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Evaluation of microvessel density by computerised image analysis in human renal cell carcinoma

Correlation to pT category, nuclear grade, proliferative activity and occurrence of metastasis

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Abstract A high microvessel density is suspected to favour tumour progression and the occurrence of metastasis. To elucidate the significance of abundant vessels for the behaviour of human renal carcinomas, the microvessel density of 110 renal cell carcinomas was correlated to pT category, nuclear grade, proliferative activity, occurrence of metastasis and relapse-free survival interval. The microvessels were quantified using CD31 immunostaining of endothelial cells and computer-aided image analysis. The rules for reproducible microvessel counting, as defined by Weidner, were strictly observed. A statistically significant relationship between the microvessel density and nuclear grade, proliferative activity, occurrence of metastasis and relapse-free survival was found; only for tumour size could no such relation be seen. Perplexingly, there is a diminution of microvessel density in association with increasing nuclear grade, proliferative activity, relapse-free survival interval and frequency of metastasis. This finding is contradictory to the hypothesis that an increasing microvessel density indicates a worsening prognosis.

Key words Renal cell carcinoma · Microvessel density · CD 31 immunohistochemistry · Image analysis · Prognostic factor

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Introduction

The fate of patients with renal cell carcinoma is determined by the occurrence of metastasis (Nurmi 1984; Hermanek and Schrott 1990; Steinbach et al. 1992). For the assessment of prognosis, various staging and grading systems have been established, but they have a restricted value in predicting the outcome of a metastatic disease (Störkel and Jacobi 1989; Yang et al. 1992).

According to the hypothesis of Folkman and Klagsbrun (1987), newly formed blood vessels are a prerequisite for the growth of solid tumours. In addition, experimental studies have demonstrated that a high microvessel density is related to tumour progression and occurrence of metastasis (Folkman et al. 1989; Liotta et al. 1974, 1991; Ellis and Fidler 1996). Therefore, the microvessel density is a hopeful and critically discussed new prognostic parameter.

A continually increasing number of studies have examined the prognostic significance of microvessel density in different carcinoma entities including renal cell carcinoma. To the best of our knowledge an evaluation of microvessel density, pT category, nuclear grade according to Syriänen and Hjelt (1978) or proliferative activity, revealed by Ki-67 antigen immunostaining in a uniform material, is not yet available. We are also not aware of studies correlating microvessel density and metastasis formation in human renal cell carcinoma.

With the aim of assessing whether or not microvessel density has a prognostic significance in renal cell carcinoma, the vascular architecture was immunohistochemically labelled with antibodies to CD31 and microvessels were counted in an image-analysis system. These findings were correlated to established prognostic factors, to the relapse-free survival and to the occurrence of metastasis.

Materials and methods

A group of 110 nephrectomy specimens of renal cell tumours (formalin-fixed and paraffin-embedded material) from the files of the Institute of Pathology, University of Jena, were selected for investigation and classified according to the UICC criteria (Hermanek et al. 1992). There were 10 pT1, 60 pT2, 27 pT3a, 10 pT3b and 3 pT4 carcinomas; 8 patients had lymph node metastases (5 pN1, 3 pN2) and 2 patients distant metastasis (pM1) at the time of operation.

The fixation time was up to 36 h, specimens with a longer formalin fixation or a restricted Ki-67 antigen immunoreactivity were excluded. At least one histological slide was available for every centimetre of tumour diameter. Subtyping according to the Mainz classification (Thoenes et al. 1986) revealed 97 clear-cell carcinomas, 8 chromophilic, 3 chromophobic and two spindle-cell/pleomorphic carcinomas. The nuclear grade was defined according to Syriänen and Hjelt (1978). Sixteen renal cell carcinomas were of nuclear grade 1, 69 nuclear grade 2, 20 nuclear grade 3, and 5 nuclear grade 4. Serial sections of 5 μm were subjected to conventional haematoxylin/eosin staining, the periodic acid/Schiff reaction, iron colloid staining according to Hale and immunohistochemistry.

Immunohistochemistry

For Ki-67 immunostaining, antigen retrieval was necessary. The sections were deparaffinized, dispersed in 10 mM citric acid (pH = 6.0) and heated in a microwave oven at 600 W (Cattoretti et al. 1992; Hindermann et al. 1997) for 45 min (the citric acid was replenished every 5 min). The sections were then allowed to cool for 30 min. Immunohistochemical staining for CD31 and Ki-67 antigen was performed using a standardised alkaline phosphatase/monoclonal anti-(alkaline phosphatase) (APAAP) method (Cordell et al. 1984). The primary antibodies against CD31 (clone JC/70A, diluted 1:30; Dako, Denmark) or against the Ki-67 antigen (clone MIB1, diluted 1:10; Dianova, Germany) were incubated for 12 h at 4°C. After washing with TRIS buffer, sections were treated with rabbit antimouse immunoglobulins (Z-259, diluted 1:70; Dako, Denmark), and then with the mouse APAAP complex (Dako, Denmark). Both incubations were done for 45 min at room temperature. To increase the staining intensity, incubation with the rabbit anti-(mouse immunoglobulins) and with the APAAP complex was repeated twice, each for 10 min (Gustman et al. 1991). 6-Bromo-2-hydroxy-3-naphthoic acid 2-methoxyanilide phosphate (naphthol AS-BI phosphate; Sigma, 2250, USA) and new fuchsin (Merck, 4040, Germany) were used as substrate and developer respectively. To inhibit endogenous tissue enzyme activity, the developing solution was supplemented with 0.25 mmol/l levamisole (Sigma, L-9756, USA). The specificity of immunostaining was controlled by replacement of the primary antibody by non-immune serum (negative control).

Image analysis

The immunoreaction for CD31 and Ki-67 antigen was quantified by image analysis (Quantimet 500, software QWin; Leica, Germany) according to the method of Barbareschi et al. (1995a). For this procedure a "cut-off level" for positive immunohistochemical staining was defined. (Barbareschi et al. 1995b). The counting fields were interactively selected to find "neovascular hot spots" and to exclude necrosis, scars, cystic tumour degeneration and haemorrhagic areas (Weidner et al. 1993; Kosmehl et al. 1995). For counting of Ki-67-antigen-positive nuclei, the region with the highest proliferative activity was selected and the number of marked nuclei was related to the tumour area (approximately 500 tumour cell nuclei counted).

For evaluation of microvascular density, the criteria demanded by Weidner (1995) were taken into consideration. The intratumoral microvessel density was assessed by scanning the sections, in a light microscope at low power, for areas of the tumour that contained the most capillaries and small venules (the so-called neovascular hot spots). In the neovascular hot spots selected by the investigator, the microvessel density was calculated by test counting, using the computer-aided image-analysis system. By this procedure the tumour areas with the true highest microvessel density within the tumour could be selected. The individual microvessel counts were done under a microscope with a magnification of 200 in the neovascular hot spots. The identification of objects to be excluded from the analysis was achieved by using an area filter that permits exclusion of objects with an area value less than or greater than a given range of values (Barbareschi et al. 1995a, b). Furthermore, the structures selected by the computer-aided image-analysis system as microvessels were verified on the computer monitor before they were counted. The microvessels were counted as the number of marked vascular structures per scanned field of counting with a size of 0.08714 mm^2 (Fritz et al 1992; Eins and Stiller 1995). The average number of vessels counted in 40 scanned fields of the neovascular hot spots was designated as the microvessel density (maximum of microvascular density within the tumour). It corresponded to a total evaluated neovascular hot spot area of 3.485 mm^2. Weidner and coworkers (1991) considered the microvessel count in a neovascular hot spot as representative upto 0.714 mm^2.

Follow-up

In 101 cases, clinical data were available with a postoperative interval of 6–60 months. In 8 patients a preoperative metastatic disease was evident (pN1/2 and/or pM0/1) and further 17 patients developed postoperative metastases.

Statistical analysis

Statistical analysis was performed using the SPSS and Exel software (Microsoft) on an IBM compatible computer.

The values of the microvessel density and Ki-67 counts were the basis of a statistical evaluation. The univariate correlation analysis was carried out between pT category, nuclear grade, cell type, occurrence of metastasis and microvessel density or proliferative activity by the Kruskal-Wallis and Mann-Whitney tests. The relation between microvessel density and proliferative activity was investigated by the Spearman rank test (Adam 1980; Kreienbrock 1995). The trend curve resulted from the first-grade regression analysis (Bronstein 1985) using the ANASTAT software (Döhler 1995). A P value of less than 0.05 ranked as significant.

The patterns of relapse-free survival were estimated by the Kaplan-Meier method on the basis of an average 24-month median follow-up period (Harms 1992), distinguishing between two patient groups: (a) renal cell carcinoma with high microvessel density (more than 50/unit) and (b) renal cell carcinoma with low microvessel density (less than 50/unit).

The association between microvessel density and other prognostic factors was assessed by the multifactorial Cox regression model (Harms 1992).

Results

All endothelial cells visible in the histological slides of the renal cell carcinomas were immunostained by CD31 antibodies. Occasionally plasma cells were positively stained as a result of a cross-reaction. However, immunolabelling of the renal cell carcinoma cells itself was not observed.

Microvessels were heterogeneously distributed in renal cell carcinomas, as a rule being more abundant at the

periphery of tumours than in their centre. The mean number of microvessels per tumour standard area amounted to 56 and ranged from 6 to 108 (Fig. 1).

When the relation between microvessel density and pT category was examined, a high number of microvessels could be observed in small primary tumours, whereas in large carcinomas the microvessel density was lower. The differences in microvessel density between the pT categories were not statistically significant ($P = 0.0605$).

With increasing nuclear grade (according to Syriänen and Hjelt 1978) a diminution of microvessel density could be demonstrated (Table 1). The statistical correlation between microvessel density and nuclear grade was tested by the Kruskal-Wallis test (significance at $P < 0.0001$).

An increasing number of Ki-67-antigen-labelled carcinoma cells were found with rising nuclear grade. The increase of proliferative activity was associated with a decrease of microvessel density. This inverse correlation between microvessel density and proliferation was statistically significant ($P = 0.007$, Fig. 2). The Spearman correlation coefficient was −0.3005.

If the cell type of the renal cell carcinoma was taken into consideration, the clear-cell renal cell carcinomas showed a microvessel density of 59.8/tumour standard area ($n = 97$), the carcinomas with spindle and pleomorphic cell types a microvessel density of 45.0/tumour

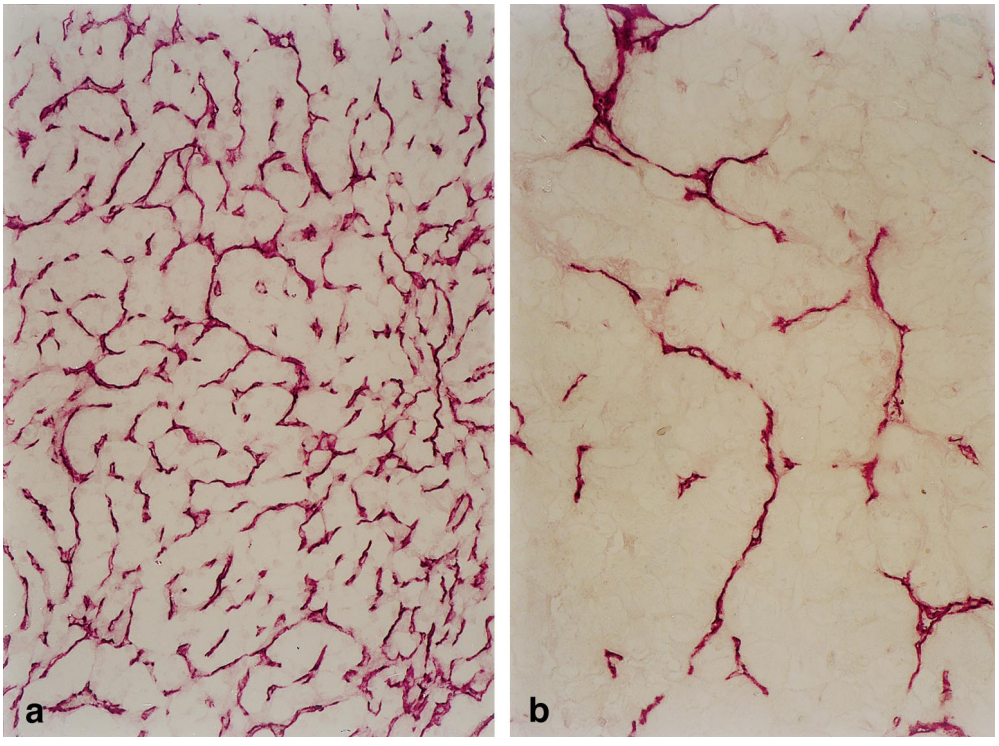

Fig. 1a, b Immunohistochemical demonstration of blood vessels with CD31 in a renal cell carcinoma of the clear-cell type (a) with high microvessel density and (b) with low microvessel density (clone JC/70A, APAAP-method, ×150)

Table 1 Results of Kruskal-Wallis univariate analysis of differences in microvessel density between various groups of nuclear grade and pT category as well as between patients with intraoperatively and postoperatively diagnosed metastasis and without metastasis of renal cell carcinoma. SD_x the standard deviation of the microvessel density

Parameter	Number of cases	Microvessel density per tumour standard area (mean values)	SD_x	P
Nuclear grade				
1	16	78.8	3.26	<0.0001
2	69	56.4	2.56	
3	20	41.4	5.54	
4	5	36.8	6.18	
pT category				
pT1	10	61.7	6.95	0.0605
pT2	60	59.2	3.11	
pT3a	27	52.1	4.43	
pT3b	10	44.8	7.01	
pT4	3	46.0	8.88	
Metastasis				
Yes	25	42.4	3.55	0.0007
No	76	60.3	2.66	

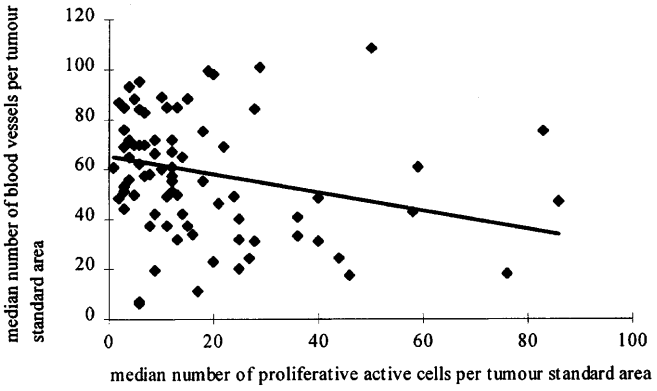

Fig. 2 Correlation of microvessel density and proliferative activity in renal cell carcinoma evaluated by means of rank test: correlation coefficient −0.3005, $P = 0.007$, resulting in a decreasing trend line after first-grade regression

standard area ($n = 2$) and the renal cell carcinomas of chromophilic and chromophobe cell type 24.9/tumour standard area ($n = 11$). In the univariate analysis (Kruskal-Wallis test) the differences between the microvessel density of the different cell types were significant ($P < 0.0001$).

In the group of renal cell carcinomas without metastasis ($n = 76$) a higher number of microvessels was found than in carcinomas with metastasis ($n = 25$). The differences in the microvessel density between these two groups was highly significant ($P = 0.0007$, Table 1). Moreover, patients with renal cell carcinomas with high microvessel density (microvessel density above 50 vessels/standard area) showed a longer relapse-free survival time than those with tumours poorer in microvessels. The difference in relapse-free survival interval between the two groups is significant ($P = 0.0017$, Fig. 3). With a cut-off value of 50 microvessels, the differences in the relapse-free survival interval between the two groups are most significant.

If the pT category was taken into account in the group of pT1 and pT2 tumours, the renal cell carcinomas with high microvessel density were associated with a significantly longer relapse-free survival (Kaplan-Meier method) than renal cell carcinomas with low microvessel density ($P = 0.0346$). For the group of pT3 and pT4 carcinomas, however, different Kaplan-Meier curves were obtained but a statistical significance could not be shown ($P = 0.1809$).

To evaluate the interaction of the various prognostic factors with respect to disease-free period, the Cox proportional-hazard regression model was subsequently applied using those parameters that were prognostically significant in the univariate analysis. pT category, nuclear grade, proliferative activity, and renal cell carcinoma cell type are identified as prognostic factors. The pT category and nuclear grade could not be introduced into the model because of instability of the single groups (high standard deviation). The solution of the multivariate model exhibits only microvessel density as an independent prognostic parameter ($P = 0.0342$), ranked before proliferative activity.

Discussion

As far as microvascular density is concerned, invasive ductal breast carcinomas belong to the best-investigated carcinomas. Numerous studies have demonstrated a clear correlation between increasing microvessel density and a worsening prognosis (Weidner et al. 1992; Van Hoef et al. 1993; Fox et al. 1994, 1995; Toi et al. 1994; Gasparini et al. 1995). But other studies were not able to confirm a relation between microvessel density and

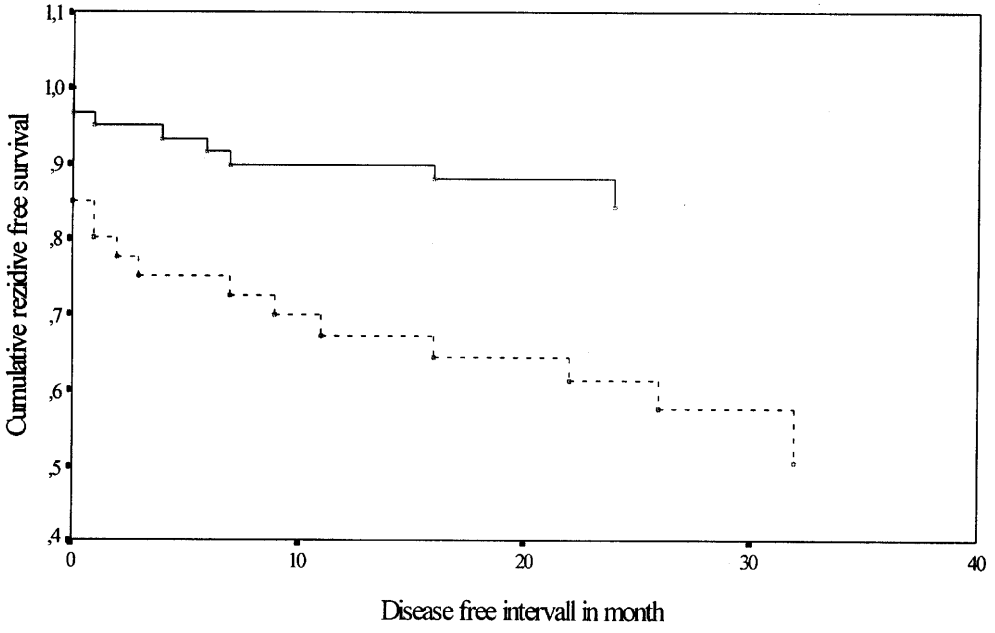

Fig. 3 Kaplan-Meier curve for the relapse-free survival interval of cases of renal cell carcinoma with high microvessel density (more than 50 blood vessels/tumour standard area, (*upper curve*) and with low microvessel density (fewer than 50 blood vessels/tumor standard area, (*lower curve*)

prognosis in breast carcinoma (Miliares et al. 1995; Axelsson et al. 1995). These different results are attributed to methodological differences in the evaluation of the microvessel density. Weidner (1995) inaugurated uniform and reproducible rules for microvessel counting, which were strictly adhered to in the present study.

For microvessel labelling the monoclonal antibody JC/70A against CD31 was used. CD31 antigen is a most sensitive and specific endothelial marker (Toi et al. 1993; Siitonen et al. 1995; Vermeulen et al. 1995; Poblet et al. 1996) and has been previously used in the counting of microvessel density (Gasparini et al. 1993; Harris et al. 1995). For quantitative evaluation of immunostained microvessels, an image analysis system was employed. This allowed reliable results to be achieved that were comparable to the results of manual microvessel counting methods, as shown by Barbareschi et al. (1995a, b) and De Jong et al. (1995).

Our study of renal cell carcinoma was able to show a correlation between the microvessel density and established prognostic factors such as pT category, nuclear grade, proliferative activity, occurrence of metastasis and relapse-free survival. This correlation was statistically significant in univariate analysis, except in the case of pT category. In multifactorial Cox regression analysis, microvessel density was the most important factor predicting the occurrence of metastatic disease, ranking before proliferative activity, shown previously to be a prognostic parameter in renal cell carcinoma (Delahunt et al. 1995; Hofmockel et al. 1995).

Microvessel density also appears to be a valuable biological parameter in renal cell carcinomas. A completely unexpected observation was the diminution of microvessel density in association with increasing nuclear grade and proliferative activity, shortening of relapse-free survival interval and frequency of metastasis. This result conflicts with the hypothesis of Folkman that increasing microvessel density is an indicator of a worsening prognosis.

In the literature, the data relating microvessel density in renal cell carcinoma to prognosis are contradictory. Yoshino et al. (1995) et al. reported that a worse prognosis for low-stage renal cell carcinomas was linked to high microvessel density. MacLennan and Bostwick (1995) examined the microvessel density in 97 cases of renal cell carcinoma and found no prognostic significance. In line with our own observations, they found an inverse correlation between microvessel density and stage as well as nuclear grade according to Fuhrmann (1982). No comment was made on these interesting findings. Recently Gerharz et al. (1996) studied microvessel density in renal cell carcinomas (79 cases; staining of endothelial cells by *Ulex europaeus* agglutinin I) and demonstrated a decrease of microvessel density in relation to a rise in nuclear grade. In view of these data from the literature and our own findings, the simple postulated relation between high microvessel density and poor prognosis is obviously not true for renal cell carcinoma (cf. Leedy et al. 1994; Reichert et al. 1995).

If the microvessel density of renal cell carcinoma is compared with that of breast carcinoma, it can be stated that breast carcinoma, with a high microvessel density and a poor prognosis, is associated with an average 136.74 vessels/mm^2 (Weidner et al. 1991). Renal cell carcinomas without metastasis revealed an average of 740.78 vessels/mm^2 and renal cell carcinomas with metastasis and a poor prognosis had still an average of 486.57 vessels/mm^2.

Mattern and Volm (1996) analysed the microvessel density of different carcinoma types (carcinomas of the lung, breast, colon, ovary and kidney) and found the value for renal cell carcinomas to be by far the highest.

In normal kidney, the tubules are closely associated with blood vessels. The renal cell carcinoma imitates the tubule differentiation (Thoenes et al. 1991). Therefore, the high microvessel density of the carcinoma tissue may be considered to reflect the normal structure, function and tissue organisation of the renal tubule system and may represent a differentiation parameter. This assumption is supported by the observation of high vascular endothelial growth factor mRNA levels in renal cell carcinoma and by the observation that microvascular endothelial cells influence tubulogenesis mediated by vascular endothelial growth factor (Nakagawa et al. 1997; Nicol et al. 1997). If the microvessel density is considered as a differentiation parameter in renal cell carcinoma, the diminution of microvascular density in relation to a high malignancy grade (increased nuclear grade and proliferative activity) becomes understandable.

In contrast to the findings of MacLennan and Bostwick (1995), the present study of 110 cases showed a statistically significant correlation of microvessel density to prognostic factors such as relapse-free survival interval or frequency of a metastastic disease. The inverse correlation implies that the relation between microvessel density and metastasis formation is more sophisticated than assumed hitherto.

So far, the contradictory results for the quantification of angiogenesis in solid human tumours have been explained by differences in the methodology and criteria of microvessel density evaluation (Vermeulen et al. 1996). Although the consensus criteria of microvessel density evaluation were applied in the present study, the results are surprising. Therefore, at least one can assume that microvessel density in renal cell carcinoma is a difficult prognostic parameter to determine.

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