportal to pericentral area may depend on functional diversity of SECs. Further study will be needed.

The study on CL-5 expression in the liver is very limited. Rahner et al. reported that CL-5 was expressed in the large vessel endothelial cells but not in SECs of rat livers[19]. In this study, CL-5 is ubiquitously expressed in the arteries and the portal veins but not in the central veins irrespective of hepatitic or fibrotic grade (Fig 1A-C). In the normal liver without hepatitis and fibrosis, CL-5 was ubiquitously expressed in SECs (Fig 1A). It remains unclear why CL-5 was detected in the human SECs in our study. The difference in species (rat vs human) may be involved in a variety of CL-5 expression in vessels of hepatic tissue. Importantly in the present study, CL-5 expression in SECs diminished with the development of hepatitis and fibrosis (Fig 1E and F, and Table 1). Downregulation of CL-5 expression in SECs correlated with increased ICG-R15 levels in this study, suggesting the dysfunction of hepatic uptake or transportation of substances by hepatocytes[20]. To our knowledge, this is the first study to show that selective CL-5 downregulation in SECs well correlates with dysfunction of the liver.

The relationship between intratumoral MVD and postoperative prognosis is controversial[3, 4]. In this study, CD34-MVD did not significantly correlate with vasculobiliary invasion or microsatellite lesions, two significant negative prognostic factors for postoperative overall survival (Tables 2 and 3). On the other hand, vasculobiliary invasion was significantly associated with lower CL-5-MVD (Tables 2 and 3). Furthermore, the multivariate analysis revealed that vasculobiliary invasion and lower CL-5-MVD are independent prognostic factors for postoperative survival. As shown in Fig. 2 and 3, some tumor vessels showing CD34 staining lacked CL-5 staining. This might be associated with the finding that lower CD34-MVD did not significantly affect postoperative survival.

CL-5 is crucial for the maintenance of epithelial tight junctions[6]. Nitta et al. reported that CL-5-deficient mice showed selective hyperpermeability of the BBB for small molecules[7]. Stamatovic et al. demonstrated that monocyte chemoattractant protein-1 (MCP-1) increases the permeability of the BBB to fluorescein isothiocyanate (FITC)-conjugated albumin, a relatively large molecule, probably through a decrease in tight junction proteins such as ZO-1, ZO-2, occludin and CL-5[21]. Extravasation of such substances may cause the interstitial fluid pressure[2]. Increased interstitial fluid pressure (IFP) is suitable for cancers in terms of the following cascade. Increased IFP causes an imbalance of blood supply, leading to hypoxia and acidosis. Hypoxia, in turn, induces genetic instability and selects for more malignant cells with increased metastatic potential[22]. Hypoxia and acidosis also compromise the cytotoxic function of immune cells[2]. Taken together, the negative correlation between CL-5-MVD and tumor size (Fig. 4) may be the cause as well as the effect of the increased IFP.

In this study, we did not perform a functional analysis of tumor vessels or SECs because of technical difficulties. Also, it remains unclear why CL-5 expression is downregulated in tumor vessels of HCCs showing a poor prognosis and in SECs in injured liver. As mentioned above, MCP-1 may be responsible for CL-5 downregulation. First, MCP-1 was verified to decrease CL-5 protein expression and increase permeability in brain endothelial cells through the activation of a Rho signaling cascade[23]. Second, MCP-1 expression is upregulated in bile duct and perisinusoidal cells with the progression of hepatic inflammation[24, 25]. Third, HCC cells as well as tumor vessels express MCP-1[26]. Other possible causes of CL-5 downregulation are genetic instability and epigenetic abnormality in endothelial

cells in tumor tissue. Hida et al. showed that endothelial cells in tumor tissues occasionally have genetic instability such as loss of heterozygosity[27]. Honda et al. demonstrated that hypermethylation as well as deacetylation of the CL-4 gene decreases CL-4 transcription in ovarian cancer cells[28]. The same transcriptional regulatory mechanism may lie behind the regulation of the CL-5 gene. Further studies will be needed to address these issues.

In conclusion, attenuated expression of CL-5 in SECs may be related to SEC dysfunction in injured liver. Downregulated CL-5 expression in tumor vessels may serve as a useful predictor for poor prognosis in HCC.

-Figure Legends-

Fig 1.

Immunostaining of CL-5 (upper panels, A-C) and CD34 (lower panels, D-F) in nontumoral liver. A and D are derived from thin serial sections of the liver showing hepatitic activity grade 0 and fibrotic grade 0. B and E are from liver with grade 1 hepatitis and grade 1fibrosis. C and F are from liver showing grade 4 hepatitis and grade 4 fibrosis. The CL-5 expression pattern was evaluated as described in Materials & Methods: 1, ubiquitous CL-5 expression in SECs (A); 2, weaker CL-5 expression in SECs when compared with those in the arteries or portal veins (B); and 3, no CL-5 expression in SECs (C). (original magnification, X60). Arrows and arrowheads indicate the portal and central veins, respectively. Scale bars = 100 μM

Fig 2.

Typical staining pattern of CD34 or CL-5 in the tumor vessels of well (A-C) and poorly (D-F) differentiated HCCs. A and D are hematoxylin and eosin-stained sections. CD34 was expressed in tumor vessels of both well (B) and poorly (E) differentiated HCCs. Most of the tumor vessels showing CD34 staining were positive for CL-5 in well differentiated HCCs (C). However, tumor vessels showing CD34 staining did not necessarily show CL-5 staining (F). T and N indicate the tumor and the adjacent non-tumor tissue, respectively. (original magnification, X40) Scale bars = 100 μM

Fig 3.

Typical CD34 and CL-5 expression in the portal venous tumor thrombus

derived from poorly differentiated HCC. Tumor vessels showing CD34 staining (A) frequently lacked CL-5 staining (B) in the portal tumor thrombus. (original magnification, X30) Scale bars = 100 μM

Fig 4.

The relationship between tumor size and MVD, highlighted by CD34 (A) and CL-5 (B). The relationship between CD34-MVD and CL-5-MVD is also shown (C).

Fig 5.

Overall survival curves after hepatectomy stratified by the existence of vasculobiliary invasion (A) and CL-5-MVD values (B). Existence of vasculobiliary invasion and lower CL-5-MVD were significantly associated with a lower overall survival rate after hepatectomy.

References

1. El-Assal, O. N., Yamanoi, A., Soda, Y., Yamaguchi, M., Igarashi, M., Yamamoto, A., Nabika, T., and Nagasue, N. Clinical significance of microvessel density and vascular endothelial growth factor expression in hepatocellular carcinoma and surrounding liver: possible involvement of vascular endothelial growth factor in the angiogenesis of cirrhotic liver. Hepatology. 27: 1554-1562, 1998.
2. Jain, R. K. Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. Science. 307: 58-62, 2005.
3. Tanigawa, N., Lu, C., Mitsui, T., and Miura, S. Quantitation of sinusoid-like vessels in hepatocellular carcinoma: its clinical and prognostic significance. Hepatology. 26: 1216-1223, 1997.
4. Sun, H. C., Tang, Z. Y., Li, X. M., Zhou, Y. N., Sun, B. R., and Ma, Z. C. Microvessel density of hepatocellular carcinoma: its relationship with prognosis. J Cancer Res Clin Oncol. 125: 419-426, 1999.
5. Schaffner, F., and Poper, H. Capillarization of hepatic sinusoids in man. Gastroenterology. 44: 239-242, 1963.
6. Tsukita, S., Furuse, M., and Itoh, M. Multifunctional strands in tight junctions. Nat Rev Mol Cell Biol. 2: 285-293, 2001.
7. Nitta, T., Hata, M., Gotoh, S., Seo, Y., Sasaki, H., Hashimoto, N., Furuse, M., and Tsukita, S. Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. J Cell Biol. 161: 653-660, 2003.
8. Morin, P. J. Claudin proteins in human cancer: promising new targets for diagnosis and therapy. Cancer Res. 65: 9603-9606, 2005.

9. Higashi, Y., Suzuki, S., Sakaguchi, T., Nakamura, T., Baba, S., Reinecker, H. C., Nakamura, S., and Konno, H. Loss of Claudin-1 Expression Correlates with Malignancy of Hepatocellular Carcinoma. J Surg Res, 2007.

10. Ramani, P., Bradley, N. J., and Fletcher, C. D. QBEND/10, a new monoclonal antibody to endothelium: assessment of its diagnostic utility in paraffin sections. Histopathology. 17: 237-242, 1990.

11. Japan Liver Cancer Study Group. General Rules for the Clinical and Pathological Study of Primary Liver Cancer (Second English ed). Tokyo, Japan: Kanehara & Co. Ltd., 2003.

12. Desmet, V. J., Gerber, M., Hoofnagle, J. H., Manns, M., and Scheuer, P. J. Classification of chronic hepatitis: diagnosis, grading and staging. Hepatology. 19: 1513-1520, 1994.

13. Resnick, M. B., Konkin, T., Routhier, J., Sabo, E., and Pricolo, V. E. Claudin-1 is a strong prognostic indicator in stage II colonic cancer: a tissue microarray study. Mod Pathol. 18: 511-518, 2005.

14. Kominsky, S. L., Argani, P., Korz, D., Evron, E., Raman, V., Garrett, E., Rein, A., Sauter, G., Kallioniemi, O. P., and Sukumar, S. Loss of the tight junction protein claudin-7 correlates with histological grade in both ductal carcinoma in situ and invasive ductal carcinoma of the breast. Oncogene. 22: 2021-2033, 2003.

15. Michl, P., Buchholz, M., Rolke, M., Kunsch, S., Lohr, M., McClane, B., Tsukita, S., Leder, G., Adler, G., and Gress, T. M. Claudin-4: a new target for pancreatic cancer treatment using Clostridium perfringens enterotoxin. Gastroenterology. 121: 678-684, 2001.

16. Cheung, S. T., Leung, K. L., Ip, Y. C., Chen, X., Fong, D. Y., Ng, I. O., Fan, S. T., and So, S. Claudin-10 expression level is associated with recurrence of primary hepatocellular carcinoma. Clin Cancer Res. 11: 551-556, 2005.

17. Ohmori, S., Shiraki, K., Sugimoto, K., Sakai, T., Fujikawa, K., Wagayama, H., Takase, K., and Nakano, T. High expression of CD34-positive sinusoidal endothelial cells is a risk factor for hepatocellular carcinoma in patients with HCV-associated chronic liver diseases. Hum Pathol. 32: 1363-1370, 2001.

18. Park, Y. N., Yang, C. P., Fernandez, G. J., Cubukcu, O., Thung, S. N., and Theise, N. D. Neoangiogenesis and sinusoidal "capillarization" in dysplastic nodules of the liver. Am J Surg Pathol. 22: 656-662, 1998.

19. Rahner, C., Mitic, L. L., and Anderson, J. M. Heterogeneity in expression and subcellular localization of claudins 2, 3, 4, and 5 in the rat liver, pancreas, and gut. Gastroenterology. 120: 411-422, 2001.

20. Hashimoto, M., and Watanabe, G. Hepatic parenchymal cell volume and the indocyanine green tolerance test. J Surg Res. 92: 222-227, 2000.

21. Stamatovic, S. M., Shakui, P., Keep, R. F., Moore, B. B., Kunkel, S. L., Van Rooijen, N., and Andjelkovic, A. V. Monocyte chemoattractant protein-1 regulation of blood-brain barrier permeability. J Cereb Blood Flow Metab. 25: 593-606, 2005.

22. Bottaro, D. P., and Liotta, L. A. Cancer: Out of air is not out of action. Nature. 423: 593-595, 2003.

23. Stamatovic, S. M., Keep, R. F., Kunkel, S. L., and Andjelkovic, A. V. Potential role of MCP-1 in endothelial cell tight junction 'opening': signaling via Rho and Rho kinase. J Cell Sci. 116: 4615-4628, 2003.

24. Marra, F., DeFranco, R., Grappone, C., Milani, S., Pastacaldi, S., Pinzani, M., Romanelli, R. G., Laffi, G., and Gentilini, P. Increased expression of monocyte chemotactic protein-1 during active hepatic fibrogenesis: correlation with monocyte infiltration. Am J Pathol. 152: 423-430, 1998.

25. Czaja, M. J., Geerts, A., Xu, J., Schmiedeberg, P., and Ju, Y. Monocyte chemoattractant protein 1 (MCP-1) expression occurs in toxic rat liver injury and human liver disease. J Leukoc Biol. 55: 120-126, 1994.

26. Yoong, K. F., Afford, S. C., Jones, R., Aujla, P., Qin, S., Price, K., Hubscher, S. G., and Adams, D. H. Expression and function of CXC and CC chemokines in human malignant liver tumors: a role for human monokine induced by gamma-interferon in lymphocyte recruitment to hepatocellular carcinoma. Hepatology. 30: 100-111, 1999.

27. Hida, K., Hida, Y., Amin, D. N., Flint, A. F., Panigrahy, D., Morton, C. C., and Klagsbrun, M. Tumor-associated endothelial cells with cytogenetic abnormalities. Cancer Res. 64: 8249-8255, 2004.

28. Honda, H., Pazin, M. J., Ji, H., Wernyj, R. P., and Morin, P. J. Crucial roles of Sp1 and epigenetic modifications in the regulation of the CLDN4 promoter in ovarian cancer cells. J Biol Chem. 281: 21433-21444, 2006.

Table 1.

Influence of hepatitic activity or fibrotic grade on the CL-5 expression pattern in SECs and ICG-R15 value according to the CL-5 expression pattern

			CL-5 expression pattern in SECs				
			1	2	3	ICG-R15 (%)	
A grade	0, 1, 2	(n)	8	18	3	16.9 ± 7.7	
	3, 4	(n)	0	7	15	16.9 ± 8.5	
					*		
F grade	0, 1, 2	(n)	8	18	2	14.9 ± 6.6	#
	3, 4	(n)	0	7	16	19.4 ± 8.9	
					*		

A grade, Hepatitic activity grade; F grade, Fibrotic grade

ICG-R15, the indocyanine green retention rate 15min after administration

ICG-R15 data are shown as the mean ± standard deviation

*:$p<0.0001$, #:$p=0.0438$

Table 2.

Relationship between pathological parameters and microvessel densitiy (MVD) highlighted by CD34 or CL-5

Factors	(n)	CD34-MVD	*p* value	CL-5-MVD	*p* value
Size					
<30	(30)	81.8 ± 26.5	0.0562	58.8 ± 27.0	0.0086
≥30	(21)	68.4 ± 20.2		38.3 ± 25.3	
Differentiation					
well / mod	(14 / 17)	83.3 ± 26.5	0.0104	65.0 ± 22.7	<0.0001
por	(20)	65.4 ± 17.5		27.7 ± 18.6	
Vasculobiliary invasion					
Present	(19)	70.4 ± 22.1	0.1966	32.4 ± 21.9	0.0002
Absent	(32)	79.8 ± 26.0		61.0 ± 25.8	
Microsatellite lesions					
Present	(12)	78.0 ± 23.0	0.7858	39.8 ± 27.4	0.1365
Absent	(39)	75.7 ± 25.6		53.6 ± 27.6	

mod, moderately differentiated; por, poorly differentiated

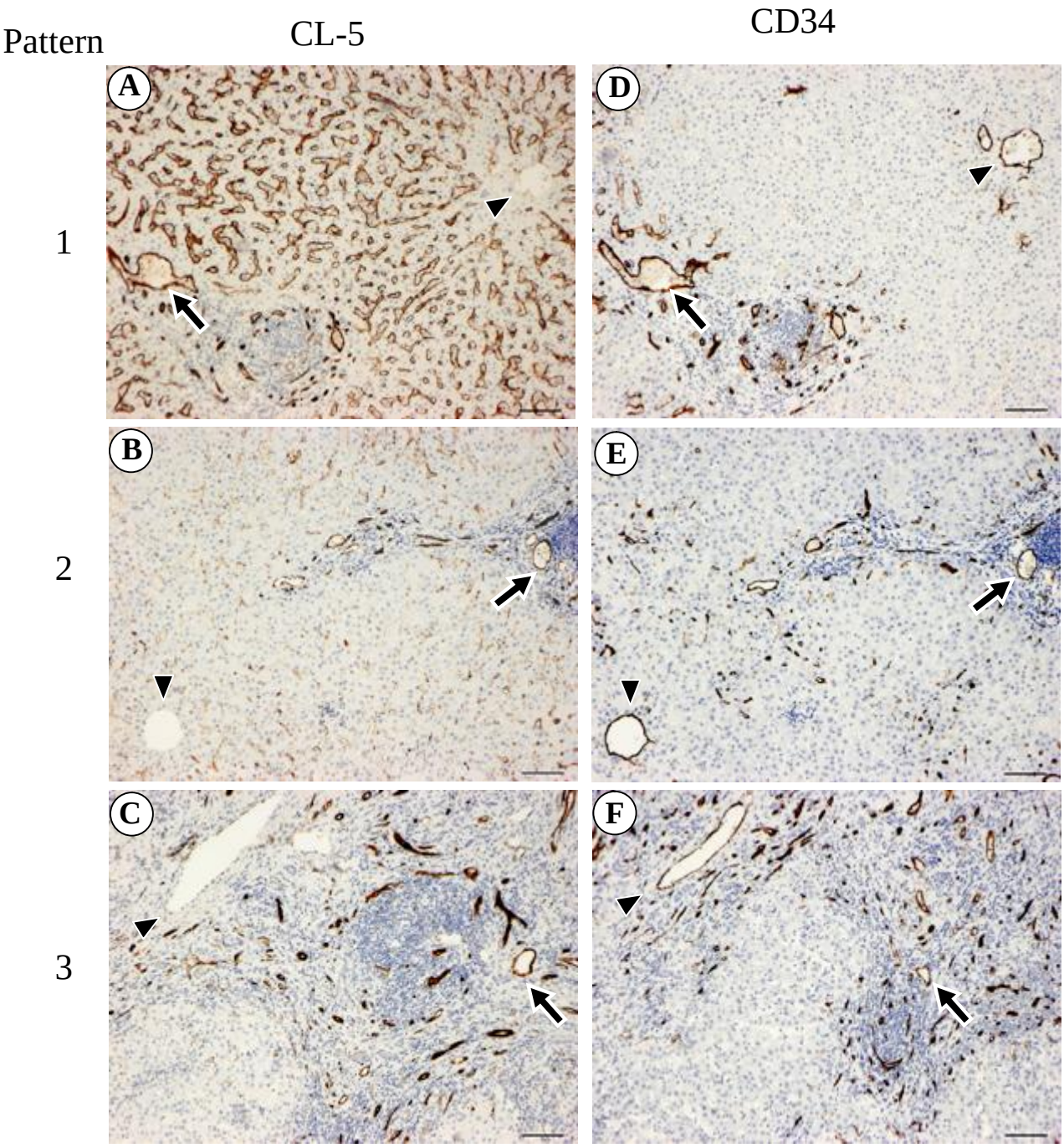
Data are shown as the mean ± standard deviation

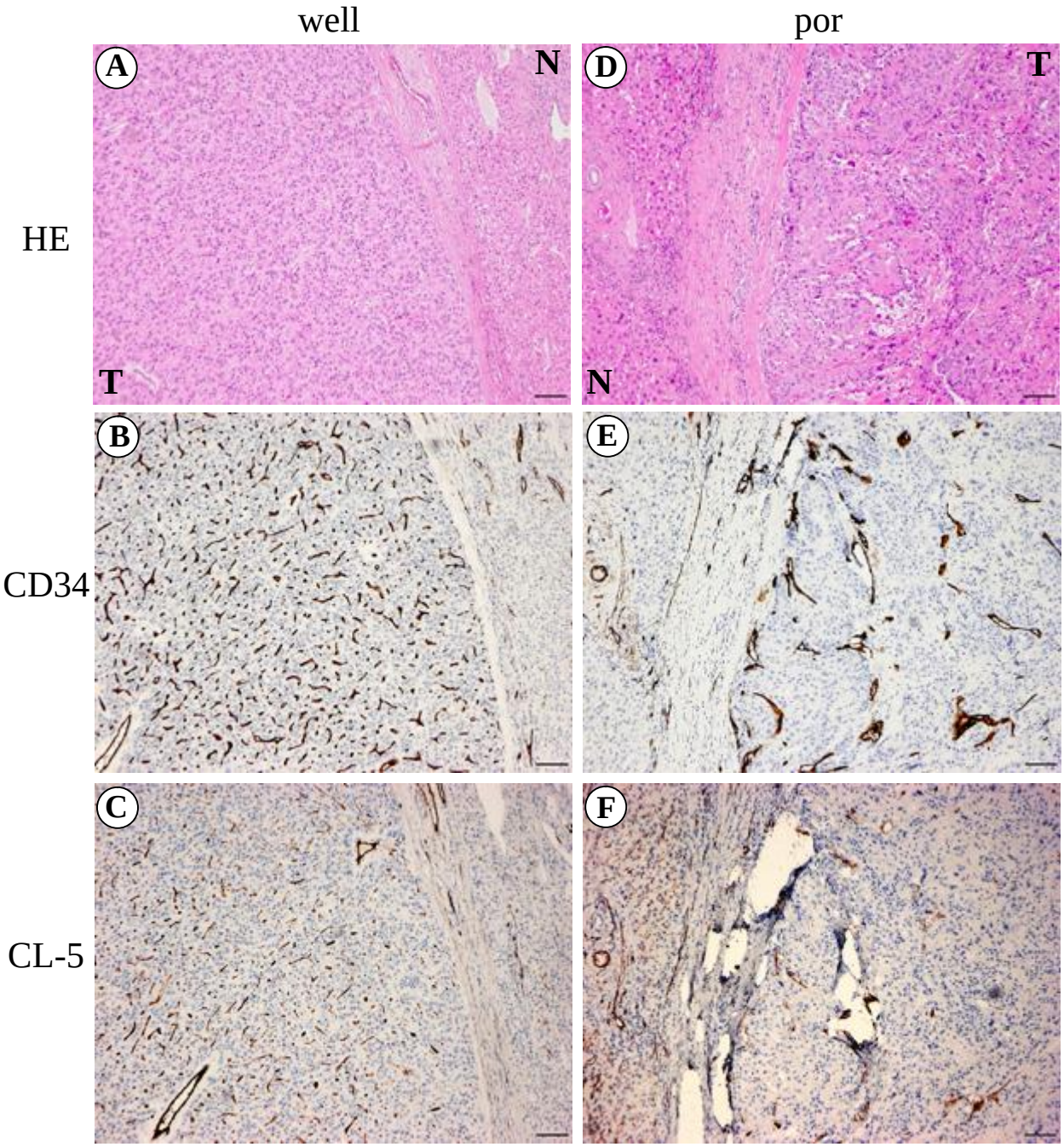
Table 3.

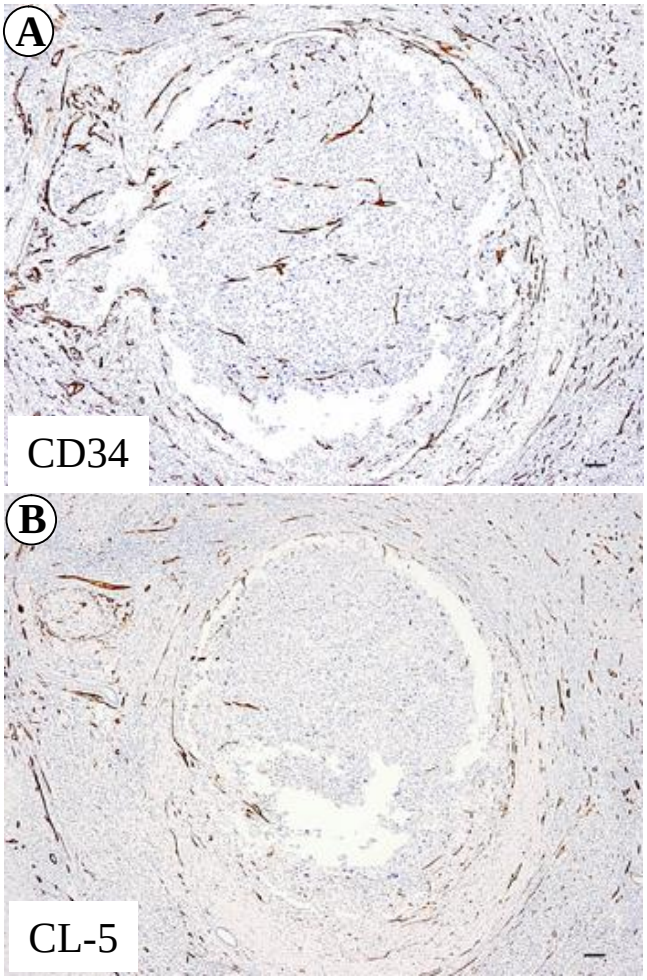
Relationship between various clinicopathological parameters and postoperative disease-free or overall survival in HCC patients

Factors	(n)	5Y-DFS (%)	*p* value	5Y-OS (%)	*p* value
Sex					
male	(42)	21.0	0.7470	58.4	0.4922
female	(9)	29.6		63.5	
Differentiation					
well / mod	(14 / 17)	28.4	0.0454	73.3	0.0769
por	(20)	14.3		33.3	
Tumor number					
Solitary	(30)	32.6	0.0121	61.9	0.2490
Multiple	(21)	9.5		55.7	
Size					
< 30	(30)	27.7	0.1820	71.6	0.0848
≥30	(21)	17.1		39.5	
Vasculobiliary invasion					
Present	(19)	11.6	0.0009	18.3	<0.0001
Absent	(32)	29.2		79.4	
Microsatellite lesion					
Present	(12)	9.4	0.0636	27.9	0.0388
Absent	(39)	26.0		67.1	
CD34-MVD					
<80	(25)	26.0	0.2260	43.1	0.0569
≥80	(26)	20.3		73.9	
CL-5-MVD					
<47	(25)	17.0	0.1005	43.5	0.0368
≥47	(26)	29.0		74.2	

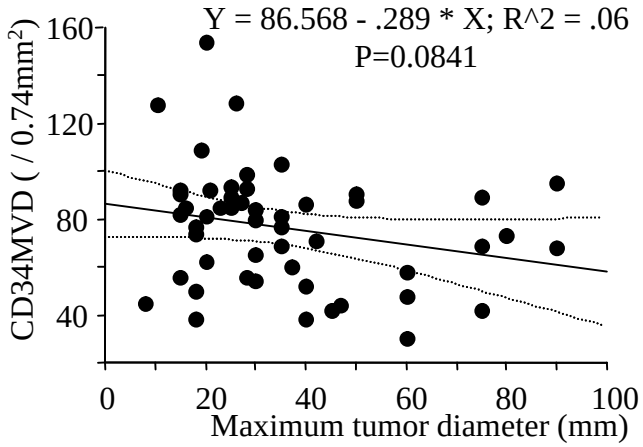
DFS, disease-free survival; OS, overall survival; mod, moderately differentiated; por, poorly differentiated**;** MVD, microvessel density


Pattern
CL-5
CD34
1
2
3
A
B
C
D
E
F

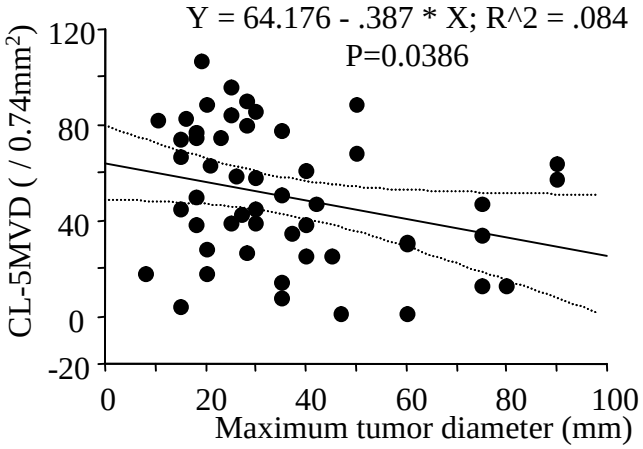

well
por
HE
CD34
CL-5
A
N
T
D
T
N
B
E
C
F


A
CD34
B
CL-5

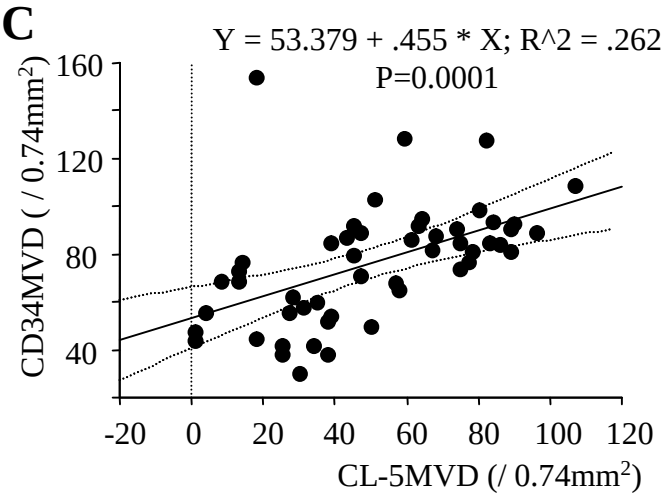
A

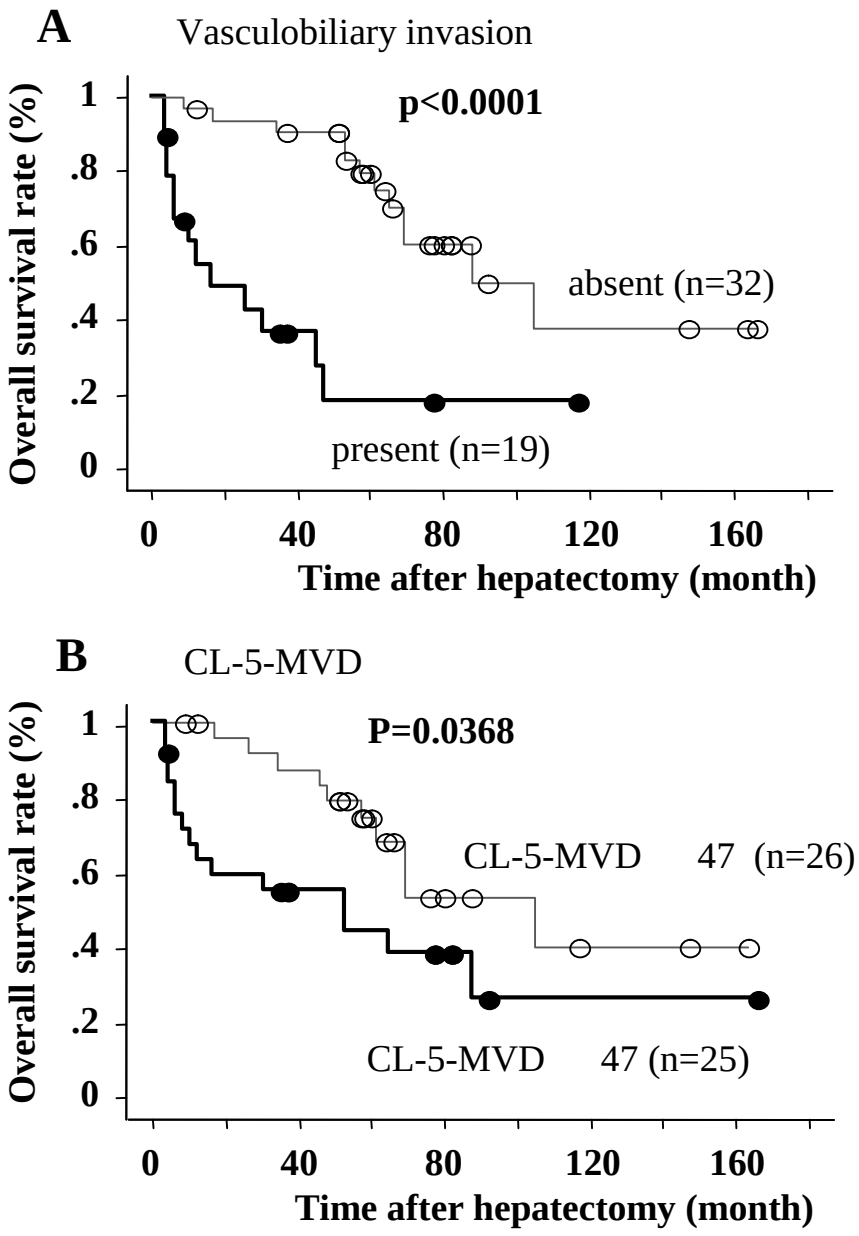

Y = 86.568 - .289 * X; R^2 = .06
P=0.0841
CD34MVD (/ 0.74mm2)
160
120
80
40
0
20
40
60
80
100
Maximum tumor diameter (mm)

B


Y = 64.176 - .387 * X; R^2 = .084
P=0.0386
CL-5MVD (/ 0.74mm2)
120
80
40
0
-20
0
20
40
60
80
100
Maximum tumor diameter (mm)

C


Y = 53.379 + .455 * X; R^2 = .262
P=0.0001
CD34MVD (/ 0.74mm2)
160
120
80
40
-20
0
20
40
60
80
100
120
CL-5MVD (/ 0.74mm2)


A
Vasculobiliary invasion
p<0.0001
absent (n=32)
present (n=19)
Overall survival rate (%)
1
.8
.6
.4
.2
0
0
40
80
120
160
Time after hepatectomy (month)
B
CL-5-MVD
P=0.0368
CL-5-MVD 47 (n=26)
CL-5-MVD 47 (n=25)
Overall survival rate (%)
1
.8
.6
.4
.2
0
0
40
80
120
160
Time after hepatectomy (month)