Original paper

Inverse correlation of phospho-KDR/Flk-1 expression and stage of colorectal cancer: implication of the significance of neoangiogenesis in activated VEGFR-2 expressing early stage colorectal adenocarcinomas

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The activation of vascular endothelial cell growth factor receptors (VEGFRs) plays an essential role in cancer progression. In this study, we investigated the expression of phosphorylated VEGFR-2 (or phospho-KDR/Flk-1), the activated form of VEGFR-2, in human colorectal adenomas and colorectal adenocarcinomas. Phospho-KDR/Flk-1 showed weak expression in the normal colorectal tissue. Phospho-KDR/Flk-1 was mainly stained in the cytoplasm of colorectal adenomas, and was stained in both the cytoplasm and nuclei colorectal adenocarcinomas. There was no indication of increased phospho-KDR/Flk-1 expression in the colorectal adenocarcinomas, as compared to that of colorectal adenomas. Furthermore, there was an inverse relationship of phospho-KDR/Flk-1 expression with cancer stage ($p < 0.0001$), lymph node metastasis ($p = 0.011$), and distant metastasis ($p = = 0.021$) of the colorectal adenocarcinomas. Our results indicate that early stage colorectal adenocarcinomas with highly activated (phosphorylated) VEGFR-2 expression may indicate the significance of neoangiogenesis of the tumors.

Key words: cancer stage, colorectal adenocarcinoma, metastasis, phospho-KDR/Flk-1, VEGFR-2.

Introduction

The growth of solid cancers requires adequate blood supply through the blood vessels. Blood vessels also provide the path for the metastatic spread of solid cancers. Vascular endothelial growth factors (VEGFs) are cytokines involved in angiogenesis and lymphangiogenesis [1]. The VEGF receptor family contains 3 members, VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1) and VEGFR-3 (Flt-4), which are transmembrane tyrosine kinase receptors expressed on the surface of endothelial cells and are involved in the regulation and formation of blood and lymphatic vessels [2, 3]. Among the VEGF receptors, VEGFR-2 is recognized to play a principal role in mediating VEGF-induced vessel formation [4]. Vascular endothelial growth factor receptor-2 is a marker for endothelial cell development and it directly regulates tumor angiogenesis, blocking

VEGF and KDR/Flk-1 interaction by monoclonal neutralizing antibody targeting KDR/Flk-1 diminished tumor vascularity and inhibited tumor angiogenesis [5]. A VEGF stimulation result in tyrosine phosphorylation and activation of VEGFR-2 and inhibition of VEGFR-2 phosphorylation significantly reduces angiogenesis in tumor xenografts *in vivo* [6, 7]. Phosphorylated VEGFR-2 has been reported to be expressed in a wide variety of normal tissues including the liver, placenta, and colon, and is not restricted to endothelia [8]. Vascular endothelial growth factors and their receptors were reported to be expressed in various tumor cells [9-14]. In addition to acting on endothelial cells to modulate angiogenesis and lymphangiogenesis, the VEGF signaling is also directly activated in tumor cells to regulate cancer progression [4]. In the study of VEGFR expression in ovarian cancer, Klasa-Mazurkiewicz *et al.* reported that the highest relative level of VEGFR-2 was detected in the early stage cancer, indicating the significance of neoangiogenesis in high VEGFR-2 expressed early stage ovarian cancer [15].

Colorectal cancer is one of the most common causes of cancer-related deaths of humans worldwide. Colorectal carcinomas spread easily to nearby tissues and display strong potential for invasion and metastasis. Metastases including lymph node metastasis and distant metastasis are crucial prognostic indicators for determining disease progression and crucial for therapeutic strategies in the work-up of colorectal cancer [16, 17]. Saad *et al*. reported a positively significant correlation of VEGF expression with the presence of lymph node metastases in colorectal cancer [17]. Phosphorylated VEGFR-2 receptors were reported to be expressed in colon cancer cells and were significantly associated with tumor diameter and poor histological differentiation [18]. We studied the activation status of VEGFR-2 in colorectal adenomas and colorectal adenocarcinomas, as well as the correlation with the metastatic status of the disease. This study suggests that there is an inverse correlation between phospho-KDR/Flk-1 expression and the metastasis of colorectal cancer. Phospho-KDR/Flk-1 expression in colorectal cancer cells may be involved in the metastasis of colorectal cancer cells, and the activity of phospho-KDR/Flk-1 is delicately regulated in metastasized colorectal adenocarcinomas. Those early stage colorectal adenocarcinomas with highly activated (phosphorylated) VEGFR-2 expression may indicate the significance of neoangiogenesis of the tumors. Our findings may be helpful for developing new therapeutic strategies for treating colorectal cancer.

Material and methods

Patients

The study was approved by the Ethics Committees of Changhua Christian Hospital (Changhua, Taiwan) and adhered to the guidelines approved by the Institutional Review Board. Colorectal tumor samples were obtained from 20 cases of colorectal adenoma and 52 cases of colorectal adenocarcinomas. The tumors were graded and categorized according to the Staging Manual (7th ed.) of the American Joint Committee on Cancer [19]. In lymph node metastasis analysis, at least 12 lymph nodes were examined in each patient. Baseline characteristics of the patients are shown in Table I. The patient group comprised 26 men and 26 women, with a mean age of 61 years (range 25 to 82 years). Eight patients had stage I tumors, 17 patients had stage II tumors, 15 patients had stage III tumors, and 12 patients had stage IV tumors. The sites of the tumors were: rectum (3), splenic flexure (1), sigmoid colon (24), hepatic flexure (5), cecum (4), transverse colon (7), appendix (1), ascending colon (4), and descending colon (5). No patients with rectal tumors ever received preoperative radiotherapy. Patients who received neoadjuvant chemoradiotherapy are excluded from the study. The study also included 3 low-grade tubular adenomas, 2 high-grade tubular adenomas, 9 low-grade tubulovillous adenomas and 6 high-grade tubulovillous adenomas. The adjacent non-tumor parts of colonic mucosa were used as healthy samples.

Immunohistochemistry

Immunohistochemistry was performed on 6-μm sections of paraffin-embedded tissue specimens as previously described [20]. The tissue sections were deparaffinized in xylene and rehydrated in graded alcohol. Antigen retrieval was performed by treatment with a boiling citrate buffer (10 mmol/l, pH 6.0) for 20 min. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide in water, and nonspecific staining was blocked by incubation with 5% bovine serum albumin in PBS for 1 h at room temperature. The samples were incubated with a 100-fold dilution of anti-phospho-KDR/Flk-1-Y996 antibody (clone RB2667, Epitomics, Burlingame, CA, USA) for 60 min at room temperature and were then washed 3 times with PBS; thereafter, the slides were incubated with a horseradish peroxidase/Fab polymer conjugate for another 30 min. The sites of peroxidase activity were visualized using diaminobenzidine (3,3'-diaminobenzidine tetrahydrochloride) as the substrate and were counterstained using Mayer's hematoxylin. In the negative control, the primary antibody was omitted and replaced by PBS.

Immunohistochemical scoring system

The results of phospho-KDR/Flk-1 immunohistochemical staining were analyzed using a semiquantitative scoring system as previously described [21]. The system combined the percentage of immunoreac-

Table I. Phospho-KDR/Flk-1 expression and clinical parameters in colorectal adenocarcinoma

	Phospho-KDR/Flk-1 expression		Total	P
	Low (0, 1+)	High (2+, 3+)		
Gender				
female	13 (50)	13 (50)	26	0.577
male	10 (38.5)	16 (61.5)	26	
Grade				
low	0	0	0	1.000
moderate	21 (43.8)	27 (56.2)	48	
high	2 (50)	2 (50)	4	
T status				
T1 + T2	2 (25)	6 (75)	8	0.278
T3 + T4	21 (47.7)	23 (52.3)	44	
Stage				
I/II	4 (16)	21 (84)	25	< 0.0001**
III/IV	19 (70.4)	8 (29.6)	27	
Lymph node metastasis				
no	8 (27.6)	21 (72.4)	29	0.011*
yes	15 (65.2)	8 (34.8)	23	
Distant metastasis				
no	14 (35)	26 (65)	40	0.021*
yes	9 (75)	3 (25)	12	
Survival				
≤ 5 years	14 (45.2)	17 (54.8)	31	1.000
> 5 years	9 (42.9)	12 (57.1)	21	
Recurrence				
no	19 (44.2)	24 (55.8)	43	0.717
yes	3 (33.3)	6 (66.7)	9	

Data are shown as number of cases (%).
, statistically significant*

tive cells (quantity score) and an estimate of staining intensity (staining intensity score). Each tissue sample was scored according to the quantity and intensity of the staining, with the rating being confirmed by two expert pathologists. We divided the phospho-KDR/Flk-1 immunohistological staining results into low-phospho-KDR/Flk-1 (phospho-KDR/Flk-1 staining 0 and 1+) and high-phospho-KDR/Flk-1 (phospho-KDR/Flk-1 staining 2+ and 3+) subgroups.

Statistical analysis

The between-groups differences for the clinico-pathologic variables were assessed using the Fisher exact test. The prognostic ability of each variable was evaluated, including tumor grade, clinical stage, T status, and lymph node metastasis. All analyses were performed using the Statistical Package for Social Sciences (SPSS) version 15.0 (SPSS, Inc, Chicago, IL, USA). A P value less than 0.05 (for a two-tailed test) was considered statistically significant.

Results

We examined the activation status of KDR/Flk-1 in colorectal adenomas and colorectal adenocarcinomas by using the anti-phospho-KDR/Flk-1-Y996 antibody, a polyclonal antibody against activated VEGFR-2. Phospho-KDR/Flk-1 showed negative or faint staining in non-neoplastic colorectal tissues

(Fig. 1). In the colorectal adenomas, phospho-KDR/Flk-1 showed a stronger expression pattern than in the non-neoplastic colorectal tissues (Fig. 1). Phospho-KDR/Flk-1 showed moderate cytoplasmic staining (2+) in 45% (9/20) of the colorectal adenomas, and showed strong cytoplasmic staining (3+) in 55% (11/20) of the same specimens. Although phospho-KDR/Flk-1 was significantly stained in colorectal adenomas, staining of phospho-KDR/Flk-1 in the vasculatures in colorectal adenomas was not observed (data not shown). Staining of phospho-KDR/Flk-1 in the vasculatures in normal non-neoplastic colorectal tissues was also not observed (data not shown). There was no significant difference in phospho-KDR/Flk-1

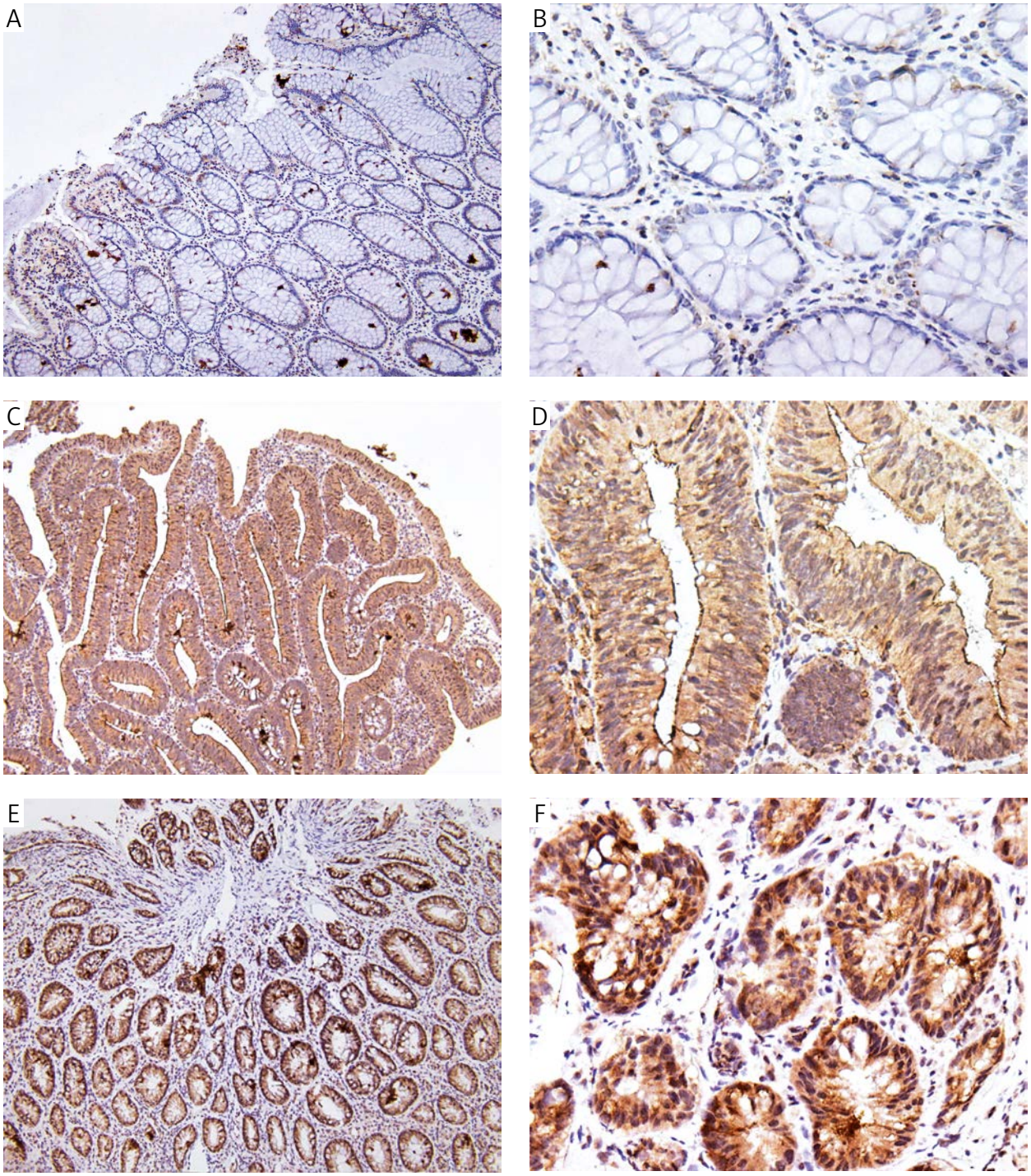

Fig. 1. Representative immunohistochemical images of phospho-KDR/Flk-1 staining in non-tumorous colorectal tissues and colorectal adenomas. **A, B)** Very weak phospho-KDR/Flk-1 staining (±) in non-neoplastic colorectal tissues. **C-F)** Phospho-KDR/Flk-1 staining in colorectal adenomas classified as moderate positive (2+; **C, D**), and strong positive (3+; **E, F**). Original magnification: A, C, and E 100×; B, D, and F 400×

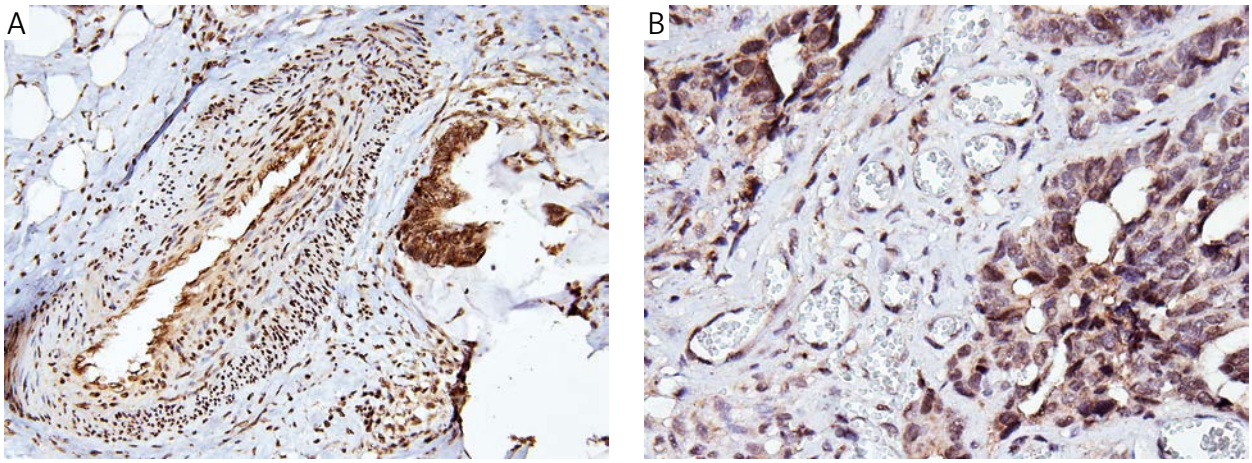

Fig. 2. Representative immunohistochemical images of phospho-KDR/Flk-1 expression in the intratumoral vasculature of colorectal adenocarcinomas. Original magnification: A 100×; B 400×

expression between tubular adenomas and tubulovillous adenomas. There was also no significant difference in phospho-KDR/Flk-1 expression between colorectal adenomas with low-grade dysplasia and adenomas with high-grade dysplasia.

In the colorectal adenocarcinomas, phospho-KDR/Flk-1 was largely expressed in the intratumoural vasculatures and colon cancer cells (Figs. 2 and 3). The colorectal adenocarcinomas showed significant cytoplasmic and nuclear phospho-KDR/Flk-1 expression (Fig. 3). Phospho-KDR/Flk-1 showed weak cytoplasmic and nuclear staining (< 2+) in 44.2% (23/52) of the colorectal adenocarcinomas, and showed moderate or strong cytoplasmic and nuclear staining (≥ 2+) in 55.8% (29/52) of the same specimens. Thus, there was no indication of increased phospho-KDR/Flk-1 expression in the colorectal adenocarcinomas compared with its expression in the colorectal adenomas, despite colorectal adenocarcinomas being malignant diseases.

We analyzed the association between phospho-KDR/Flk-1 and the clinicopathologic features including tumor grade, clinical stage, T status, lymph node metastasis, distant metastasis, disease recurrence, and patient survival of colorectal adenocarcinomas. The results showed an inverse relationship of phospho-KDR/Flk-1 expression and the clinical stage of the colorectal adenocarcinomas ($p < 0.0001$) (Table I). The results also showed that phospho-KDR/Flk-1 expression correlated inversely with lymph node metastasis ($p = 0.011$), and distant metastasis ($p = 0.021$) of the colorectal adenocarcinomas (Table I). No statistically significant association was found between the level of phospho-KDR/Flk-1 expression and the grade, T status, patient survival, or recurrence of the colorectal adenocarcinomas (Table I). These results indicated that phospho-KDR/Flk-1 is involved in colorectal cancer metastasis, and its activity is elaborately regulated during colorectal cancer metastasis.

Discussion

Although KDR/Flk-1 is generally recognized to play essential roles in angiogenesis and lymphangiogenesis and, thus, the metastasis of tumors, our results indicated that phospho-KDR/Flk-1 expression was not positively correlated with the metastasis of colorectal cancer. Conversely, our results showed that phospho-KDR/Flk-1 expression was correlated negatively with the metastatic status of the disease (Table I). Our results also showed no indication of increased phospho-KDR/Flk-1 expression in colorectal adenocarcinomas, as compared to the level of phospho-KDR/Flk-1 expression in colorectal adenomas, despite colorectal adenocarcinomas being malignant tumors and colorectal adenomas being benign tumors. However, we cannot exclude the essential role of KDR/Flk-1 activation in the malignant progression of colorectal tumors. Our results also showed no indication of increased phospho-KDR/Flk-1 expression in colorectal adenocarcinomas, as compared to the level of phospho-KDR/Flk-1 expression in colorectal adenomas, despite colorectal adenocarcinomas being malignant tumors and colorectal adenomas being benign tumors. Because the colorectal adenocarcinomas still expressed a very significant phospho-KDR/Flk-1 level, we cannot exclude the essential role of KDR/Flk-1 activation in the malignant progression of colorectal tumors. However, our results indicated that the activation status of KDR/Flk-1 in the colorectal adenomas and colorectal adenocarcinomas might be different. The study included only a limited number of samples, so a further study with a large number of samples is needed to confirm the finding. We also observed that phospho-KDR/Flk-1 mainly showed cytoplasmic staining in adenomas while both nuclear and cytoplasmic staining is noted in carcinomas. Fox and Blazquez *et al.* reported that phosphorylated KDR can be located in the nucleus of neoplastic cells

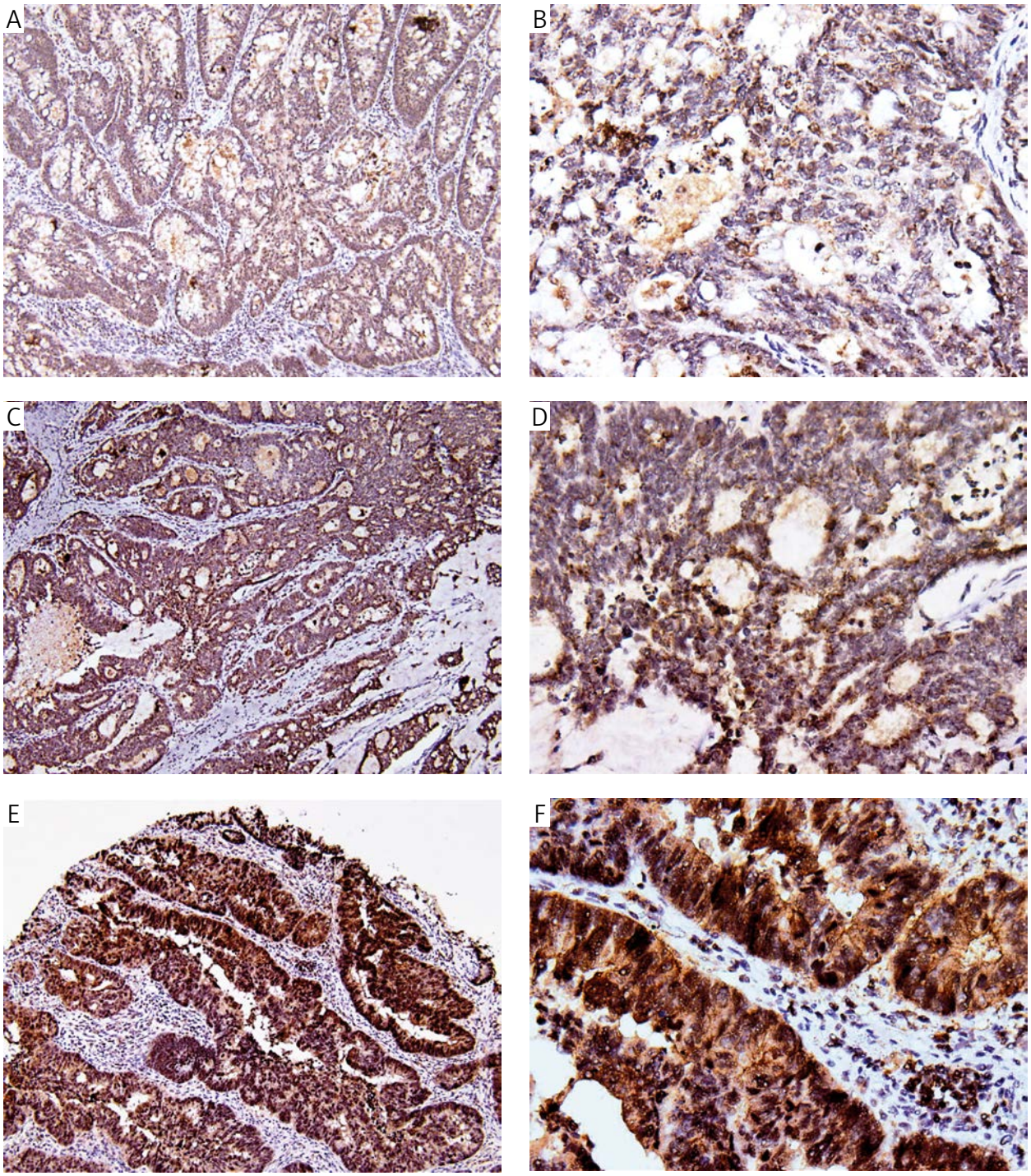

Fig. 3. Representative immunohistochemical images of phospho-KDR/Flk-1 staining in colorectal adenocarcinomas. Colorectal adenocarcinomas samples were classified as weak positive (1+; **A, B**), moderate positive (2+; **C, D**), and strong positive (3+; **E, F**) for phospho-KDR/Flk-1 staining. Original magnification: A, C, and E 100×; B, D, and F 400×

[13, 22]. The differential expression and distribution of phospho-KDR/Flk-1 in colorectal adenomas and adenocarcinomas and its significance need further study.

We found that phospho-KDR/Flk-1 was significantly expressed in colorectal adenomas and colorectal adenocarcinomas, and that an inverse relationship exists between its expression and the metastasis of colorectal adenocarcinomas. Thus, in addition to acting on intratumoral endothelial cells to modulate the angiogenesis and lymphangiogenesis of cancer, KDR/Flk-1 activation in colorectal cancer cells may also be involved in colorectal cancer progression, and it seems that its activation is elaborately regulated in metastasized colorectal cancer. Anjomshoaa *et al.* reported an inverse relationship between colon cancer progression and tumor-proliferative activity; those with established metastases had lower Ki-67 expres-

sion and significantly lower proliferative activity than did colorectal cancers that were non-metastatic [23]. Angiogenesis and lymphangiogenesis are complex processes that depend on angiogenic factors secreted by tumor cells and stroma cells [24, 25]. The multistep processes of lymphangiogenesis and angiogenesis accompany the multistage development of tumors [26, 27]. The development of a non-metastasized tumor into a metastatic tumor causes numerous changes within the tumor microenvironment [28]. Studies have reported that tumors in more advanced stages do not rely on a unique angiogenesis driver [29, 30]. A network of other growth factors, such as fibroblast growth factor (FGF), and cytokines, such as interleukin 1 and interleukin 8, create a crosstalk within the tumor microenvironment and are involved in tumor angiogenesis [29, 31, 32]. The microenvironments in lymphatic nodes and the sites of a metastasized tumor obviously are different from the microenvironment in the original tumor site. Because VEGF receptors are also expressed in colorectal cancer cells, the activation status of VEGFR-2 might be regulated by changing the levels of VEGF and other growth factors within the tumor microenvironment in the development of a non-metastasized tumor into a metastatic tumor, as described. This may explain why there was an inverse relationship between phospho-KDR/Flk-1 expression and cancer stage, as well as the metastasis of the colorectal adenocarcinomas in our studies. Klasa-Mazurkiewicz *et al.* have reported previously that the highest relative level of VEGFR-2 was detected in the early stage cancer, indicating the significance of neoangiogenesis in high VEGFR-2 expression early stage ovarian cancer [15]. Thus, it is also possible that the development of a non-metastasized tumor (i.e. stage I-II tumors) into a metastatic tumor (i.e. stage III-IV tumors) might have resulted in decreased KDR/Flk-1 expression, thus causing an inverse relationship between phospho-KDR/Flk-1 expression and cancer stage. Both findings suggest that VEGFR-2 plays a role in the neoangiogenesis of those early stage cancers that express high VEGFR-2 expression levels.

Conclusions

In this study, we found that phospho-KDR/Flk-1 was significantly expressed in colorectal adenomas and colorectal adenocarcinomas, and that there was an inverse relationship of phospho-KDR/Flk-1 expression and cancer stage, lymph node metastasis, and distant metastasis of colorectal cancer. Our results indicate that phospho-KDR/Flk-1 may play a crucial role in the neoangiogenesis of early stage cancers with high phospho-KDR/Flk-1 expression levels. However, the results are based on a small number of cases so they should be confirmed by larger studies.

The authors declare no conflict of interest.

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