Immunohistochemical expression of vascular endothelial growth factor (VEGF) does not correlate with microvessel density in renal cell carcinoma

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The aim of present study was to investigate the relationship between the immunohistochemical expression of vascular endothelial growth factor (VEGF) and microvessel density (MVD) assessed by CD31 and endoglin (CD105) in renal cell carcinoma (RCC).

Specimens from 45 cases of RCC. were formalin-fixed, paraffin embedded, and sections were stained with H&E. Additional sections from each case were stained for VEGF, CD31, CD105, and alpha smooth muscle cell actin (SMA). VEGF immunohistochemical expression was estimated as negative (0), weak positive (+1), moderate positive (+2), and intense positive (+3). Microvessel density (MVD) was estimated on 5 hot spots (x400) from each case, and the arithmetic media was the final result. MVD was separately calculated on slides stained with CD31 and CD105. The rate between mature and immature blood vessels was calculated on slides stained with CD31/CD105/SMA. Statistic analysis was performed with SPSS10.0.

The immunoreaction for VEGF was positive in epithelial cells of the renal tubules, and occasionally, in endothelial cells. In RCC, tumor cells were positive in 34 from 45 cases (75.5%). 11 cases were negative, 14 were slightly positive (+1), 13 moderate (+2), and 7 intense (+3). No relationship was found between the expression of VEGF and pathological form and nuclear grade, excepting for the chromophilic variant (3 cases, all positive). CD31 was positive in all cases, and CD105 in 39 cases. The mean values of MVD on slides stained for CD31 and CD105 were: 31.68 (range 9.8-60.2)/20.66 (range 4.2-52.8). The rate CD31/SMA positive blood vessels was 1/0.62.

VEGF was expressed in 75.5% of 45 cases with RCC, and the mean value of MVD CD31/CD105 was 31.68/20.66. The immunohistochemical expression of VEGF does not correlate with MVD performed on slides stained for both CD31 and endoglin. The majority of blood vessels in the tumor area are of mature type, with perivascular cells positive for SMA.

Key words: Renal cell carcinoma (RCC), Angiogenesis, Microvessel density (MVD), Vascular endothelial growth factor (VEGF), Immunohistochemistry

Angiogenesis is the process of new blood vessels formation from preexisting vessels, and is essential for tumor growth and progression. As Folkman demonstrated three decades ago, a malignant tumor cannot grow over 2-3 mm in the absence of blood vessels [1, 2]. Even when mechanisms of tumor angiogenesis is not completely understood, the vasculature became a target for therapy with antiangiogenic drugs [3, 4, 5]. These drugs target the endothelial cells, like angiostatin or endostatin, or inhibit the most powerful angiogenic agent, the vascular endothelial growth factor (VEGF) [6, 7]. During the angiogenic switch, tumor cells acquire the property to secrete angiogenic factors. Current trials with antiangiogenic drugs gave some conflicting results probably because patients included were not tested for the angiogenic potential of tumor cells.

Renal cell carcinoma (RCC) is well known since many years as a highly vascular tumor. On the other hand, the behavior of this tumor is unpredictable and there is known large tumors without metastasis and relative small tumors with metastasis. The growth of malignant cells in RCC is strictly angiogenesis-dependent, because the density of blood ves-

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Table 1.Immunohistochemical technique used in the study

Antibody	VEGF	CD105	CD31	αSMA
Clone	VG-1	SN6h	JC70A	1A4
Dilution	1:30	1:50	RTU	RTU
AR	Microwave, 20', pH9	Proteinase K, 15', RT	Proteinase K, 25', RT	No
Incubation	30 min	30 min	30 min	30 min
Working system	LSAB+/HRP	LSAB+/HRP	EnVision/HRP	LSAB2/HRP

AR – antigen retrieval, RTU – ready-to-use, RT – room temperature

sels correlated inversely with necrosis [8]. Microvessel density (MVD) was investigated in RCC by many authors, but results varied within large limits [9, 10, 11]. Almost all studies investigated MVD using only the most specific endothelial marker CD31 and just three teams used CD105 (endoglin) that seems to stain only activated endothelial cells [12, 13, 14]. Actually, it is not yet clear which is the prognostic value of MVD in RCC. Moreover, to the best of our knowledge, the rate between mature and immature blood vessels in RCC was not investigated.

The immunohistochemical expression of VEGF was demonstrated in about 70% of cases with RCC and VEGF mRNA was found in tumor cells [15]. On the other hand, conflicting results were published about the expression of VEGF and microvessel density (MVD) [16, 17, 11]. Few studies were performed on the immunohistochemical expression of VEGF in RCC, and conflicting results have been reported on its relation to nuclear grade, different RCC types or MVD.

Our purpose was to investigate the relationship between the immunohistochemical expression of VEGF and MVD, and to evaluate the rate between immature, intermediate, and mature blood vessels in the tumor area of RCC.

Material and methods

Patients. 45 cases with RCC were investigated, aged between 45 and 72 years, without distant metastasis, and without neoadjuvant therapy. Radical nephrectomy was performed in all cases by transperitoneal route. The largest diameter of the tumors was between 4.2 and 12.5 cm.

Specimen processing. Biopsies were formalin-fixed, paraffin-embedded, and sections were stained with haematoxylin-eosin. Conventional clear cell carcinoma was found in 31 cases (3 cases with chromophilic variant), papillary carcinoma in 8 cases, chromophobe cell carcinoma in 2 cases, collecting duct carcinoma in one case, and sarcomatoid carcinoma in 3 cases. The differentiation of the tumors was estimated using the nuclear grade, according Fuhrman criteria [18], and there were found 11 cases with grade 1, 21 cases with grade 2, 10 cases with grade 3, and 3 cases with grade 4.

Immunohistochemistry. 5μm thick slides were deparaffinized with xylene and then hydrated by graded ethanol. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide for 5 minutes at room temperature. Slides from each case were immunostained for VEGF, CD31, CD105, and αSMA *(Table 1).* The final reaction product was visualized with diaminobenzidine dihidrochlorid (10 minutes), and nuclei were stained with Lillie's modified haematoxylin. All reagents were from DakoCytomation, Denmark. The immunohistochemical procedure was performed with DakoCytomation Autostainer, excepting for the antigen retrieval.

VEGF scoring. VEGF was considered positive when the final reaction product was found in the cytoplasm of tumor cells, with granular pattern. Based on the intensity of reaction and percentage of positive tumor cells, VEGF reaction was noted as negative (0), weak positive (+1, weak reaction in less than 10% of tumor cells), moderate positive (+2, weak-moderate reaction in 10 to 50% of tumor cells), and intense positive (+3, strong or moderate intensity in more than 50% of tumor cells). Scattered intense positive tumor cells included in large areas with weak or moderate positive cells were excluded, and the predominant pattern was taken into account.

MVD. MVD was separately performed for each case on slides stained for CD31 and CD105. There were counted only blood vessels in the tumor area according to the procedure of Weidner et al [19]. Five high power fields (x400) of highest microvessel density (hot spots) were selected, and there were counted all positive individual signals with and without evident lumen. Positive staining for CD31 and CD105 was considered a countable vessel, whether or not a distinct lumen was visible. In tumors where the microvasculature consisted of a dense network, each distinct branch was considered a single vessel. In many cases with clear cell carcinoma was found a complex network of vessels, and therefore, there were counted the branching points. The same procedure was applied on slides stained with αSMA. Vessels with perivascular cells positive for αSMA were considered mature, according to criteria published by Gee et al [20].

Statistical analysis. The data were analyzed using the SPSS10.0 software package. The correlation between the immunohistochemical expression of VEGF and MVD was determined using the Kruskal-Wallis test. $P<0.05$ was considered to be statistical significant.

Results

The immunohistochemical reaction for VEGF was intensely positive in epithelial cells of the tubules of the remnant kid-

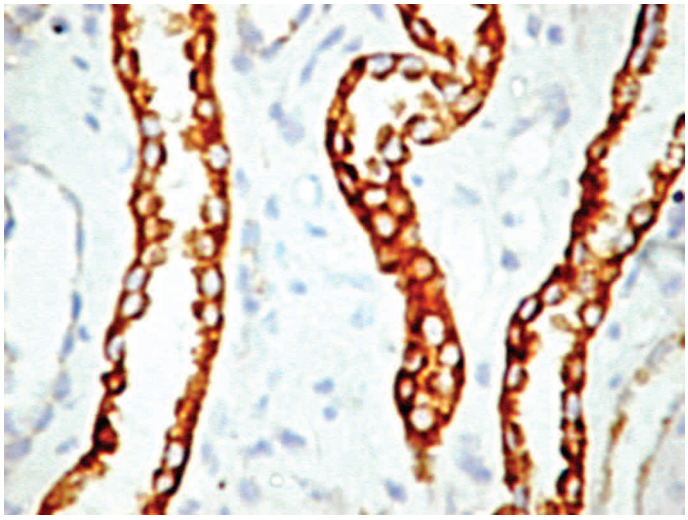

Fig. 1. VEGF expression in tubules of the kidney tissue. X400.

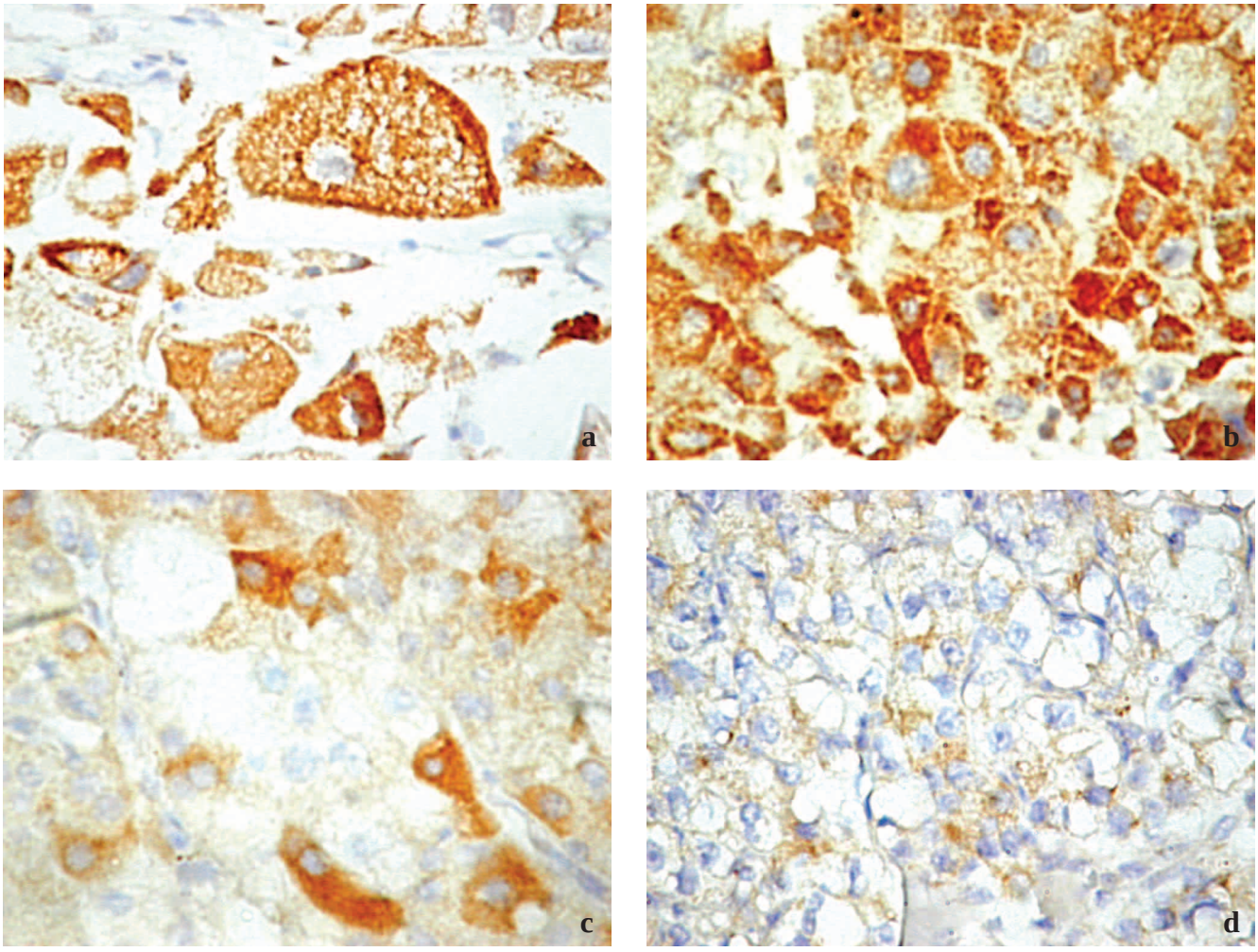

Fig. 2. The final product of reaction in the cytoplasm of tumor cells, with granular pattern (a). Reaction noted with +3 (b). Isolated intense and weak stained cells, +2 (c). Weak reaction with granular pattern, +1 (d). VEGF, LSAB+, DAB, x400.

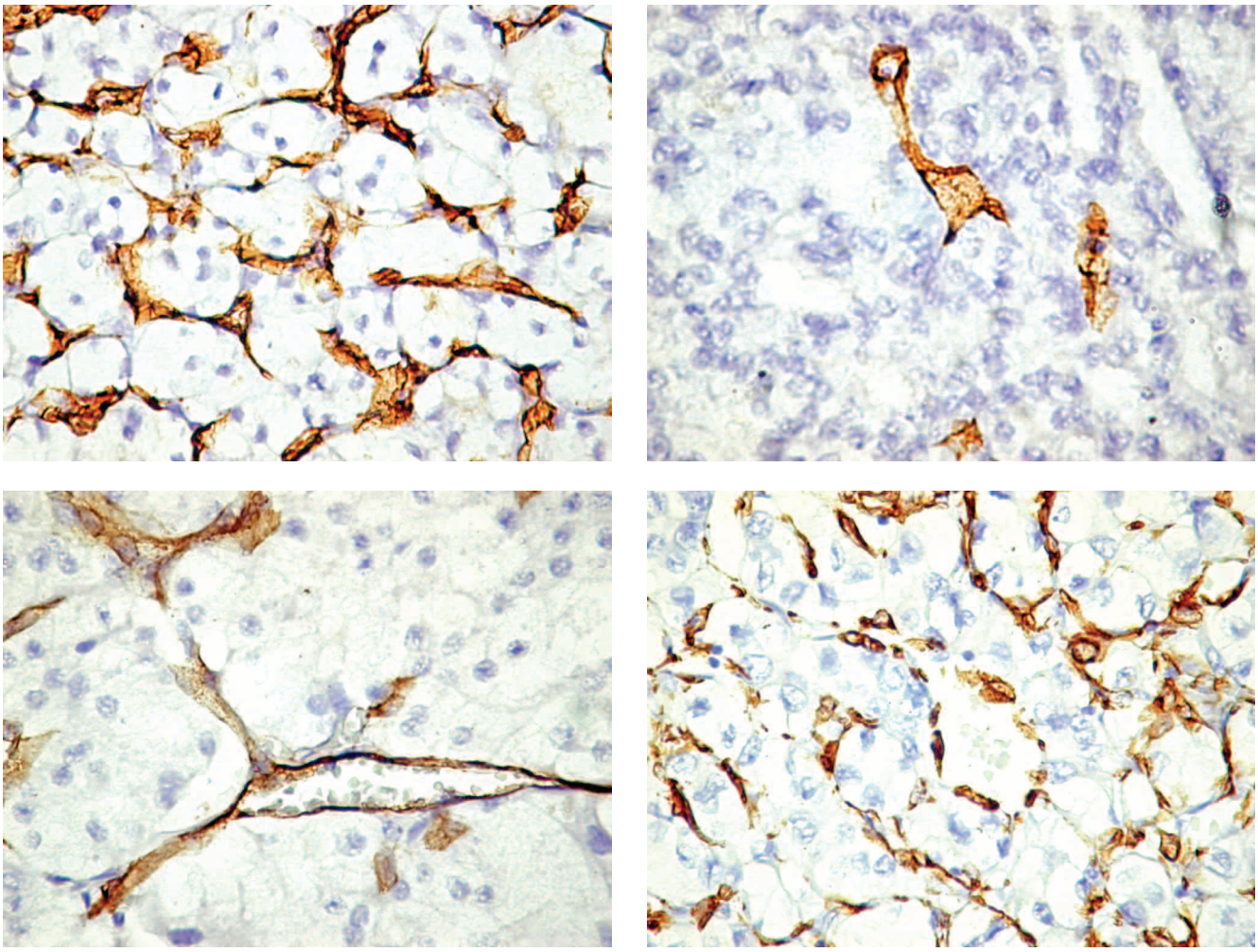

Fig. 3. CD31 expression in clear cell carcinoma (a). CD31 expression in papillary carcinoma (b). CD105 (c). Clear cell carcinoma, αSMA (d). x400.

ney parenchyma, close to the tumor *(fig.1)*. Only rare endothelial cells were positive. The positive reaction in the renal parenchyma was considered as internal positive control, and it was found in all cases. In RCC, only tumor cells were positive in 34 from 45 cases (75.5%). The final reaction product was cytoplasmic, with granular pattern *(fig.2a)*. In cases noted with +3 (n=7) almost all tumor cells were intensely stained *(fig.2b)*. Thirteen cases were noted with +2 (n=13), and 14 with +1) *(fig.2c and d)*. There were found positive 26 from 31 cases with clear cell carcinoma, 6 from 8 cases with papillary carcinoma, 1 from 2 cases with chromophobe cell carcinoma, and 1 from 3 cases with sarcomatoid carcinoma. No correlation was found between VEGF expression and the nuclear grade ($p<0.2$).

CD31 was positive in all cases, and CD105 in 39 cases. Both reactions were strongly and strictly positive for the endothelium *(fig.3a and b)*. αSMA was positive in the perivascular cells *(fig.3c)*, and in three cases in the stroma in cells with myofibroblast morphology. The mean values of MVD on slides stained for CD31 and CD105 were: 31.68 (range 9.8-60.2)/20.66 (range 4.2-52.8). Values of MVD calculated on slides stained for CD105 was lower than for CD31 in all but 4 cases, and in 6 cases the reaction was negative in the tumor area (5 clear cell carcinoma, 1 chromophobe cell carcinoma). MVD was significantly lower in cases with extensive necrosis, and intermediate values were obtained in clear cell carcinoma with cystic transformation.

Significant differences were found between pathological types of renal cell carcinoma. In papillary carcinoma, CD31/CD105 MVD was 18,56/11.36, in sarcomatoid carcinoma, 27.13/15.8. Intermediate values were obtained for chromophobe cell carcinoma (34.6/18.1), and highest values were noticed in clear cell carcinoma (34.02/27.54).

Mature blood vessels showed positive reaction for αSMA in the cytoplasm of perivascular cells. The number of αSMA positive vessels was significantly lower in papillary carcinoma

Table 2. Overview on the methods and MVD values in RCC

Authors	Year	Cases	Antibody	Magnify	MVD mean value (range)
Delahunt et al	1997	150	FVIII	X400	33.0 (1-238)
Paradis et al	2000	62 ccc 12 pap	CD34 CD34	X200 X200	22+/-9.3 6.5+/-5
Kawata et al	2001	71	FVIII	X50	1.98 (0.01-58.5)
Hemmerlein et al	2001	58	CD31	X400	19.2 (without necrosis) 8.2 (with necrosis)
Zhang et al	2002	70	CD31	X200	39.91 (12-93)
Yagasaki et al	2003	84	CD105	X400	17.99 (6.05-29.93)
Imao et al	2004	70	CD34	X400	98+/-63 (11-301)
Hemmerlein et al	2004	24	CD31	X250	19.4 (without necrosis) 8.2 (with necrosis) (3.25-42.75)
Sandlund et al	2006	210	CD105	X250	6.0 (0-66)
Present study	2006	45	CD31 CD105	X400 X400	31.68 (9.8-60.2) 20.66 (0-52.8).

ccc – clear cell carcinoma, pap – papillary carcinoma

(2.4/HPF), chromophobe cell carcinoma (15.42/HPF) and sarcomatoid carcinoma (12.44/HPF) than values obtained with CD31/CD105. No significant differences were obtained in cases of conventional clear cell carcinoma. The lowest number of mature vessels was found in cases of sarcomatoid carcinoma. The overall rate CD31/αSMA positive blood vessels were 1/0.62. No correlation was found between the immunohistochemical expression of VEGF and MVD ($p<0.32$).

Discussion

Angiogenesis is an important factor for malignant tumor progression, and is regulated by activators and inhibitors. Tumor angiogenesis has been well documented in experimental and clinical studies, but data for RCC are controversial. Angiogenesis was reported as the only significant predictor of prognosis in low stage RCC in some studies [17, 21], and it was found no relation with patient survival in another [22].

VEGF is a well-known activator of angiogenesis, and it was shown that VEGF mRNA in RCC is higher than in the normal kidney tissue [15]. We noticed the positive and strong reaction for VEGF in both normal renal tubule cells (tubules of the nephron and collecting ducts) and RCC cells, confirming previous reports [23, 24]. There was no VEGF expression in other structures of the normal kidney tissue. This is in contrast with data published by Paradis et al [11] that found VEGF expression in glomerular epithelial cells and epithelial cells of the proximal tubule.

In RCC, VEGF is expressed in the cytoplasm of tumor cells in 71% to 74.3% of cases [11, 25]. We found VEGF-positive reaction in 75.5% that is in accord with previous findings. Using a four-grade scale, Yagasaki et al [13] found 20.2% negative cases, 32.2% +1 cases, 25% +2 cases, and 22.6% +3 cases. A significant correlation was found between the expression of VEGF and nuclear grade. Despite of obtaining similar results regarding the immunohistochemical expression of VEGF, we were not able to demonstrate a correlation with the nuclear grade. There was no difference in VEGF expression among the different RCC types or nuclear grade in the study published by Jacobsen et al [23], proved also by our results. Recently, Jacobsen et al [26] reported different isoforms pattern among RCC types, and concluded that $VEGF_{189}$ is the only protein isoform involved in progression. This finding might be another explanation for the lack of correlation with MVD found in our study that used VEGF clone VG-1.

Many authors investigated MVD in RCC, and obtained values were significantly different. Yagasaki et al [13] found a mean MVD value of 17.99±11.94, using CD105, on 30 consecutive fields. Using this method, a significant correlation was found with VEGF expression. Hemmerlein at al [27] found a significant difference between cases with and without necrosis (7.9 versus 19.4, CD31, x250). In RCC tumor necrosis is associated with low MVD value and an increase in VEGF expression within the perinecrotic rim [8]. This finding could explain why the immunohistochemical expression of VEGF does not correlate with MVD in our study, contrary to data published by others [25]. Furthermore, these conflicting results might be caused by different methods used to stain and/or to count vessels *(Table 2)*.

The prognostic value of MVD in RCC is controversial. A positive correlation was noticed between high MVD value and a good prognosis, but most studies have shown an increased MVD to be related to a poor prognosis. Some authors reported that the patient survival was correlated with MVD [9, 11], and others found no correlation with cancer-specific survival [16, 28]. Moreover, MVD seems to decrease as the grade of the tumor decreases [8, 22]. In a study published by Imao et al [29], MVD ranged between 11 and 301, with significant difference between non-metastatic and metastatic cases (109±67 versus 58±35). They also found a significant higher value of MVD in small tumors (142±54/x400), but a correlation with grade was not found ($p=0.32$), and this finding was confirmed also by our observations. Imao et al (2004) reported that high MVD is related to the non-metastatic status and decreased MVD correlate with metastasis and is associated with reduced survival rate.

We noticed a significant difference between MVD values assessed by CD31 and CD105 (31.68 versus 20.66). CD31 is

a pan-endothelial marker and endoglin (CD105) is a membrane glycoprotein, especially expressed in tumor-associated vascular endothelium, and could be a marker of tumor angiogenesis. This is why lower MVD values with CD105 are usually expected. In our study, endoglin was positive in 39 from 45 cases (86.6%). There are few studies on the expression of endoglin in RCC, and it is accepted that about 75% of cases are positive. Our results of MVD with CD105 are similar with those published by Yagasaki et al [13], but significantly higher than mean values recently reported by Sandlund et al.[12]. Significant differences were found between different types of renal cell carcinoma, with lowest values in papillary carcinoma, and highest in clear cell carcinoma, for both CD31 and CD105. This finding strongly suggests an active angiogenesis in the tumor area, because CD105 is mainly expressed by tumor-associated endothelium.

α-SMA was positive in perivascular cells of the mature blood vessels. We noticed few mature vessels in sarcomatoid carcinoma, and significant differences between α-SMA and CD31/CD105 positive vessels in papillary and chromophobe cell carcinoma. In cases with clear cell carcinoma, the large majority of CD31 positive vessels were also positive for α-SMA. It might be hypothesized that in clear cell carcinoma, small blood vessels undergo a rapid maturation, and this could be an explanation for the non-significant differences we found between values of MVD (CD31/CD105) and the number of mature vessels. To the best of our knowledge, a comparison between different types of vessels in renal cell carcinoma was not yet performed. On the other hand, a substance that could induce rapid maturation of newly formed vessels is not known, but it could explain formation of mature vessels with large lumen, as they are found in tumors of the kidney parenchyma. The rate between immature, intermediate, and mature vessels deserves further investigation, because mature vessels are no longer accepted as target for specific therapy.

Conclusions

VEGF was expressed in 75.5% of 45 cases with RCC, and the mean value of MVD CD31/CD105 was 31.68/20.66. The immunohistochemical expression of VEGF does not correlate with MVD performed on slides stained for both CD31 and endoglin. The majority of blood vessels in the tumor area are of mature type, with perivascular cells positive for SMA.

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