VEGFR3 and CD31 as Prognostic Factors in Renal Cell Cancer

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Abstract. *Aim: To evaluate the expression levels of vascular endothelial growth factor receptor-3 (VEGFR3) and CD31 and assess their associations with grade, stage and survival in patients with renal cell cancer (RCC). Patients and Methods: Our study included 224 consecutive patients who received treatment during the years 1985-1995 in Tampere Finland but had not been treated with modern anti-angiogenesis drugs. All tumor samples were re-classified and investigated using immunohistological techniques. Data were collected from patient records and the Finnish Cancer Registry. Results: In total, 54.2% and 98.2% of the tumor samples tested positive for VEGFR3 and CD31 expression, respectively. CD31 expression levels were classified into two groups according to the median level revealing that its high expression was nearly significantly associated with low tumor stage (p=0.069). In an age- and gender-adjusted analysis, low expression of CD31 associated with poorer survival. Grade 3 and grade 4 tumors had significantly higher mortality rates compared to those of grades 1-2 (hazard ratio (HR)=4.91; 95% confidence interval (CI)=1.12-20.4; p=0.029 for grade 3 and HR=9.31; 95% CI=2.23-38.8; p=0.002 for grade 4). In addition, stage 2, 3 and 4 tumors revealed that they possessed significantly higher mortality hazard ratios compared to those of stage 1 tumors (HR=2.62; 95% CI=1.27-5.41; p=0.009 for stage 2, HR=4.37;95% CI=2.29-8.3; p<0.001 for stage 3 and HR 13.8; 95% CI=7.18-26.7; p<0.001 for stage 4). Conclusion: High CD31 expression associated significantly with better survival and VEGFR3 had no association with survival. Both higher tumor grade and stage were associated with a decreased survival time.*

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Vascular endothelial growth factor (VEGF) is an important regulator of angiogenesis (1). It has also been associated with pathological angiogenesis in tumors and ischemic, inflammatory and pathological intraocular conditions (2-4). There are five mammalian VEGF ligands, each of which occurs as several different variants. These variants bind to vascular endothelial factor receptors (VEGFRs) and induce biological responses (2). The main lymphangiogenic receptor VEGFR3 is widely expressed in blood vessels and it is essential for the development of circulation during early embryogenesis (2, 5). VEGF-C and VEGFR3 signaling are important for lymphangiogenesis and this process is activated in individuals with cancer and inflammation, while it is inactive under normal physiological conditions (5). The *VEGF* genotype +936 has been found to be associated with age-related macular degeneration (6). VEGF expression has been correlated with tumor size and stage and poor survival in renal cell cancer (RCC) patients by univariate analysis (7). Yang *et al*. have found positive VEGF expression in RCC tumor cells but negative expression in normal renal cells (8). The same study showed that positive VEGF expression is correlated with grade, lymph node involvement and vascular invasion. VEGFR-1 has been shown to be up-regulated in endothelial cells in vascular tumors (1).

Anti-angiogenic therapy inhibits the generation of new blood vessels and blocks the growth and metastasis of cancer cells (5). VEGFR3 is a highly interesting therapeutic target because it plays a role in angiogenesis, as well as in lymphatic maintenance (2). Knowledge of RCC biology has improved over the recent years. At least two cellular signaling pathways for molecular-targeted therapy, the VEGF and mammalian target of rapamycin (mTOR) pathways, are known (9). Von Hippel-Lindau (VHL) disease is associated with an increased

Table I. *Clinical and pathological characteristics*.

Patients (n=224)	132 men (58.9 %)
	92 women (41.1 %)
Median age at the time of nephrectomy	65 (IQR 55.9-71.9)
Stage	
1	79 (35.3%)
2	43 (19.2%)
3	61 (27.2%)
4	39 (17.4%)
Histology	
Clear cell renal cell carcinoma	202 (90.2%)
Papillary renal cell carcinoma	12 (5.4%)
Chromophobe renal cell carcinoma	5 (2.2%)
Sarcomatoid	2 (0.9%)
Unclassified	1 (0.4%)
Grade	
1-2	22 (9.8%)
3	114 (50.9%)
4	88 (39.3%)

IQR, Interquartile range.

Table II. *Association of tumor stage with CD31 and VEGFR3 expression according to expression level*.

	Stage				
	1 n (%)	2 n (%)	3 n (%)	4 n (%)	*p*-Value
CD31					0.069
Low	28 (37.8%)	21 (51.2%)	35 (57.6%)	22 (59.5%)	
High	46 (62.2%)	20 (48.8%)	25 (42.4%)	15 (40.5%)	
VEGFR3					0.899
Low	32 (43.8%)	19 (46.3%)	29 (49.2%)	16 (42.1%)	
High	41 (56.2%)	22 (53.7%)	59 (50.8%)	38 (57.9%)	

VEGFR3, Endothelial growth factor receptor-3.

Table III. *Age- and gender-adjusted univariate analysis of VEGFR3 and CD31 with tumor grade and stage by using Cox proportional hazard models*.

	Adjusted			
Grade	n	HR	(95 % CI)	*p*-Value
1-2	22	1		
3	114	4.91	(1.12-20.4)	0.029
4	88	9.31	(2.23-38.8)	0.002
Stage				
1	79	1		
2	44	2.62	(1.27-5.41)	0.009
3	61	4.37	(2.29-8.35)	<0.001
4	39	13.8	(7.18-26.7)	<0.001
VEGFR3 high	115	1		
VEGFR low	97	1.04	(0.69-1.56)	0.087
CD31 high	106	1		
CD31 low	106	1.53	(1.01-2.33)	0.044

VEGFR3, Endothelial growth factor receptor-3; HR, hazard ratio; CI, confidence interval.

risk of RCC. Inactivation of VHL can lead to over-production of VEGF, thereby inducing formation of highly vascular tumors, such as those observed in RCC (10). Mutations in the *VHL* gene have been reported in up to 80% of RCC patients (11). After treatment with multi-targeted tyrosine kinase inhibitor, marked changes in VEGF, VEGFR2 and VEGFR3 plasma levels have been observed in metastatic RCC patients exhibiting objective tumor responses compared to those presenting with stable or progressive disease (12). Another study found that a marked decrease in the soluble VEGF2 concentration in patients with metastatic RCC is correlated with a higher objective response rate and longer progression-free survival (13). Several promising biomarkers for VEGF-targeted therapy have been studied but none fulfilled the criteria for level I evidence (14).

CD31 is a member of an immunoglobulin superfamily that is expressed on the surfaces of circulating platelets, neutrophils, monocytes and naïve B lymphocytes. It plays a major role in tissue regeneration and its expression has been detected in vascular tumors (15). CD31 is a ligand for CD38. One previous study has shown that low CD31 and CD38 expression levels are correlated with better survival in patients with B-cell chronic lymphocytic leukemia (16). Increased CD31 expression has been demonstrated in clear cell RCC (ccRCC) compared to papillary RCC (pRCC). The same study has associated low CD31 expression with higher tumor stage and nuclear grade but has suggested that its expression is not an independent prognostic factor (17). Biswas *et al*. have demonstrated an association between elevated CD31 expression, low tumor grade and improved survival (18).

High tumor stage and grade have been correlated with decreased survival in our larger study of RCC patients treated at the Pirkanmaa Hospital District (19). VEGF is a biomarker that has been independently associated with survival in a previous study of RCC (20). There are few studies evaluating the association of VEGFR3 or CD31 with prognosis in RCC patients. None of the patients evaluated in our study cohort had been treated with the specific angiogenesis inhibitors. The aim of the present study was to evaluate VEGFR3 and CD31 expression levels as prognostic factors in RCC and to assess their associations with tumor stage and grade.

Patients and Methods

RCC. A total of 224 patients with primary RCC were included in this study. The clinical and pathological characteristics of the patients are summarized in Table I. This study included the same patients' materials as our previous study, with the exception of the

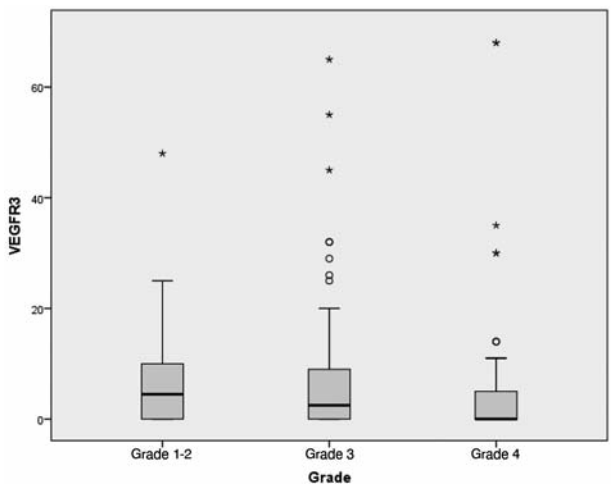

Figure 1. *Differences of vascular endothelial growth factor receptor-3 (VEGFR3) expression between tumor grades. Values are shown by median (black line), interquartile range (box) and range (line bar). Outliers and extreme cases are expressed as dots or stars.*

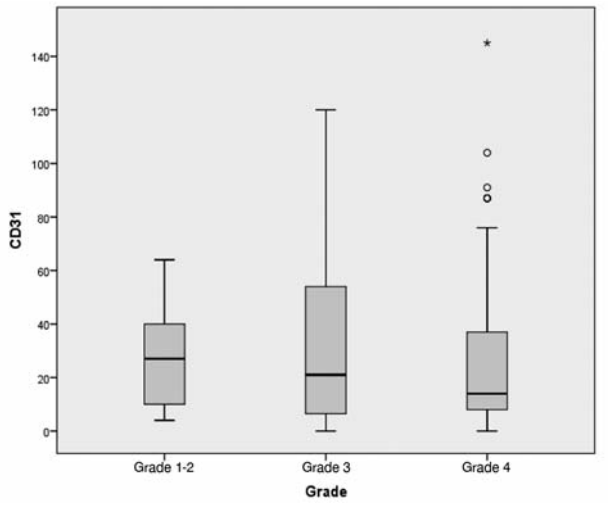

Figure 2. *Differences of CD31 expression between tumour grades. Values are shown by median (black line), interquartile range (box) and range (line bar). Outliers and extreme cases are expressed as dots or stars.*

autopsy samples (21). Patients underwent surgery between 1985 and 1995 at the Tampere University Hospital or Tampere Hospital, Tampere, Finland. RCC was pathologically staged according to the TNM 2002 classifications (22). Patients' data were collected from records at two hospitals and retrospective analysis was performed. The median follow-up time was 5.4 years, with an interquartile range (IQR) of 1.41-11.9. After nephrectomy, patient follow-up and treatment were performed according to standard clinical practice. The research protocol and use of tumor samples were approved by the ethics committee at the Tampere University Hospital and the National Authority for Medicolegal Affairs.

Histopathology. All of the tumors were re-evaluated and re-classified using the Heidelberg classification and Fuhrman grading system (23, 24) by a uropathologist (PK). A multi-tissue block was obtained from the region of each 1-mm biopsied RCC specimen with the highest grade and used for immunohistochemical analysis

Immunohistochemistry. Immunohistochemistry to assess CD31 (1:200, Novocastra Laboratories Ltd., Newcastle upon Tyne, UK) was performed on formalin-fixed, paraffin-embedded tissue sections as part of a tissue microarray (TMA). Briefly, sections were deparaffinized with xylene and rehydrated in graded alcohol, treated in an autoclave in 10 mmol/l sodium citrate (pH 5.0) for 2 min and washed with phosphate-buffered saline. They were then incubated with a primary antibody at 4˚C overnight and antibody binding was detected by a Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, USA). Diaminobenzidine (DAB) was used as the chromogen. The slides were counterstained with hematoxylin and eosin and mounted. VEGFR3 was stained with the 9D9 antibody (a mouse monoclonal antibody against the extracellular domain of human VEGFR3; a kind gift from Professor Kari Alitalo, Helsinki, Finland) at a concentration of 10 μg/ml as detailed previously (25).

The mean vessel density (MVD) was quantified as the number of CD31-positive or VEGFR3-positive microvessels per high-powered field at 250× (field of view of 0.407 mm^2, including the entire TMA core) using a Leitz Laborlux 12 bright-field microscope (Leitz GmbH, Wetzlar, Germany. The two fields with the highest vessel densities were counted and an average of the two scores was reported. Scoring was performed in a blinded manner.

Statistical analyses. Statistical analyses were performed using IBM SPSS Statistics for Windows (version 21.0, Armonk, NY, IBM Corp., released 2012). The differences between the categorical variables were tested using the Pearson Chi-square test or Fisher's exact test. Continuous variables were tested by the independent Kruskal-Wallis test due to skewed distribution. Age- and gender-adjusted univariate survival analyses were performed using the Cox proportional hazards models. Survival was illustrated by Kaplan-Meier's survival estimation methods. *p*-Values under 0.05 were considered as statistically significant.

Results

Patients. The median age of the 224 patients was 65 years (IQR, 55.9-71.9) at the time of diagnosis. The most typical tumor type observed in our study was ccRCC (90.2%). Low-grade tumors (grades 1-2) were rare (22 patients, 9.8%) and we classified the tumor grades into three groups as follows: grades 1-2, 3 and 4. Patients' basic characteristics were the same as those reported in our previous study, excluding the five autopsy samples (21) described in Table I.

Expression of VEGFR3. Negative VEGFR3 staining was observed in 97 (45.8%) of the tumors and positive staining (>0 vessels) occurred in 115 (54.2%) Twelve (5.4%) samples had poor immunostaining and were excluded from further analyses. The median number of VEGFR3-positive vessels was 2 (range=0-68). The distribution of VEGFR3 expression according to grade nearly reached statistical

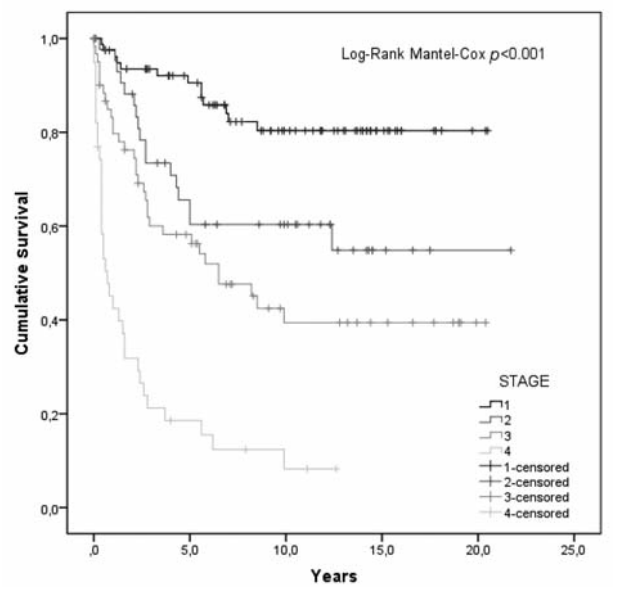

Figure 3. *Kaplan-Meier survival analysis according to tumor stage.*

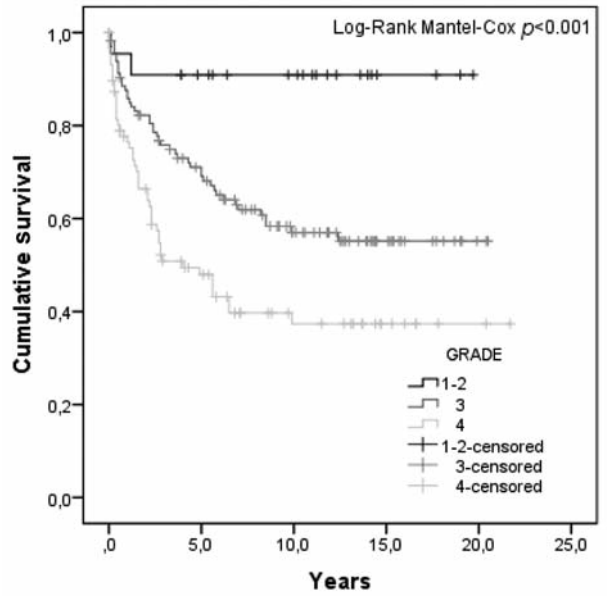

Figure 4. *Kaplan-Meier survival analysis according to tumor grade.*

significance, as shown by the independent Kruskal-Wallis test (p=0.058) but no dependence on stage (p=0.87) was observed. The VEGFR distribution and tumor grade are shown in Figure 1.

VEGFR3 expression and clinicopathological characteristics. We categorized the VEGFR expression levels into low (no positive vessels) and high (>0, positive vessels) groups. Ten (90.9%) pRCC samples showed low expression and it was high in only one (9.1%, p=0.02). Both types of sarcomatoid RCCs exhibited reduced expression and one collecting duct RCC sample showed high expression. Differential VEGFR3 expression was not observed in either the chromophobe RCC or ccRCC samples. Of the grade 1-2 tumors, six (27.3%) showed low expression and 16 (72.7%) had high expression; however, the Pearson Chi-square test showed no statistical significant association (p=0.14). Higher tumor grade did not affect VEGFR3 expression. The expression of this protein was not associated with tumor staging, as revealed by cross-tabulation (p=0.90, n=211) shown in Table II.

Expression of CD31. Negative CD31 staining was observed in only four samples (1.8%). Mean vessel density detected by CD31 expression varied from 0-145. The median expression level was 18. Twelve samples (5.4%) were of poor quality and were excluded from the analysis. The independent Kruskal-Wallis test showed no association between CD31 expression and tumor stage (p=0.31) or grade (p=0.50).

CD31 expression and clinicopathological characteristics.

The CD31 expression values were divided into two groups (low and high) using the median cut-off value of 18. All four (100%) chromophobe RCC samples exhibited low expression. The only collecting duct RCC showed high expression; reduced expression was observed in the only unclassified RCC sample. Ten (83.3%) pRCC samples exhibited low vessel density, while high vessel density was elevated only in two samples (16.7%). A total of 89 (46.6%) ccRCC samples showed a reduction in expression and an increase was observed in 102 (53.4%). A cross-tabulation of the different types of RCC samples *versus* the CD31 expression levels revealed significant differences (p=0.04) according to the Pearson Chi-Square test.

A total of 8 (38.1%) and 13 (61.9%) grade 1-2 tumor samples showed low and high CD31 expression, respectively, in addition to, 52 (48.1%) and 56 (51.9%) grade 3 tumors, and 46 (55.4%) and 37 (44.6%) grade 4 tumors, respectively. The Pearson chi-square test showed no association of low or high CD31 expression with tumor grade (p=0.35) (Figure 2).

The CD31 expression was low in 28 (37.8%) and elevated in 46 (62.2%) stage 1 tumor samples. Furthermore, its expression was low in 22 (59.5%) and high in 15 (40.5%) stage 4 tumor samples. Low CD31 expression showed a nearly statistically significant association with high tumor stage (p=0.069, n=211) shown in Table II.

Survival. The median survival time of the whole patient population was 5.6 years (IQR=1.6-11.9). Both high tumor

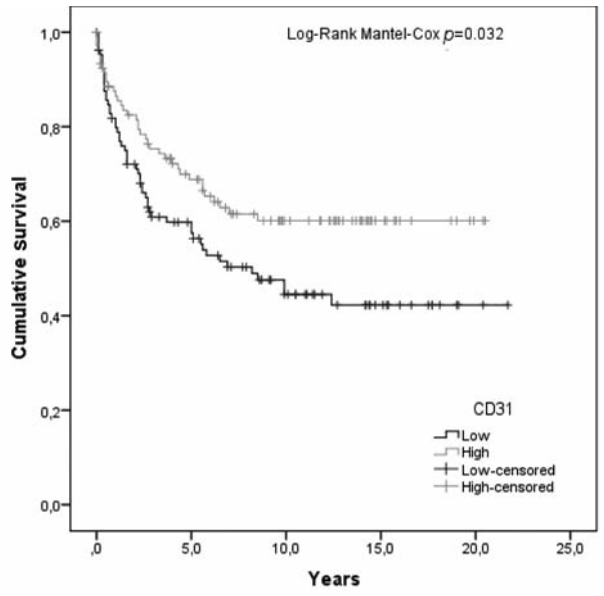

Figure 5. *Kaplan-Meier survival analysis according to CD31 intensity.*

stage and high grade were associated with decreased survival time, as determined by the age- and gender-adjusted Cox regression univariate analysis shown in Table III; (Grade 3: HR 4.91; 95% CI 1.12-20.4; and p=0.029; and grade 4: HR 9.31; 95% CI 2.23-38.8; and p=0.002 (compared to grades 1-2). Stage 2: HR 2.62; 95% CI 1.27-5.41; and p=0.009; stage 3: HR 4.37; 95% CI 2.29-8.35 and p<0.001; and stage 4: HR 13.8; 95% CI 7.18-26.7 and p<0.001, (compared to stage 1).

When the VEGFR3 and CD31 expression values were divided into low (0 and <18, respectively) and high (>0 and ≥18, respectively) groups, Cox regression univariate analysis showed that low CD31 expression associated with longer survival (low CD31 HR 1.53; 95% CI 1.01-2.33; and p=0.044 compared to high CD31, while VEGFR3 expression showed no association (low *versus* elevated VEGFR3 expression HR 1.04; 95% CI 0.69-1.56; and p=0.87), shown in Table III.

With regard to VEGFR3 expression, the RCC-specific survival (RCC-SS) was 44.5% in the low-expression group and 55.5% in the high-expression group (n=212). A total of 44.2% of the patients with RCC-SS and local tumors (stages 1-3, n=173) possessed low VEGFR3 expression and its expression was elevated in 55.8%. The Pearson Chi-square test showed no association of the five-year RCC-SS with VEGFR3 expression for any of the tumor types (p=0.78) or the local tumors (p=0.52). The RCC-SS rates were 43.3% and 56.7% for the patients with low *versus* high CD31 expression, respectively, for all of the tumor types (p=0.037) and it was 43.0% and 57.0%, respectively, for local tumors (p=0.11).

Kaplan-Meier survival analysis. Kaplan-Meier survival analysis was used to assess tumor grade, stage, as well as VEGFR3 and CD31 expression. Higher tumor stage and grade were associated with increased mortality (log-rank Mantel-Cox test p<0.001 and p<0.001, respectively) as shown in Figures 3 and 4. CD31 expression showed statistically significant association with survival (p=0.03), as shown in Figure 5. VEGFR3 expression had no association with survival in Kaplan-Meier analysis (p=0.96.)

Discussion

The aim of the present study was to evaluate VEGFR3 and CD31 expression levels as potential prognostic factors of RCC and to assess their correlations with known prognostic factors in RCC, *e.g*., tumor stage and grade. All tumor samples were re-classified and re-evaluated by one experienced uropathologist (PK). We retrospectively analyzed a series of 224 consecutive patients with RCC tumors. Immunostaining of all tumor samples was performed using TMAs. Our data included patients treated between the years 1985-1995. At that time, no specific anti-angiogenic drugs existed, although twenty-three patients were treated with interferon, which has some antiangiogenic activity.

Our understanding over the molecular mechanisms underlying tumor angiogenesis has recently increased. It has been shown that this process is a result of the interactions of several components of the tumor microenvironment (26). New targeted-therapies, such as those involving VEGF/VEGFR and mTOR pathways, have improved the survival of advanced RCC patients (27). Knowledge with regard to lymphangiogenesis in RCC is limited but some data pertaining to survival and VEGFR3 and CD31 expression in these patients are available (17, 18, 28, 29). Studies have been mainly performed on patients with metastatic renal cell cancer who have been treated with a tyrosine inhibitor or VEGF/VEGFR-blocking agents. Low VEGFR3 expression has been associated with poor survival in patients treated with sunitinib (28). Bieber *et al*. have shown no association of VEGFR3 expression with tumor stage, grade or survival in RCC patients (29). VEGFR3 expression has been found to correlate with histological grade, lymph node status and distant metastasis in one previous study of 82 patients (30). However, it has not been found to be correlated with gender, age, tumor size or TNM staging. Harmon *et al*. have shown that a low baseline plasma level of the soluble form of VEGFR3 is associated with improved progression-free survival but they found that it is not associated with OS in advanced-stage RCC patients treated with sunitinib (31).

Most patients in our study had ccRCC (90.2%), which is the most common form of RCC. A total of 97 (45.8%) of the tumors tested positive for VEGFR3 expression and the majority of the tumors (98.2%) also tested positive for CD31 expression. CD31 is known to be expressed in highly

vascular tumors (15), such as those found in RCC. Most of our tumor samples (80%) exhibited low (0-10) VEGFR3 expression, which may have been because of its general down-regulation in RCC. Immunostaining of VEGFR3 and CD31 failed in twelve cases in each group (5.3%). All tumor sections were evaluated and the most representative area of each patient's tumor sample was selected by two individuals. The samples with failed immunostaining had, presumably, only minor effects on our main results; but they were excluded from our analysis.

In a previous study, CD31 has been shown to be more highly expressed in ccRCC compared to pRCC tumors (17). Similarly, we observed in our materials its reduced expression in pRCC tumors compared to ccRCC tumors. Bieber *et al*. have reported VEGF-C and VEGF-D up-regulation in pRCC compared to ccRCC but no differential VEGFR3 expression (29). In a previous study, expression levels were divided into four groups according to staining intensity (29). Our study showed that classification of VEGFR3 expression into two groups (low and high staining intensities) resulted in the association of low expression with pRCC. Low VEGFR3 and CD31 expression levels were associated with both the chromophobe RCC and pRCC samples. The association between low CD31 expression and high tumor stage was almost statistically significant. High VEGFR3 expression has been shown to be associated with the improved survival of RCC patients after treatment with sunitinib (31). Low CD31 expression in follicular lymphoma patients is significantly correlated with increased OS and progression-free survival (32).

We categorized the VEGFR3 and CD31 expression levels into two groups. High CD31 expression associated with better survival, while VEGFR3 expression showed no association with survival. Two known prognostic factors, tumor grade and stage, were associated with survival in the RCC patients. Patients with stage 4 RCC show poor survival despite recent medical advancements. Therefore, we explored the expression levels of VEGFR3 and CD31 and assessed their correlations with survival in patients with local or metastatic RCC. Our study indicated that local RCC patients with elevated tumor CD31 expression tended to have better RCC-SS rates. However, we found no statistically significant correlation of survival with VEGFR3 expression in local or metastatic RCC patients. Lymphangiogenesis, which is a process involving signaling *via* VEGFR3, plays a role in tumor progression and metastasis (33). Further studies may be performed to assess expression levels of different marker(s), alone or in addition to those of VEGFR3 and/or CD31, to predict survival and to estimate patient responses to novel targeted-therapies. However, detection of low VEGFR3 and CD31 expression may have an additional value in differentiating between chromophobe RCC and PRCC patients.

Conclusion

Low CD31 expression levels associated with poorer survival of the RCC patients and were nearly significantly correlated with high tumor stage. Tumor grade and stage were shown to be powerful prognostic factors. Detection of the expression levels of VEGFR3, CD31 and other lymphangiogenic markers and assessments of their correlations with the survival of RCC patients require further investigation.

Conflicts of Interest

There are no conflicts of interest to be declared.

Acknowledgements

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