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Original Article

Proliferative activity, angiogenesis and nuclear morphometry in renal cell carcinoma

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Abstract

Background: Prognostic parameters other than tumor stage and grade are essential for renal cell carcinoma (RCC) patients. This study was undertaken to determine the usefulness of cellular proliferation, angiogenesis and nuclear morphometry in predicting the biological aggressiveness of RCC.

Methods: Surgical specimens of 70 patients with RCC were investigated by conventional histology, Ki-67 immunostaining and stereological assessment of angiogenesis and mean nuclear volume.

Results: There was no difference in disease-specific survival with respect to sex, age and histopathological type (except sarcomatoid and other types). The survival was significantly lower and the chance of metastases was higher in the group with higher proliferative activity ($P = 0.007$). There was no relation between angiogenesis, mean nuclear volume, stage and survival. There was a significant relation between both Fuhrman and WHO grades, tumor stage and survival. Histopathological type, grade, angiogenesis and mean nuclear volume failed to predict recurrences and/or metastases. In multivariate analysis, only TNM stage and proliferative activity were found to be independent prognostic factors.

Conclusions: In addition to tumor grade and stage, proliferative activity of a given RCC may have the potential to identify patients with an impaired prognosis.

Key words angiogenesis, Ki-67, kidney, morphometry, renal cell carcinoma.

Introduction

The pathological stage and histological grade of a tumor are considered the most important predictors of prognosis in patients with renal cell carcinoma (RCC).[1] However, in most patients, staging and grading are not sufficient to predict the course of disease. Patients with the same stage and grade may have considerable differences in survival. Therefore additional prognostic factors are necessary to predict tumor aggressiveness. This may help us to define subgroups of patients with different prognoses.

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The purpose of this study is to assess the proliferative activity, angiogenesis and mean nuclear volume in patients with RCC and to search for a prognostic factor.

Methods

Between 1989 and 1998, 70 patients (46 male, 24 female) with RCC were treated by radical nephrectomy at our institution. The age of the patients ranged between 26 and 78 years (median, 56 years). The patients were followed up for a median of 36 months (minimum 3 months and maximum 103 months). Overall survival and disease-free survival were evaluated. The disease-free survival was defined as no evidence of recurrence and/or metastases by physical examination and imaging (i.e. abdominal ultrasonography, computed tomography and chest X-ray). Patient

Table 1 Distribution of RCC cases age, sex, grade and stage

Histopathological type	*n*	Age (mean)	Sex		Fuhrman grade				WHO grade			TNM stage			
			M	F	1	2	3	4	1	2	3	I	II	III	IV
Clear cell	42	26–74 (53.1)	28	14	7	25	9	1	13	24	5	20	10	8	4
Chromophilic	5	43–62 (52.8)	4	1	2	2	1	0	2	2	1	2	1	2	0
Chromophobe	14	39–73 (59.2)	11	3	2	10	2	0	2	11	1	5	3	4	2
Sarcomatoid	9	51–78 (63.2)	3	6	0	0	3	6	0	0	9	1	1	3	4
Total	70	26–78 (56.6)	46	24	11	37	15	7	17	37	16	28	15	17	10

RCC, renal cell carcinoma.

and tumor characteristics are shown in Table 1. Eighteen patients (26%) died of disease. Four of them had clear cell RCC, three chromophobe RCC, nine sarcomatoid RCC and two chromophilic RCC. Two patients died of other causes. Thirteen patients (eight clear cell, three chromophobe and two chromophilic RCC) developed metastases. Seventeen patients were lost to follow up, but all were alive with no evidence of disease at the time of last follow up.

Histology

Formalin-fixed, paraffin-embedded surgical specimens of all patients were re-evaluated. Pathological staging was done according to the criteria of the International Union Against Cancer (UICC) TNM staging system.[2] The histological classification of Thoenes and Störkel[1] was used and grading was done according to Fuhrman *et al*. and World Health Organization (WHO)[3,4] systems.

Immunohistochemistry

Tissue sections (5 μm thick) were deparaffinized through xylene to alcohol and to distilled water. They were then placed in a microwave-resistant container with 10 mmol/L citrate buffer and exposed to microwaves for 10 min at 750 W. Sections were incubated for 1 h with the monoclonal antibodies CD31 (dilution 1 : 40; M0823, DAKO, Glostrup, Denmark) and Ki-67 (dilution 1 : 50; M0722, DAKO). Primary antibody was detected by means of the avidin–biotin–peroxidase complex technique using a commercially available reagent kit (LSAB 2 System, DAKO).

Proliferative index

Immunohistochemical staining of Ki-67 by the MIB-1 monoclonal antibody was used to calculate the Ki-67 labeling index. The number of Ki-67 positive cells per 500–1000 tumor cells were counted. The Ki-67 index was defined as the percentage of Ki-67 positive tumor cell nuclei (Fig. 1).[5]

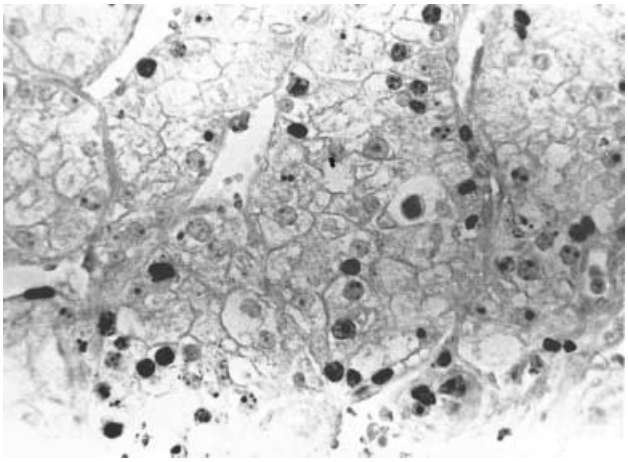


Fig. 1 Ki-67 immunostained nuclei in a clear cell RCC case (original magnification ×200).

Angiogenesis

A square lattice was superimposed on the video screen and the number of intersections (NI) between the test lines and vessel walls and the points hitting the stroma (PS) were counted. The number of vessels (NV) with the same measuring field was counted as defined (Fig. 2).[6] Vascular surface density (VSD) is equivalent to the vascular surface per unit tissue volume:

$$\mu m^2 / \mu m^3 = \mu m^{-1}$$

Microvessel number (NVES) is the number of vessels per mm^2 stroma (mm^{-2}).[6]

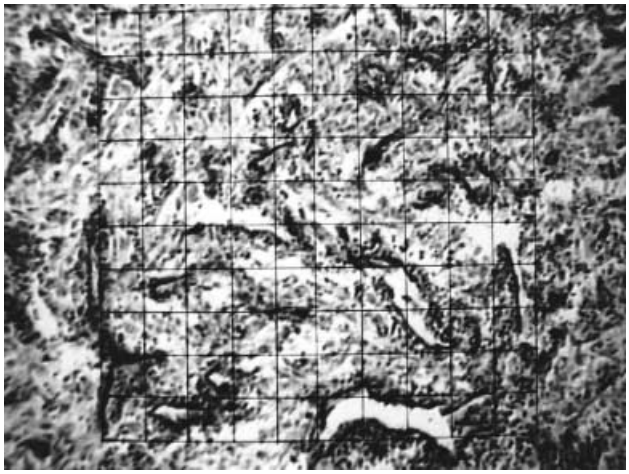


Fig. 2 The test system on the screen of the monitor for assessing angiogenetic parameters.

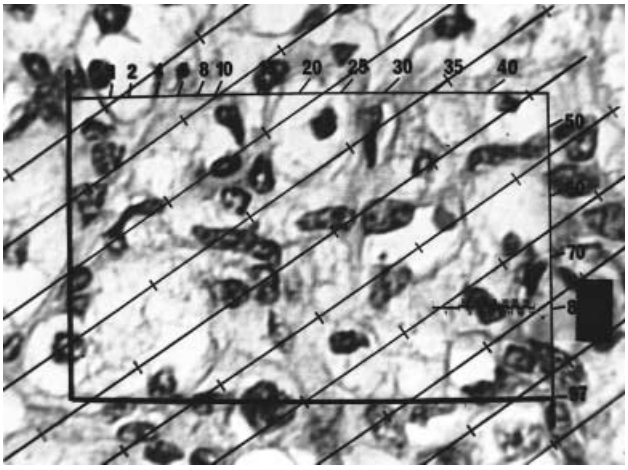


Fig. 3 The test grid and the ruler for assessing mean nuclear volume.

Mean nuclear volume

Volume–weighted mean nuclear volume (MNV) was estimated using the video-microscope system described. The test grid and the 25 mm l_3^0 ruler are shown in Fig. 3. The area of tumor demonstrating the highest grade was considered to be the most appropriate field and MNV was estimated as defined.[7]

Statistical methods

The relationship between independent prognostic factors (stage and prognosis) and various parameters (age, sex, grade, Ki-67 score, MNV, NVES, VSD, histopathological type) were evaluated by one-way ANOVA and χ^2 tests. The correlation between two parameters was evaluated by Spearman's rank correlation coefficient. Ki-67 indices, MNV, NVES and VSD, were compared according to clinicopathologic parameters using Kruskal–Wallis and Mann–Whitney *U*-tests.

The continuous variables were grouped to be applied to these analysis according to the best cut-off values obtained from corresponding single-factor receiver operating characteristics (ROC) curves with respect to the end-points. Cut-off levels were 5%, 2.5 mm^{-1}, 13 mm^{-2}, and 250 μm^3 for Ki-67 index, VSD, NVES and MNV, respectively.

Survival was measured from the time of surgical treatment until tumor-related death or last known follow up. Patients were considered censored if they were alive after the last follow up or died from a cause other than RCC. The Kaplan–Meier method was used to estimate the survival function in a group. The significant parameters were further evaluated by Cox proportional hazards model and independent prognostic factors were defined. For statistical analysis, the commercially available software Scientific Package for Social Sciences (SPSS) was used.

Results

The Ki-67 score ranged from 1.24 to 56.09% (mean ± SD, 10.31 ± 12.13).The patients were divided into two groups and the estimated survival proportion was 92% in the group with a Ki-67 score less than 5%; and 63% in the group whose Ki-67 score was more than 5% ($P = 0.007$).

Ki-67 score, MNV, NVES and VSD values and their distribution in patients that are alive with no evidence of disease, with recurrences, with metastatic disease and died of disease are shown in Table 2 and Fig. 4.

Survival decreased significantly with the increase in stage ($P = 0.001$) (Fig. 5). There was no difference in disease-specific survival with respect to sex and age. There was a significant difference between sarcomatoid and the other histopathological types ($P < 0.05$; Fig. 5).

Survival decreased with increasing Fuhrman and WHO grade ($P = 0.001$ and $P = 0.0027$, respectively). Tumor stage was directly proportional to increasing Fuhrman and WHO grade ($P = 0.0041$ and $P = 0.009$, respectively). The survival was significantly lower in the group with higher Ki-67 score ($P = 0.007$; Fig. 5). The stage and the chance of metastases increased with

Table 2 Ki-67 score, MNV, NVES and VSD values

	Min	Max	Mean ± SD
Ki-67 score (%)	1.24	56.09	10.31 ± 12.13
MNV (μm^3)	80.00	1121.79	312.23 ± 194.89
NVES (mm^{-2})	4.51	147.15	23.19 ± 20.75
VSD (mm^{-1})	1.04	8.29	3.23 ± 1.49

MNV, mean nuclear volume; NVES, microvessel number; VSD, vascular surface density.

the increase in Ki-67 score ($P = 0.027$ and $P = 0.002$, respectively). There was no difference between NVES, VSD and MNV value, stage and survival.

Table 3 summarizes the statistical analysis between the various parameters.

Disease-specific survival according to prognostic factors is given in Table 4.

No difference was observed in disease-specific survival with respect to the histopathological type, age, sex, VSD and NVES values and MNV values ($P > 0.05$). However, there was a significant difference in survival between patients with Fuhrman grade 4 and grade 2–3 ($P = 0.0011$), WHO grade 1 and 2 ($P = 0.001$), WHO grade 2 and 3 ($P = 0.0002$) (Fig. 5), Ki-67 score less than 10% and more than 11% ($P = 0.0004$), stage T2 and T3-4 ($P = 0.0003$).

Cox proportional hazards model was applied with those parameters that were prognostically significant in the univariate analysis to evaluate the interaction of these factors with respect to survival and disease-free period. Thus, Fuhrman and WHO grade, stage, Ki-67 score and histopathological type entered the multivariate model. The multivariate analysis showed that only TNM stage and Ki-67 score were independent prognostic factors.

Discussion

Renal cell carcinoma is the most common malignant kidney tumor and five-year survival rate is 30–60%.[8] Currently, tumor stage and grade are regarded as the major prognostic factors of survival in RCC.[9] Although these histological parameters provide independent prognostic information, the outcome after surgery remains uncertain and attempts to find better prognostic criteria are still ongoing.

Histopathologically, sarcomatoid RCC is well known to have a poor prognosis.[8,9] Chromophobe RCC has a better prognosis than clear cell RCC.[10] In our

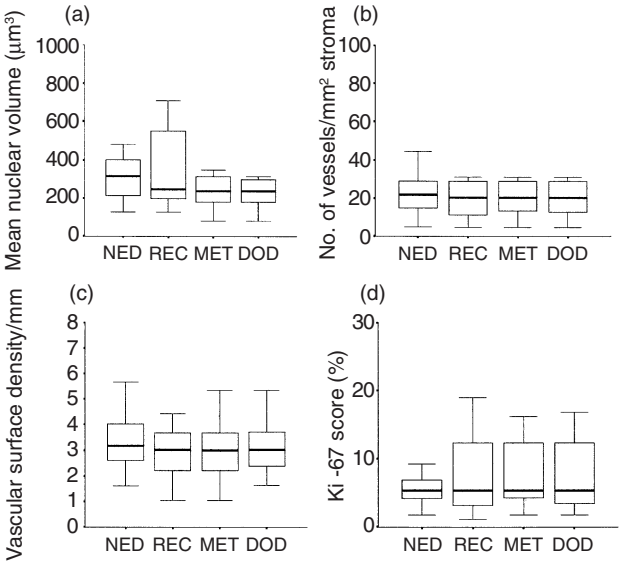


Fig. 4 The boxplot showing the median, interquartile range, outliers and extreme cases of (a) mean nuclear volume (μm^3), (b) number of vessels per mm^2 stroma, (c) vascular surface density per mm and (d) Ki-67 score (%) in patients that are alive with no evidence of disease (NED), with recurrences (REC), with metastatic disease (MET) and died of disease (DOD).

series, there were 14 chromophobe, 42 clear cell, five chromophilic (papillary) and nine sarcomatoid RCC. Sarcomatoid RCC was associated with significantly worse disease-free survival than other subtypes, but there was no difference in disease-free survival between patients with other subtypes. The prognosis for patients with chromophobe RCC was worse than that for patients with clear cell RCC. Nuclear grade of chromophobe RCC was 1 in two patients, 2 in ten and 3 in two patients, but the majority of clear cell RCC were nuclear grade 1 and 2. This might cause the difference in prognosis in these subgroups. Three of the sarcomatoid RCC cases were nuclear grade 3 and the other six were nuclear grade 4.

In some studies, there was no significant difference in survival between stage 1 and 2 disease[11] so the cut-off between these two categories was shifted from a tumor diameter of 2.5 cm to 7 cm in the last edition of the classification. In our series, stage was a significant prognostic factor.

Various grading systems have been proposed for RCC using a variety of nuclear, cytoplasmic and architectural features and most of these have been shown to have some degree of correlation with survival.[3,4] In our series, both Fuhrman nuclear grade and WHO grade were significant prognostic factors for both survival and tumor stage. There was a good correlation

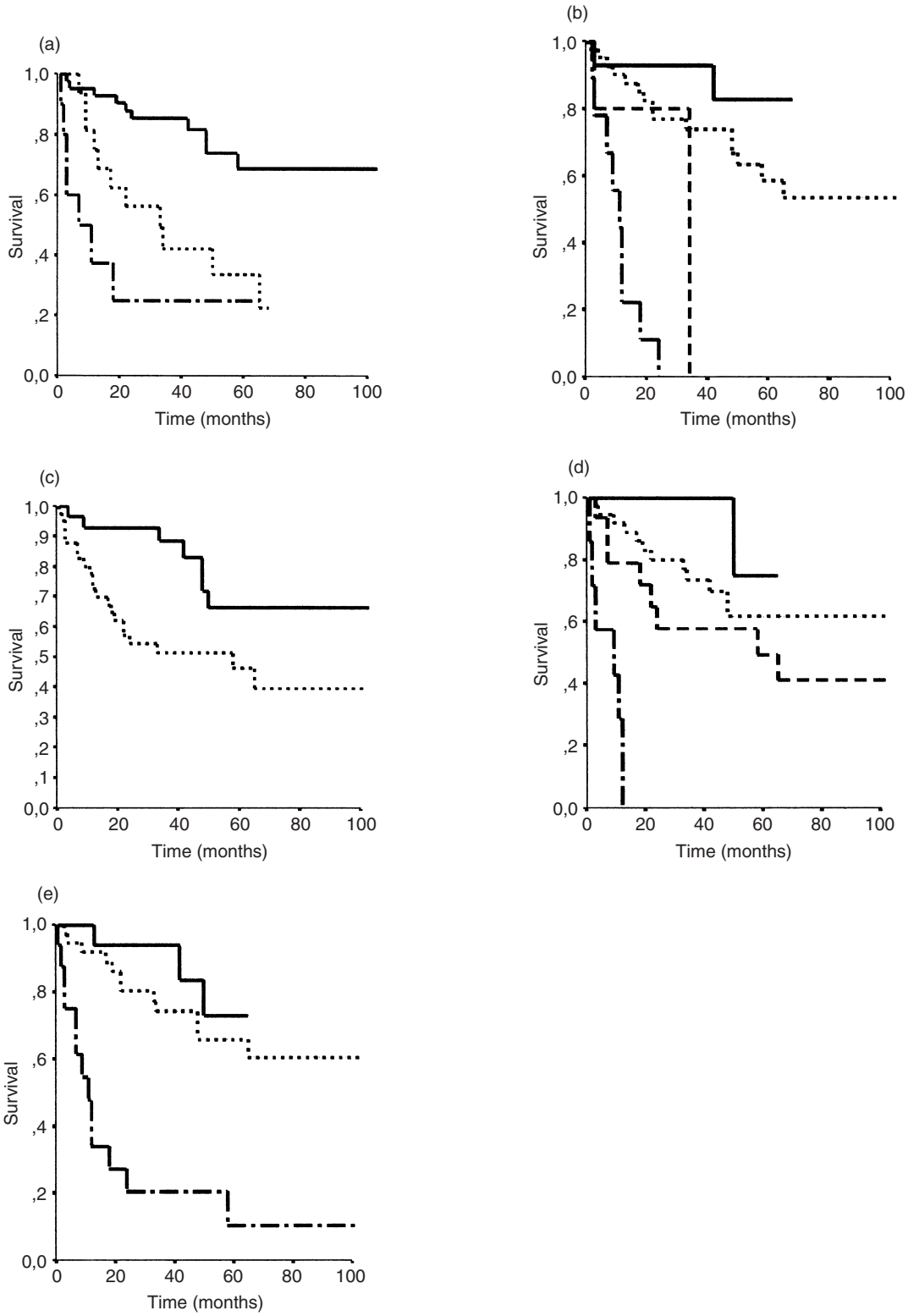


Fig. 5 Actuarial disease-related survival curves with (a) stage: —·—, stage 4; ····, stage 3; ——, stage 2. (b) Histopathological type: —·—, sarcomatoid; – –, chromophil; ····, clear cell; ——, chromophobe. (c) Ki-67 score: ····, Ki-67 > 5; ——, Ki-67 < 5. (d) Fuhrman nuclear grade: —·—, grade 4; – –, grade 3; ····, grade 2; ——, grade 1. (e) WHO grade: —·—, grade 3; ····, grade 2; ——, grade 1.

Table 3 Spearman's correlation coefficients (*r**) of statistical analysis between variables

	Fuhrman grade	WHO grade	MNV	Histopathological type
Ki-67 score	0.82	0.82	0.59	0.62
MNV	0.53	0.22	—	0.18
Histopathological type	0.72	0.63	0.18	—

MNV, mean nuclear volume; * represents good correlation, if it is greater than 0.50, moderate correlation, if it is between 0.25 and 0.50, no correlation if it is less than 0.25.

Table 4 Median survival times for patients with Fuhrman grade, Ki-67, histopathological type, VSD, NVES and MNV values

	Median survival time (months)
Fuhrman grade	
1	89
2	71
3	41
4	38
Ki-67 score	
< 5%	100
> 5%	44
Histopathological type	
Chromophobe	60
Clear cell	100
Chromophilic	39
Sarcomatoid	12
VSD	
$< 2.5\ mm^{-1}$	100
$> 2.5\ mm^{-1}$	67
NVES	
$< 13\ mm^{-2}$	100
$> 13\ mm^{-2}$	67
MNV	
$< 250\ \mu m^3$	72
$> 250\ \mu m^3$	60

VSD, vascular surface density; NVES, microvessel number; MNV, mean nuclear volume.

between WHO grade and Fuhrman nuclear grade. In multivariate analysis, neither grading system was found to be an independent prognostic factor. Most of our cases were WHO grade 1 and 2 and Fuhrman nuclear grade 1 and 2. This relative homogeneity may have affected the results. Also, the lack of reproducibility of tumor grading for RCC should be emphasized.[12]

There is an association between proliferative activity and survival for various malignancies.[13] Evaluation of cell proliferation kinetics has been undertaken using a variety of cell cycle markers.[5] Ki-67 antigen is closely associated with active cell proliferation being expressed in all phases of the cell cycle except G0.[5,13] Studies of Ki-67 antibody labeling in RCC using the MIB-1 clone, have shown Ki-67 expression to correlate to the biological behavior of the tumor, defining late recurrences.[14] The Ki-67 index in our cases were significantly higher in higher stages, grades and recurrent and metastatic tumors. There was a good correlation between Ki-67 indices and grade. This may indicate that higher grade tumors have a rapid proliferative rate. There was no difference between Ki-67 score and the histopathological types, but it was higher in sarcomatoid RCC. This significance may be dependent on the grade, as all of the sarcomatoid RCC were, naturally, high grade. In multivariate analysis, Ki-67 score was found to be an independent prognostic factor, along with tumor stage. Although only a limited number of cases have been investigated in this study, Ki-67 index seems to be an 'important' prognostic factor. In some series, this significant association was not observed.[15] This may be the result of variations in counting methodology and is, in part, because of sample size.

Angiogenesis is necessary for tumor growth and the development of metastases.[16] Angiogenesis has been correlated to tumor growth and survival for some human malignancies and may be valuable for patient selection for aggressive therapies.[17] There are various models to assess angiogenesis. Automated morphometry is an expensive and time-consuming technique, the method can not be routinely processed and is dependent on the observer's experience and skill. Vascular surface density and NVES, assessed by stereology, was shown to be reproducible and provided significant parameters for the prediction of survival and tumor stage.[6] The method can also be processed in all pathology laboratories in routine practice.

Results on the prognostic significance of angiogenesis for RCC are inconclusive. One study found a correlation between tumor microvessel density and patient outcome and tumor microvessel density has been shown to be an independent predictor of survival for

T1 and T2 tumors.[18] In another study there was no association between tumor microvessel density and survival.[19]

In this study, angiogenesis was assessed by stereology. There was no relation between VSD, NVES and patient survival, tumor stage and grade. These results correlate to the findings of Gelb *et al*.[14] Inconsistency of the results may be due to both, the different methods and to the sinusoidal vascular pattern of RCC differing from other solid tumors that have microvessel structures. This different vascular pattern of RCC also disables the use of 'hot-spot' methods to define angiogenetic properties. Therefore, impact of angiogenesis in RCC should be assessed in larger and relatively homogeneous series by measuring absolute vascular surface area within a defined volume of tumor tissue (e.g. VSD and NVES).

The other parameter tested in this analysis was mean nuclear volume. There was no correlation between MNV and patient sex, age, tumor stage, histopathological types and patient survival. Mean nuclear volume was higher in high-grade tumors ($P = 0.005$). These results concord with our previous series.[20] In this study, MNV also failed to predict metastases and recurrences.

In summary, we found that tumor stage and Ki-67 immunostaining are significantly correlated to various clinicomorphological parameters and the clinical course of RCC. High Ki-67 index and tumor stage had the potential to identify patients with impaired prognosis who thus might be candidates for early aggressive adjuvant treatment.

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