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Urokinase Receptor and Vascular Endothelial Growth Factor Are Synergistically Associated with the Liver Metastasis of Colorectal Cancer

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Considering recent findings that the urokinase plasminogen activation (PA) system is involved in invasion and vascular endothelial growth factor (VEGF) is involved in angiogenesis of colorectal cancer, we evaluated these factors in the liver metastasis of primary colorectal cancer. Cancer tissues from 71 colorectal cancer patients were assayed quantitatively for antigen levels of urokinase type plasminogen activator (uPA), uPA receptor (uPAR), and plasminogen activator inhibitor-1 and -2 (PAI-1, PAI-2), and were also assayed immunohistochemically for expression of VEGF protein. Among the PA system factors, both the levels of uPAR and PAI-1 were significantly higher in larger tumors than in smaller ones, and were also significantly higher in tumors that invaded subserosa, serosa or adjacent organs than in mucosal, submucosal tumors or in tumors that invaded the muscle layer. The uPAR levels were significantly higher in tumors with liver metastasis than in those without. VEGF overexpression was significantly more frequent in tumors with lymph node involvement or liver metastasis than in those without. Among the PA system factors, the uPAR levels were significantly higher in tumors with VEGF overexpression and a multivariate analysis revealed that high uPA level and VEGF overexpression were independent risk factors for liver metastasis. The combination of high uPAR level and overexpression of VEGF was associated with the worst prognosis in patients with colorectal cancer. These results suggest that uPAR and VEGF might contribute synergistically to the liver metastasis of colorectal cancer.

Key words: Urokinase-type plasminogen activator (uPA) — uPA receptor (uPAR) — Plasminogen activator inhibitor type 1 (PAI-1) — Vascular endothelial growth factor (VEGF) — Microvessel density (MVD)

Invasion is essential for tumor progression. Among various factors involved in tumor invasion, the plasminogen activation (PA) system plays an important role, as do other proteolytic enzymes such as matrix-metalloproteinases (MMPs).[1–3] Similarly, angiogenesis is also crucial for tumor growth and metastasis.[4,5] Vascular endothelial growth factor (VEGF) is a specific mitogen for vascular endothelial cells and plays an important role in tumor angiogenesis.[6,7] Both the PA system and VEGF have been shown to contribute to metastasis.[8,9]

Plasminogen is a zymogen activated by plasminogen activators (PAs) to active plasmin, which then degrades matrix protein directly or indirectly through the activation of MMPs. Among the PAs, urokinase-type PA (uPA) is reported to play an important role in tumor progression and invasion via the uPA receptor (uPAR) expressed on the tumor cell surface.[9,10] uPAR is a glycosylphosphatidylinositol-anchored cell surface protein that specifically binds both single-chain and double-chain uPA and strongly enhances the activation of surface-bound plasminogen into plasmin.[11] The relevance of uPA in tumor progression has been demonstrated by the poor prognosis of patients with a high content of uPA in tumor tissue.[11–15] uPA activity in tumor tissue is also regulated by two physiologic PA inhibitors, type 1 (PAI-1) and type 2 (PAI-2), both of which belong to the serine protease inhibitor superfamily.[16] Higher levels of PAI-1 have been found in larger tumors and in tumors of patients with a poorer prognosis.[17] A possible promoting function of PAI-1 in tumor growth is suggested by its potential to modify cell adhesion capacity,[18,19] which is independent of uPA-inhibitory activity.

Angiogenesis is essential for the growth of a solid tumor.[5] The overexpression of VEGF has been reported in various tumors.[20–24] VEGF protein binds to specific receptors, VEGF receptor-1 and VEGF receptor-2, expressed on endothelial cells that induce endothelial cell migration, proliferation and tubule formation,[25–27] thereby enhancing tumor neovascularization. Aside from the induction of tumor angiogenesis, VEGF has several additional functions that serve to enhance tumor progression, including enhancing the permeability of tumor vessels,[28] and inhibiting either apoptosis of endothelial cells[29,30] or the matura-

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tion of dendritic cells.[31] Both an association between the PA system and angiogenesis,[32, 33] and an association of the PA system and VEGF have been reported.[33, 34]

Liver metastasis is the most crucial problem affecting the prognosis of the patient with colon cancer.[35] In the present study, we investigated the correlation among the PA system, VEGF and several clinicopathologic factors, with emphasis on the relationship between raised uPAR level and overexpression of VEGF, and their association with liver metastasis.

MATERIALS AND METHODS

Patients and assay for PA system antigen levels Tumors and adjacent normal specimens were obtained from 71 patients who had undergone surgery for colorectal cancer at our institute from January 1994 through April 1996. All specimens were examined for clinicopathologic factors including histologic diagnosis, degree of differentiation, and lymph node involvement. The specimens were rapidly frozen and stored at −80°C until homogenized. The tissue samples were homogenized with a 10-fold volume of buffer (0.1 *M* Tris-HCl, pH 7.5, containing 0.15 *M* NaCl and 1% Triton X-100) using a Physcotron microhomogenizer (NITI-ON, Tokyo). After incubation at 4°C for 12 h, the samples were centrifuged at 4°C at 12 000*g* for 10 min. The supernatants were stored at −80°C until assayed.

The antigen levels of uPAR, uPA, PAI-1, and PAI-2 in the supernatant of the homogenized tissue samples were assayed with commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the methods recommended by the manufacturers (uPAR, PAI-2: American Diagnostica, Greenwich, CT; uPA, PAI-1: Biopool, Ume, Sweden). The antigen levels are expressed as ng/mg protein of the tissue extracts. Protein concentrations were determined using the BCA Protein Assay Reagent kit (Richmond, CA).

Immunohistochemical staining for VEGF Immunohistochemical staining for VEGF in formalin-fixed, paraffin-embedded tissue sections was performed using the streptavidin-biotin method. The sections were mounted on silanized slides, deparaffinized, and rehydrated through graded alcohol to water. The sections were immersed in pepsin solution for 30 min at 37°C. Endogenous peroxidase activity was blocked by incubation with 0.6% H_2O_2. The sections were then treated with 10% normal rabbit serum for 10 min to block nonspecific protein binding. Anti-human VEGF mouse monoclonal antibody (IBL Co., Ltd., Gunma) at a 1:100 dilution was added to the tissue sections and incubated for 60 min at room temperature. After a brief rinse, the sections were treated with biotinylated antimouse IgG for 10 min at room temperature. After washing, the sections were incubated with diaminobenzidine and H_2O_2 for 15 min, washed, lightly counterstained with hematoxylin, dehydrated in graded alcohols, cleared in xylene, and mounted. Negative controls were similarly processed using normal IgG as the primary antibody. When there were unequivocally positively stained cancer cells, this was defined as positive expression. The VEGF protein was detected homogenously in the cytoplasm of the tumor cells (Fig. 1)

Microvessel density (MVD) MVD was determined by immunohistochemical staining with an antihuman Factor VIII-related antigen polyclonal antibody (A082, Dako Japan, Tokyo), as described elsewhere. The sections were immersed in pepsin solution for 20 min at 37°C. Endogenous peroxidase activity was blocked by incubation with 0.3% H_2O_2 in methanol and nonspecific protein binding was blocked by treatment with 10% normal goat serum for 10 min. Positive staining was observed in vascular endo-

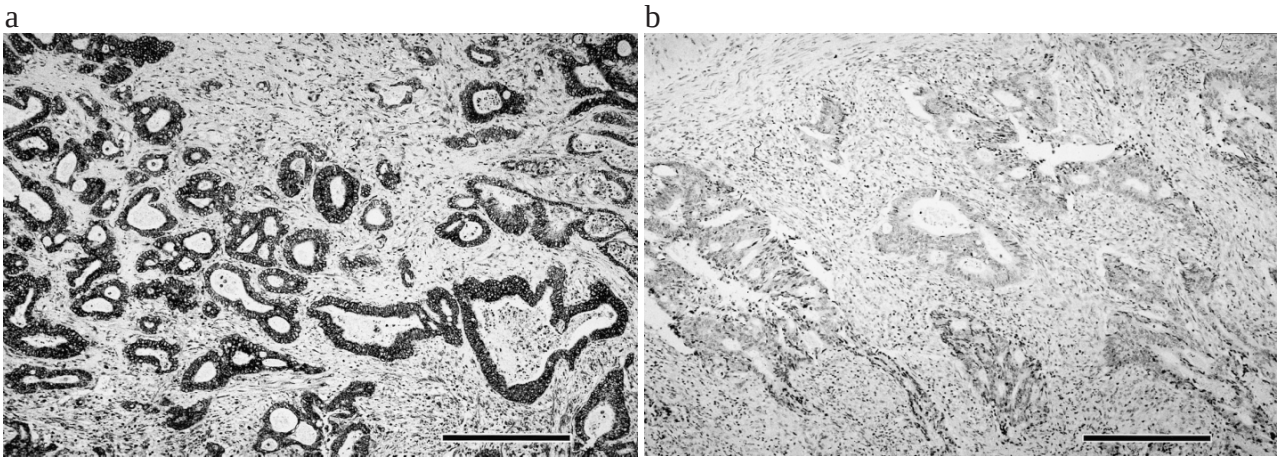

Fig. 1. Immunohistochemical staining with anti-VEGF antibody in colorectal cancers. a: Positive staining; staining is seen diffusely within the cytoplasm of the tumor cells. b: Negative staining. Bars: 250 μm.

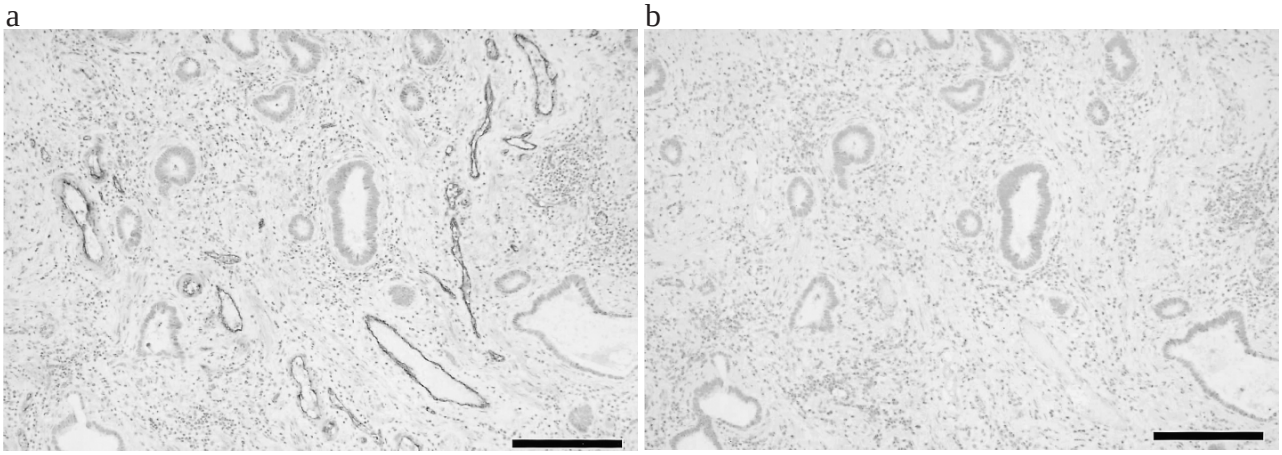

Fig. 2. Immunohistochemical staining with anti-Factor VIII associated antigen antibody in colorectal cancers. a: Positive staining; staining is seen in the vascular endothelial cells. b: Negative staining (control). Bars: 250 μm.

Table I. Correlation between Antigen Levels and Clinicopathological Variables

Variable	*n*	uPAR	uPA	PAI-1	PAI-2
Tumor size					
≤50 mm	43	2.39±1.02	7.81±4.74	2.96±3.00	0.73±1.22
>50 mm	28	3.32±1.54[a)]	8.65±4.04	6.93±8.13[a)]	1.19±2.28
Depth of infiltration					
≤m.p.[c)]	21	2.17±0.97	7.97±5.75	2.21±2.72	1.06±2.38
>m.p.	50	3.00±1.39[b)]	8.22±3.87	5.50±6.58[b)]	0.93±1.58
Tumor differentiation					
Differentiated	68	2.77±1.33	8.29±4.44	3.95±3.97	0.94±1.75
Others	3	2.42±1.36	4.89±4.69	17.66±20.45[a)]	0.27±0.25
Lymph node involvement					
Negative	41	2.51±1.10	8.26±4.94	4.88±6.96	0.62±0.92
Positive	30	3.09±1.54	7.97±3.80	4.05±4.10	1.32±2.38
Lymphatic involvement					
Negative	41	2.75±1.25	8.67±4.82	4.92±6.73	1.01±1.96
Positive	27	2.78±1.45	7.56±4.00	3.84±4.26	0.86±1.40
Vascular involvement					
Negative	44	2.70±1.35	7.97±5.05	5.01±6.86	1.07±1.97
Positive	24	2.88±1.30	8.72±3.37	3.54±3.26	0.74±1.23
Liver metastasis					
Negative	63	2.60±1.23	8.29±4.54	4.01±4.20	0.87±1.70
Positive	8	3.99±1.51[a)]	6.97±3.86	8.06±13.22	1.23±1.93
MVD					
≤35/field	17	2.88±1.66	9.02±6.04	3.20±3.68	0.91±1.59
>35/field	50	2.74±1.25	8.06±3.92	4.95±6.60	0.89±1.78

All values are expressed as ng/mg protein.
a) Significant difference between two groups ($P<0.005$).
b) Significant difference between two groups ($P<0.05$).
c) m.p.: muscularis propria.

thelial cells (Fig. 2). MVD was evaluated by counting the number of endothelial deposits/field by light microscopy at 500 magnification without knowledge of patients' details. The mean of four counts for each specimen was calculated and statistically analyzed.

Statistical analysis Statistical analysis was performed using a statistical software package (Statview 4.5, Abacus Concepts, Berkeley, CA). The data were analyzed by using Student's *t* test, the χ^2 test, or the Mann-Whitney *U* test. Survival was calculated from the date of surgery to the

Table II. Correlation between VEGF Expression and Clinicopathological Factors

Variable	VEGF (–) n=41	VEGF (+) n=30	P value
Tumor size (diameter)			
≤50 mm	24	19	
>50 mm	17	11	NS
Depth of infiltration			
≤m.p.	14	7	
>m.p.	27	23	NS
Tumor differentiation			
Differentiated	40	28	
Others	1	2	NS
Lymph node involvement			
Negative	29	12	
Positive	12	18	0.0096
Lymphatic involvement			
Negative	26	15	
Positive	13	14	NS
Vascular involvement			
Negative	26	18	
Positive	13	11	NS
Liver metastasis			
Negative	41	22	
Positive	0	8	0.0004
MVD			
≤35/field	11	6	
>35/field	28	22	NS

date of death or of the last follow-up. The survival rate was estimated by the Kaplan-Meier method and analyzed by means of the log-rank test. To define independent risk factors for liver metastasis, multivariate analysis was performed with a logistic regression model. Differences were considered significant when P values were less than 0.05.

RESULTS

Relationship between PA system antigen levels, VEGF overexpression and clinicopathological parameters Antigen levels of both uPAR and PAI-1 were significantly higher in large (>50 mm in diameter) tumors than in small (≤50 mm) tumors and were similarly significantly higher in tumors that invaded the subserosa, serosa or adjacent organs than in either mucosal or submucosal tumors or in tumors that invaded the muscle layer. The PAI-1 antigen levels were significantly lower in differentiated carcinoma. The uPAR antigen levels were significantly higher in tumors with liver metastasis than in those without. MVD was not affected by any factor in the PA system, and the clinicopathological variables did not affect the antigen levels of uPA or PAI-2 (Table I).

Table III. Correlation between VEGF Expression and Levels of PA System Factors

Variable (ng/mg protein)	VEGF (–) n=41	VEGF (+) n=30	P value
uPAR	2.44±1.13	3.19±1.47	0.0176
uPA	8.73±4.43	7.34±4.46	NS
PAI-1	4.12±3.79	5.01±7.97	NS
PAI-2	0.82±1.27	1.04±2.21	NS

Table IV. Correlation of Variables to Liver Metastasis Determined by Univariate Analysis

Variable	Metastasis (–) n=63	Metastasis (+) n=8	P value
Tumor size (mm)			
≤50 mm	42	1	
>50 mm	21	7	0.0027
Depth of infiltration			
≤m.p.	21	0	
>m.p.	42	8	NS
Tumor differentiation			
Differentiated	61	7	
Others	2	1	NS
Lymph node involvement			
Negative	38	3	
Positive	25	5	NS
Lymphatic involvement			
Negative	36	5	
Positive	24	3	NS
Vascular involvement			
Negative	40	4	
Positive	20	4	NS
CEA			
≤5 ng/mg protein	35	2	
>5 ng/mg protein	28	6	NS
VEGF			
Negative	41	0	
Positive	22	8	0.0004
uPAR			
≤2.8 ng/mg protein	40	0	
>2.8 ng/mg protein	23	8	0.0006
uPA			
≤10 ng/mg protein	43	5	
>10 ng/mg protein	20	3	NS
PAI-1			
≤5 ng/mg protein	44	5	
>5 ng/mg protein	19	3	NS
PAI-2			
≤1.26 ng/mg protein	51	6	
>1.26 ng/mg protein	12	2	NS
MVD			
≤35/field	17	0	
>35/field	42	8	NS

Table V. Risk Factors Affecting Liver Metastasis Determined by Multivariate Analysis Using Logistic Regression

Variable	Coefficient (95%CI)	*P* value
VEGF	0.222 (0.085–0.360)	<0.001
uPAR	0.155 (0.090–0.301)	<0.001

VEGF overexpression was observed in 30 of 71 patients (42.3%). VEGF overexpression was significantly more frequent in tumors with lymph node involvement or liver metastasis than those without (P=0.0096, P=0.0004) (Table II). There was no correlation between VEGF overexpression and MVD, although MVD tended to be higher in tumors with VEGF overexpression than in those without (data not shown).

Correlation between VEGF expression and the PA system As shown in Table III, only uPAR levels among the factors in the PA system were significantly higher in tumors with VEGF overexpression (P=0.0176).

Factors in the PA system, VEGF and clinicopathological parameters in relation to liver metastasis Liver metastases were detected in 8 of 71 patients (11.3%). In univariate analysis, three factors (tumor size, VEGF expression, uPAR antigen levels) were significantly associated with liver metastasis (Table IV). In a multivariate analysis, tumor size, depth of infiltration, tumor differentiation, lymph node involvement, lymphatic involvement, vascular involvement, carcinoembryonic antigen (CEA) levels, uPAR levels, uPA levels, PAI-1 level, VEGF expression and MVD were taken into account. Among these factors, uPAR levels and VEGF expression were identified as independent risk factors for liver metastasis (Table V).

Impact of uPAR level and/or VEGF overexpression on survival High levels of uPAR (>2.8 ng/mg protein) and VEGF overexpression were significantly associated with shorter overall survival (uPAR, P=0.0248; VEGF, P=0.0261) (Fig. 3, a and b). The overall survival of patients with high uPAR levels and VEGF overexpression was lowest among 4 subgroups {A group (n=28), the low uPAR and negative VEGF group; B group (n=12), the low uPAR and positive VEGF group; C group (n=13), the high uPAR and negative VEGF group; D group (n=18), the high uPAR and positive VEGF group) (Fig. 3c). At 3 years, the overall survival rates were 95.5%, 91.7%, 92.3% and 72.2%, respectively.

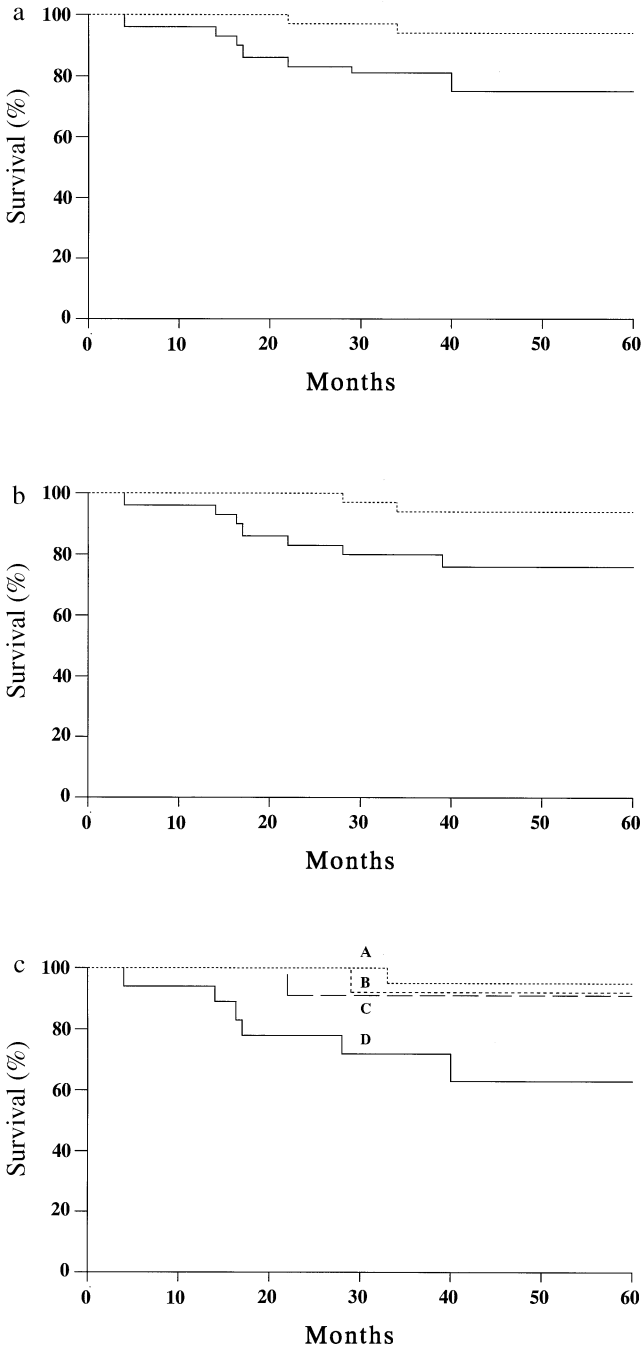

Fig. 3. Overall survival after surgery shown for all 71 patients (a) according to uPAR antigen levels. ····· low uPAR group (≤2.8 ng/mg protein, n=40), —— high uPAR group (>2.8 ng/mg protein, n=31). (b) According to VEGF expression. ····· VEGF negative group (n=41), —— VEGF positive group (n=30). (c) According to both uPAR levels and VEGF expression. A, the low uPAR and VEGF negative group (n=28); B, the low uPAR and VEGF positive group (n=12); C, the high uPAR and VEGF negative group (n=13); D, the high uPAR and VEGF positive group (n=18).

DISCUSSION

Both the PA system and VEGF play an important role in the progression of colorectal cancer. Considering that uPAR and PAI-1 levels have been shown to be directly related to tumor growth, and that uPAR levels have also

been shown to be the most substantial determinant of the overall survival rate in patients with colorectal cancer,[36] we focused on the role of the PA system and VEGF in the liver metastasis of colorectal cancer.

The levels of both uPAR and PAI-1 correlated with tumor size and depth of infiltration, and the uPAR levels also significantly correlated with liver metastasis. The PAI-1 antigen levels tended to be higher in the tumors with liver metastasis than in those without, although the difference was not significant (P=0.0712). Although high levels of uPA have shown a close relationship with poor outcome in various tumors including breast cancer[13] and colorectal cancer,[14] no clinicopathological factor correlated with the uPA level in this study. Other studies on tumor uPA levels and clinical prognosis[3] have also reported similar findings. Since uPA activity is fully expressed after binding to uPAR on the tumor cell surface, antigen levels of uPAR might be more crucial than those of uPA. PAI-1 has also been shown to promote tumor growth and invasion by potentiating tumor cell detachment from the matrix.[18, 19] The direct association of uPAR with integrins has been demonstrated[37] and PAI-1 has been shown to dissociate the binding between matrix vitronectin and either integrin $\alpha V\beta 3$[19] or uPAR.[18] Such a modification of cell adhesion to the matrix by uPAR and PAI-1 may be intimately involved in tumor growth, invasion and metastasis.

VEGF is a potent angiogenic factor and promotes tumor angiogenesis. In the present study, we evaluated the role of VEGF immunohistochemically, because a positive correlation between immunohistochemical overexpression of VEGF and hematogenous metastasis in early gastric cancer was demonstrated in our previous study.[38] VEGF overexpression was significantly associated with liver metastasis and was the most significant among the clinicopathological factors by univariate analysis. Several previous studies have indicated that VEGF overexpression in a primary tumor is significantly associated with hematogenous metastasis[39, 40] and is correlated with relapse[41] or poor outcome in various tumors including colorectal cancer.[20–24] It has also been reported that VEGF mRNA expression is high in cancer tissue with vascular involvement, and the VEGF protein has been strongly detected in cancer cells invading blood vessels.[42] Taken together, these results suggest that VEGF increases the chance of hematogenous metastasis.

In this study, the uPAR level was significantly higher in tumors with VEGF overexpression. VEGF has been shown to increase uPA and uPAR expression on endothelial cells,[33, 34] and degradation of the extracellular matrix by the PA system or MMP is essential for angiogenesis, followed by endothelial cell migration induced by various factors, including receptor-bound uPA.[32, 33] Recently, it has been reported that uPA stimulated human vascular smooth cell migration.[43] These results suggest that both uPAR and VEGF might synergistically induce tumor angiogenesis. High uPAR levels and VEGF overexpression may indicate abundant metastatic potential in the tumors. This was supported by our multivariate analysis, where both uPAR and VEGF were independent risk factors for liver metastasis. Although Nakata *et al.* reported a positive correlation between uPAR mRNA expression and VEGF mRNA expression in colorectal cancer,[42] this is the first report to demonstrate that both uPAR and VEGF are independent risk factors for liver metastasis. The synergistic effect of uPAR and VEGF on the progression of liver metastasis may account for the worst survival rate in the group with high uPAR levels and VEGF overexpression among four groups. Although the precise roles of the PA system and VEGF in colorectal cancer are still unclear, the present observations may contribute to the management of patients with colorectal cancer.

Although high uPAR and VEGF overexpression might enhance angiogenesis in the tumor, a positive relationship between VEGF overexpression and MVD was not observed. In some studies, a positive correlation between MVD and VEGF expression has been reported using an immunohistochemical analysis with antibody to Factor VIII associated antigen, CD31 or CD34.[40, 44] However, conflicting results in breast or ovarian cancer regarding the correlation between MVD, and VEGF expression or clinicopathologic factors in tumor tissue have been reported.[45, 46] Particularly since specific antigens localized in the tumor vessels have not been identified, MVD measured by antibody to Factor VIII associated antigen in the present study did not necessarily reflect all tumor vasculature. If immunohistochemistry targeted to tumor vasculature-specific antigens is possible, the correlation between MVD and VEGF expression may could be examined precisely. It is also possible that microvessel formation in colon cancer may be regulated by other angiogenic factors.

In the present study, VEGF overexpression was more frequent in tumors with lymph node involvement. Both VEGF and VEGF-C have been shown to play important roles in angiogenesis and lymphangiogenesis in human malignant mesotheliomas.[47] Although it is not clear whether lymphatic involvement is associated with lymphangiogenetic activity, VEGF might function to promote not only hematogenous metastasis, but also lymphatic metastasis.

In conclusion, high antigen levels of uPAR and the overexpression of VEGF may synergistically contribute to the liver metastasis of colorectal cancer, resulting in a very poor outcome for patients with both factors.

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