Prognostic Impact of Midkine Expression and Microvessel Density in Colorectal Carcinoma

Kolorektal Karsinomda Midkin Ekspresyonu ve Mikrodamar Sayısının Prognoza Etkisi

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Geliş Tarihi/*Received:* 17.01.2012
Kabul Tarihi/*Accepted:* 10.07.2012

This work partly presented as a poster in 22nd European Congress of Pathology, Florence, Italy, 4-9 Semptember, 2009.

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ABSTRACT Objective: Midkine (MK) is a heparin-binding growth factor that plays important roles in cell transformation and angiogenesis. Recent studies indicated that MK was involved in genesis and development of colorectal carcinomas (CRC). However in these tumors the relationship among MK expression, angiogenesis and prognosis has not been evaluated. The purpose of this study was to investigate whether MK expression was associated with angiogenesis and survival in patients with CRC. **Material and Methods:** Tumor specimens from 61 patients diagnosed as CRC were included in this study. Serial sections from paraffin embedded tissues were stained with anti-midkine and anti-CD34 antibodies. Angiogenesis was assessed as microvessel density (MVD). Chi-square test, Kaplan-Meier method and Cox regression analysis were used for statistical analysis. **Results:** MK expression was observed in 36 of the cases. Non-neoplastic mucosa was consistently negative. Any relationship was not observed between MK expression and clinicopathologic parameters, so MK expression failed to predict tumor behavior. Moreover MK expression was not associated with MVD. On the other hand the prognosis was significantly worse in patients with high MVD (>5.8). Survival analysis revealed that although MK expression had no impact on prognosis, MVD was an independent prognostic variable. **Conclusion:** Our results revealed that MK expression has no prognostic relevance in CRC. However MVD could be reliable indicator of prognosis. Although our data needs to be clarified with further molecular studies, the lack of correlation between MK expression and MVD suggests that MK has no impact on CRC related angiogenesis.

Key Words: Colorectal neoplasms; midkine; prognosis

ÖZET Amaç: Midkin (MK) hücre transformasyonu ve anjiogenezde önemli bir rol alan 'heparin-bağlayıcı-büyüme faktörü' dür. Yakın geçmişde yapılan çalışmalarda MK'nin kolorektal karsinom oluşumunda (KRK) ve gelişiminde rol oynadığı gösterilmiştir. Ancak bu tümörlerde MK ekspresyonu, anjiogenez ve hastalığın seyri arasındaki ilişki araştırılmamıştır. Bu çalışmanın amacı KRK olgularında MK ekspresyonunun, anjiogenez ve prognoz ile ilişkisinin olup olmadığının araştırılmasıdır. **Gereç ve Yöntemler:** Bu çalışmaya KRK tanısı alan 61 hastanın tümör spesimeni dahil edildi. Parafin kesitler anti-midkine ve anti-CD34 antikorları ile boyandı. Anjiogenezis mikrodamar yoğunluğu (MVD) olarak değerlendirildi. İstatistiksel analiz için Ki-kare testi, Kaplan-Meier metodu, ve Cox regresyon analizi uygulandı. **Bulgular:** MK ekspresyonu 36 olguda gözlendi. Non neoplastik mukoza daima negatifti. MK ekspresyonu ile klinik ve patolojik parametreler arasında hiçbir ilişki gözlenmedi. Bu nedenle tümör davranışını belirlemede MK ekspresyonu yetersizdi. Dahası MK ekspresyonu ile MVD arasında ilişki bulunmadı. Diğer bir yandan MVD'si yüksek olan (>5,8) hastalarda prognoz belirgin olarak kötüydü. Her ne kadar MK ekspresyonunun hastalığın seyrine bir etkisi olmasa da çok değişkenli sağkalım analizinde MVD'nin bağımsız prognostik değişken olduğu gözlendi. **Sonuç:** Bulgularımız KRK'da MK ekspresyonunun prognostik bir değeri olmadığını göstermiştir. Buna karşın MVD hastalığın seyrinin belirlenmesinde önemli bir belirteçtir. Her ne kadar bulgularımızın daha ileri moleküler çalışmalar ile desteklenmesi gerekse de, MK ekspresyonu ve MVD arasında bir ilişkinin gözlenmemesi MK'nin KRK ile ilişkili anjiogenezde rolü olmadığını düşündürmektedir.

Anahtar Kelimeler: Kolorektal tümörler; midkine; prognoz

Turkiye Klinikleri J Gastroenterohepatol 2012;19(1):15-22

Midkine (MK) is a secreted basic heparin-binding growth factor, initially found as a product of a retinoic acid-responsive gene that is located at chromosome 11p11.2.[1] Similar to other growth factors MK play fundamental roles that are closely related to biological processes during development, including angiogenesis.[2] In vitro studies demonstrated that, soluble MK promotes growth of fibroblasts and induction of their extracellular matrix synthesis.[3,4] It also stimulates growth and glycosaminoglycan synthesis of endothelial cells and induces their fibrinolytic activity.[5,6] MK provides survival of embryonic neurons.[7] MK expression is controlled both spatially and temporally during development.[3] MK is most intensely expressed during midgestation, while its expression in normal human adult tissues is weak or undetectable.[3,4] Recently MK expression has been documented in malignant tumors and accumulated data indicated that this factor might also contribute to carcinogenesis and tumor progression.[3,8-13] Moreover, the relationship between angiogenesis and MK expression was observed in some tumors.[14-17] In spite of the recent data concerning about the influence of MK on endothelial cells and its proangiogenic effect, the exact role of MK in angiogenesis, especially in tumors is poorly defined.[18]

In colorectal tumors, previous studies have demonstrated that MK was involved in genesis and development of colorectal carcinomas (CRC).[19-22] Besides, in an elegant experimental study Takei et al. demonstrated that antisense oligodeoxynucleotide targeted to MK might suppress tumorigenicity of mouse rectal carcinoma cells.[23] However in these tumors the relationship among MK expression, angiogenesis and prognosis has not been evaluated. The purpose of this study was to investigate whether or not any relationship exists between MK expression, angiogenesis and survival in patients with CRC.

MATERIAL AND METHODS

A total of 61 patients diagnosed as CRC in the Department of Pathology, School of Medicine Akdeniz University were included in the study. The patients were surgically treated at the Department of Surgery, from 1998 to 2008. This study was approved by the Akdeniz University ethics committee according to declaration of Helsinki. The clinicopathologic characteristics of the patients are summarized in Table 1 and Table 2.

The median age of the 36 men was 59.6 years (range 17-78 years) at the time of operation, and that of the 25 women, 63.2 years (range 36-84 years). Survival data were available on all patients and were obtained from case records. Forty-one of 61 patients (67%) have survived, with mean survival duration of 75 months (range 43 to 120 months). Twenty cases died in 8 to 70 months (mean 34 months).

Four micrometer thick haematoxylin and eosin stained tissue sections from the surgical specimens fixed in 10% formalin and embedded in paraffin were reviewed and representative tissue blocks were selected. Slides were immunostained with anti-midkine (sc-46701, 1: 50 dilution, Santa Cruz, USA) and CD34 (QBEnd 10,1:50 dilution; Dako, Glostrup, Denmark) by the avidin-biotin immunoperoxidase technique. Briefly, sections from each primary tumor, adjacent mucosa were deparafinized and heated in a microwave oven for 20 min to retrieve antigens. Endogenous peroxidase was blocked using 0.3% hydrogen peroxide in methanol for 10 min. Each step of incubation was followed by thorough washing of the slides in distilled water and phosphate-buffered saline (PBS; 0.001%, Sigma). Slides were incubated with primary antibody to MK over night at 4°C. For CD34 staining slides were incubated with primary antibody for 30 min. Whole sections were allowed to react with the secondary biotinylated antibody for 15 min and streptavidin for 15 min. Finally, all slides were treated with DAB reagent to develop color and counterstained with haematoxylin. Negative controls were performed by using non-reactive IgM of the same concentration as the primary antibodies. In all series, relevant positive controls for MK included sections from duodenum.

Angiogenesis was assessed as microvessel density (MVD). MK expression and MVD were estimated without knowledge of the clinical data or prognostic outcome. For determination of MVD the Chalkley counting procedure was applied (Figure 1).[24]

TABLE 1: Distribution of midkine expression, percentage and intensity of staining among clinicopathological factors.

	Midkine Expression		Percentage of staining *				Intensity of staining †				
	Absent	Present	0	1	2	3	0	I	II	III	p values
Age											
0	9	19	9	5	8	6	9	4	5	10	ns
1	17	6	17	9	3	4	17	3	2	11	
Gender											
Male	14	22	14	6	7	9	14	4	4	14	ns
Female	12	13	12	8	4	1	12	3	3	7	
Location											
Right side	10	15	9	1	1	2	9	-	1	3	ns
Left side	16	21	17	13	10	8	17	7	6	18	
Grade											
Well differentiated	1	3	1	2	-	1	1	-	-	3	
Moderately differentiated	20	30	20	12	10	8	20	7	6	17	ns
Poorly differentiated	5	2	5	-	1	1	5	-	1	1	
Level of Invasion											
T1+T2	8	9	8	4	3	2	8	2	3	4	ns
T3+T4	18	26	18	10	8	8	18	5	4	17	
Lymph node involvement											
Absent	15	24	15	9	8	7	15	6	7	11	ns
Present	11	11	11	5	3	3	11	1	-	10	
Stage											
SI+SII	7	9	7	3	4	2	7	1	3	5	ns
SIII+SIV	19	26	19	11	7	8	19	6	4	16	
Distant metastasis											
Absent	9	13	9	4	4	5	9	2	1	10	ns
Present	17	22	17	10	7	5	17	5	6	11	
Recurrence											
Absent	22	30	22	12	9	9	22	6	6	18	ns
Present	4	5	4	2	2	1	4	1	1	3	
MVD											
<5.84	10	19	10	9	4	6	10	5	5	9	ns
5.84 ≤	16	16	16	5	7	4	16	2	2	12	

* 0: negative, 1: 1-29%, 2: 30-49, 3: more than 50%, † I: weak, II: moderate staining, III: strong staining, ns: not significant.

For the evaluation of MK expression the staining pattern was classified semi quantitatively as follows: (-): tissue specimens without staining, (1): tissue specimens with 1 to 29% of the cancer tissue stained, (2): tissue specimens with 30 to 50 % of the cancer tissue stained, (3): tissue specimens with more than 50% of the cancer tissue stained.

The data were analyzed by SPSS 10.0 for Windows software. Chi-square test was used for univariate analysis of categorical data. Univariate and multivariate survival analysis were performed by using Kaplan-Meier method and Cox regression analysis, respectively. Tests were considered significant when their p-values were <0.05.

RESULTS

MK immunoreactivity was detected in 36 of the cases (Figure 2). Non-neoplastic mucosa was consistently negative. Any relation was not observed between MK expression and clinicopathologic pa-

TABLE 2: Correlation between clinicopathological factors and MVD.

	Hypovascular MVD<5.84	Hypervascular 5.84 ≤ MVD	Mean MVD±SD	p value
Age				
0	15	13	5.39±2.57	ns
1	14	19	6.24±3.11	
Gender				
Male	18	18	5.67±2.73	ns
Female	11	14	6.12±3.12	
Location				
Right side	5	8	6.54±3.17	ns
Left side	24	24	5.69±2.80	
Grade				
Well differentiated	4	0	3.50±1	
Moderately differentiated	23	27	5.90±2.89	ns
Poorly differentiated	2	5	6.86±3.07	
Level of Invasion				
T1+T2	12	5	4.71±1.64	0.025
T3+T4	17	27	6.30±3.14	
Lymph node involvement				
Absent	23	16	5±2.32	0.002
Present	6	16	7.36±3.2	
Stage				
SI+SII	11	5	4.83±2.06	4.83±2.06
SIII+SIV	18	27	6.20±3.07	
Distant metastasis				
Absent	24	15	4.67±2.04	0.001
Present	5	17	7.95±2.93	
Recurrence				
Absent	25	27	5.79±2.89	ns
Present	4	5	6.22±2.94	
Midkine Expression				
Absent	10	16	6.04±3.26	ns
Present	19	16	5.71±2.60	

ns: not significant.

rameters (Table 1). In addition MK expression was not correlated with MVD and failed to predict survival. Cases were divided into two groups according to the mean value of MVD for further analysis. The hypervascular group consisted of 32 tumors with MVD 5.84 or higher, and hypovascular group consisted of 29 tumors with MVD less than 5.84. Lymph node involvement, advanced stage and the presence of distant metastasis were more frequent in hypervascular group ($p<0.05$) (Table 2). Univariate analysis based on the log rank test revealed that the prognosis of patients in hypervascular group was significantly worse than hypovascular group. The 5-year survival rates of the hypovascular and hypervascular groups were 72% and 42%, respectively (Figure 3). Multivariate analysis with covariates that showed statistical significance in the univariate analysis, MVD was found to be an independent prognostic factor together with the presence of distant metastasis ($p<0.05$).

DISCUSSION

In previous studies on CRC, MK expression was detected more frequently in primary tumors when

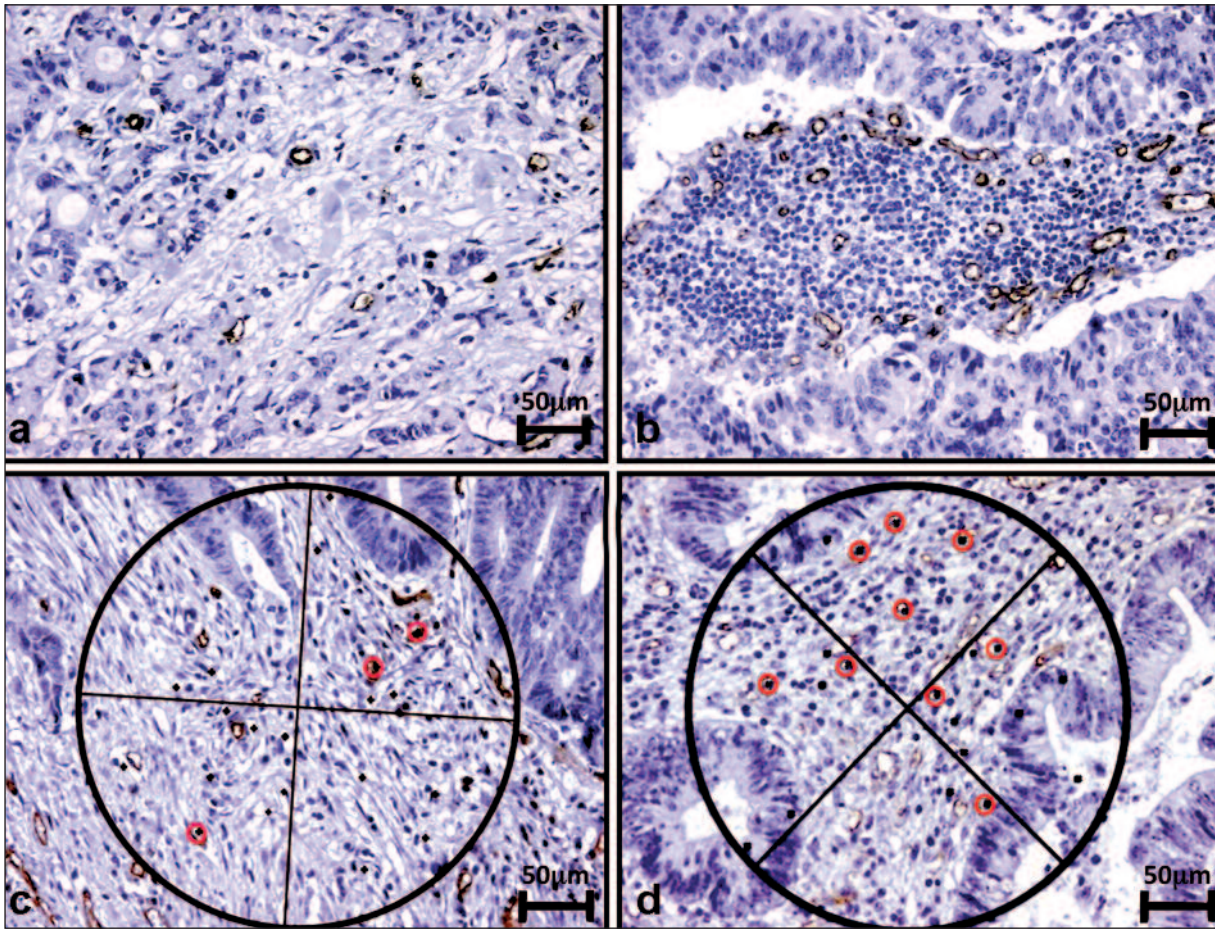

FIGURE 1: Immunohistochemical expression of CD34 in hypovascular **(a)** and hypervascular groups **(b)**. Examples of Chalkley counts in hot spots with low **(c)** and high **(d)** numbers of vessel profiles. Hitting Chalkley dots (black) are marked by red circles. The Chalkley grid area is 0.196 mm2 (magnification x 200).

compared to normal mucosa.[20,22] Parallel to this finding in our series MK expression was identified in 59% of CRC, whereas the corresponding normal mucosa remained negative. Recently it has been indicated that although MK expression in adult tissues is restricted, it is highly expressed in a number of malignancies originated from different organs. From this point of view our observation coincides with the previous data supporting that MK could be a general marker of human tumors rather than tissue specific.[3,8,12,13,17]

In human colorectal adenomas the staining intensity of MK was found to be associated with the severity of dysplasia suggesting that MK might play a role in the early stage of carcinogenesis.[20] The relationship between cell proliferation and MK expression has been also evaluated.[22] Results demonstrated that MK might be involved in tumorigenesis by facilitating cell proliferation in sporadic CRC. However, it remains unknown whether MK expression is associated either with tumor behavior or survival in these tumors. In the present study we did not observed any association between MK expression and tumor progression suggesting that evaluation of MK expression by immunohistochemistry is not valuable to predict the prognosis of patients with CRC.

On the other hand since MK is considered as a strong candidate for participation in carcinogenesis, the significance of both serum MK (S-MK) levels and MK expression in tumor tissues have been studied together or separately in various malignancies with different conclusions. Whereas S-MK is a good marker of prognosis in many cancers, its level

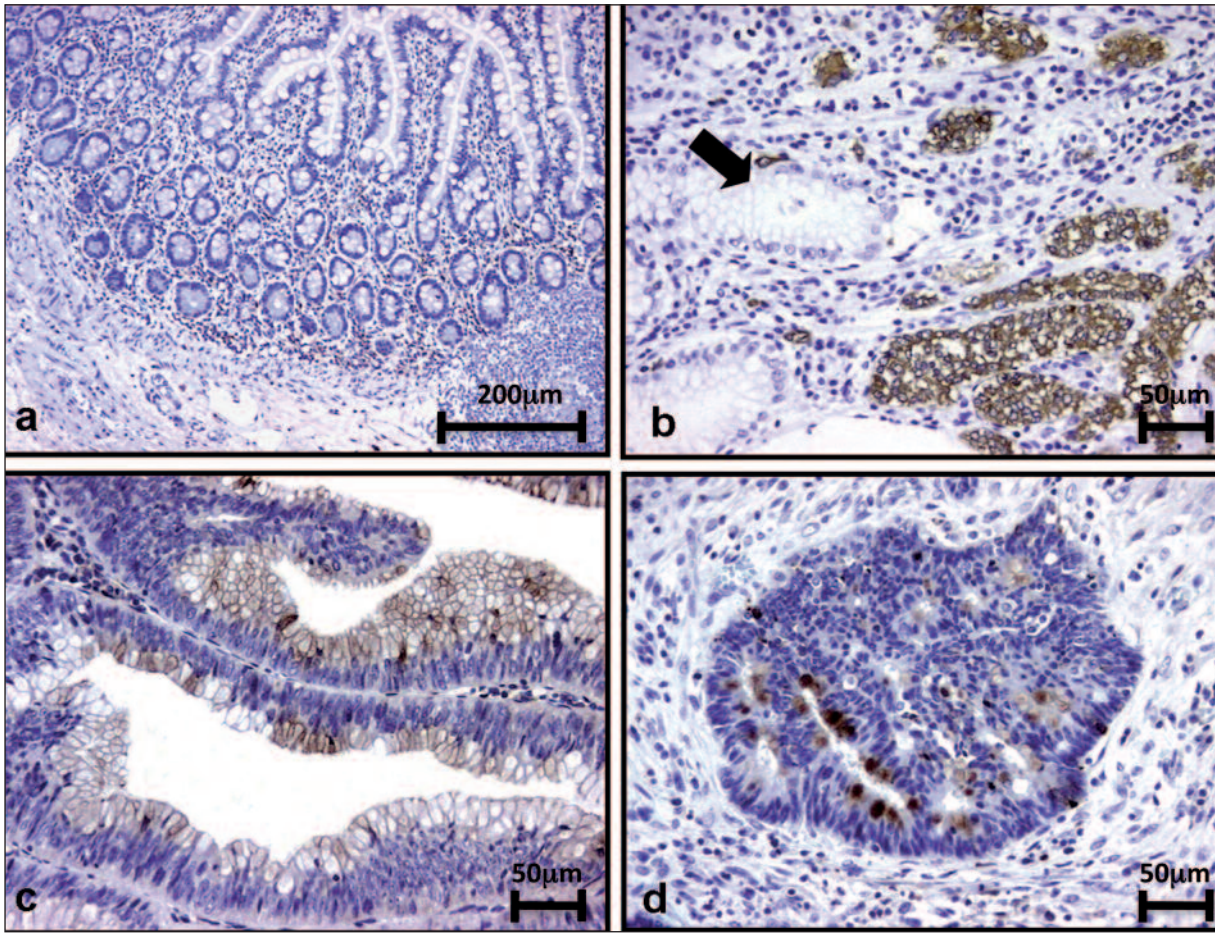

FIGURE 2: Midkine staining in normal colon mucosa **(a)** and in CRC **(b, c and d)**. While tumor cells show cytoplasmic staining, normal mucosa remains negative (a and b: arrow). (magnifications **a:** x100; **b, c and d** x 200).

does not invariably correlate with tissue MK expression.[9-13,25-28] Recently an elevated S-MK level is detected in CRC.[19] However, the relation between S-MK and CRC prognosis is not being documented. Because the present work was performed retrospectively from archival tissues, S-MK level has not been evaluated warranting further prospective studies. For this reason the results of our study does not exclude the possible significance of S-MK in CRC progression and it is not convenient to conclude with our findings that MK has no effect on prognosis unless the impact of S-MK levels in CRC prognosis is completely clarified.

Because MK is involved in neovascularization it's thought to be a novel mediator of tumor angiogenesis, in another word tumor behavior.[8,12,14-17] In the results of some previous studies on salivary gland tumors, pancreatic head carcinoma and oral SCC a relationship between MK and angiogenesis has been reported.[12,15,17] These findings prompted us to investigate the relation between

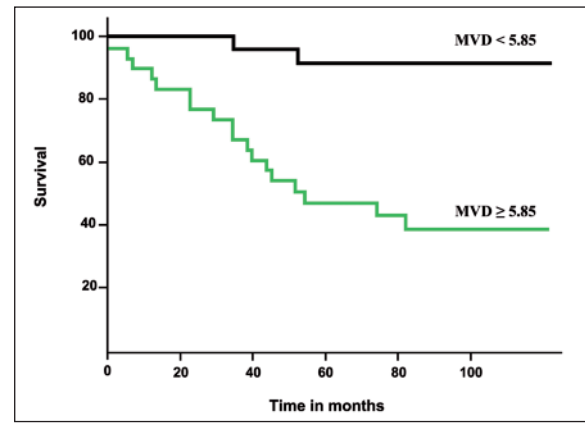

FIGURE 3: Survival curves according to MVD status. Patients were stratified by the mean microvessel count of the series.

MK expression and angiogenesis in CRC. However, in contrast to our expectation, we did not observed a significant difference between MK expression of hypervascular and hypovascular tumors suggesting that MK expression is not related with angiogenic activity in CRC. The role of MK on angiogenesis has been investigated in a few studies but different results have been reported and at present the effect of MK on angiogenesis is not completely clarified.[6,18,29] Some studies in mouse myocardial ischemia models demonstrated that the potent angiogenic activity of MK and suggested that this activity might be explained primarily by the PI3K-Akt signaling axis.[29] In another elegant study Sumi et al. performed a blood vessel model to investigate the effect of MK on endothelial cells.[6] Although MK induced the proliferation of endothelial cells and their glycosaminoglycan synthesis, it had no effect on these cells when they were cultured separately from smooth muscle cells. Their results indicated that the target of MK was smooth muscle cell, which secretes factors such interleukin-8 acting on the endothelial cells. Besides in a more recent study, it has been demonstrated that MK can abrogate the vascular endothelial growth factor A (VEGF-A)-induced proliferation of human microvascular endothelial cells through the down-regulation of proangiogenic cytokines and through the upregulation of the antiangiogenic factor, tissue inhibitor of metalloproteinase 2.[18] Moreover, phosphorylation of vascular endothelial growth factor receptor 2 (VEGFR-2) and of downstream signaling molecules, such as phosphatidylinositol-3-kinase and mitogen-activated protein kinases, is also impaired, indicating that MK might be also a negative regulator of angiogenesis.[18] Therefore further molecular studies are necessary to conclude the exact role of MK in angiogenesis.

On the other hand, angiogenesis as expressed by MVD was significantly associated with parameters of advanced disease (Table 2). In our series, the prognosis of patients with hypovascular tumor was more favorable than the hypervascular ones ($p<0.005$). Furthermore, multivariate analysis showed that microvessel count was a significant and independent prognostic factor to predict the probability of survival. The majority of studies in CRC do find a correlation between higher MVD, more aggressive tumor behavior and poor prognosis, but other reports illustrate discordant results.[30-36] This may be explained by the different methods used for quantification of MVD and variable staining techniques. In our study Chalkley point counting was used as a method of determining MVD.[24] Recent study indicated that this is an appropriate method because it is reproducible, accurate and correlates with other measures of MVD in colorectal cancer.[35,37,38]

In conclusion, our results revealed that MK expression has no prognostic relevance in CRC. However MVD could be a reliable indicator of tumor behavior and prognosis. Although our data needs to be clarified with further molecular studies, the lack of correlation between MK expression and MVD suggests that MK has no impact on CRC related angiogenesis.

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