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# The clinical significance of lymphangiogenesis in renal cell carcinoma

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**Background:** The formation of lymphatic vessels (lymphangiogenesis) occurs in tumor tissues and is crucial for tumor development and progression in some cancers. Lymphangiogenesis and its clinical effect on renal cell carcinoma have been less thoroughly investigated in comparison with angiogenesis. The aim of this study was to evaluate the role of lymphangiogenesis as a prognostic factor in renal cell carcinoma (RCC).

**Material/Methods:** The expression of peritumoral/intratamoral lymphatics was studied by immunohistochemical methods in paraffin-embedded nephrectomy specimens from 133 patients with clear cell carcinoma. Patients were divided into 3 groups depending on postoperative follow-up: I) patients without metastases, II) patients with metastases during follow-up, and III) patients with metastases during the operation. Peritumoral lymphatics (PTL) and intratumoral lymphatics (ITL) were immunostained with a D2-40 antibody.

**Results:** The mean number of PTL present in each group was I=14.1, II=10.6, III=12.1. The mean number of ITL present in each group was I=0.7, II=2.3, III=2.3. The 3 groups showed statistically significant differences only in the case of ITL. A mean count of ITL  $\geq 1$  is significantly associated with an increased risk of regional lymph node involvement and distant metastasis. Patients with expression ITL  $> 0.2$  and PTL  $\leq 15.2$  had a significantly shorter cancer-specific survival.

**Conclusions:** The number of ITL showed an association with more aggressive cases of RCC and progression of disease. Therefore, the level of expression ITL, together with stage and histological grading, may provide valuable predictive information about the outcome of treatment.

**Key words:** renal cell carcinoma • lymphangiogenesis • lymphatic vessels • immunohistochemistry • prognosis

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## Background

Almost 30% of renal cancer patients will die after radical treatment because of metastases formation during follow-up [1]. Such recognized prognostic factors as pathological staging (pT) and histological grading (G) are not sufficient for prognosis. Therefore, it is crucial to identify risk factors to determine which patients have a high risk of disease progression. This would allow us to establish the group of patients who need accurate follow-up and adjuvant therapy [2].

Angiogenesis is a critical step in the growth, invasive progression, and metastatic spread of solid tumors [3–6]. The formation of lymphatic vessels (lymphangiogenesis) also occurs in neoplastic tissues and is significant for tumor progression in some cancers [7–10]. As opposed to angiogenesis, lymphangiogenesis has been less thoroughly investigated in RCC, the reason being the lack of markers that can distinguish lymphatic vessels from vascular vessels. The D2-40 antibody reacts with lymphatic endothelium and may be useful in the identification of lymphatic vessels [11–13].

There are reports presenting low density of lymphatic vessels that can be observed within renal cancer. However, according to some research, their presence is connected with higher aggressiveness of the tumor and worse patient survival [14,15].

The aim of this study was to evaluate the role of lymphangiogenesis as a prognostic factor in clear cell renal cell carcinoma (ccRCC).

## Material and Methods

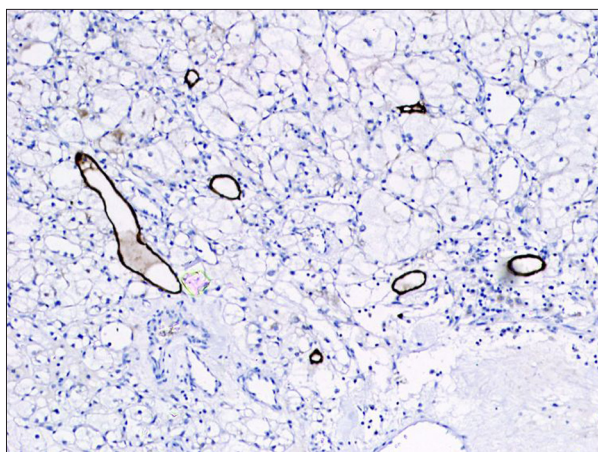
### Patients

A total of 133 patients with clear cell renal cell carcinoma, with complete documentation and follow-up, were randomly selected for study. Patients underwent surgery (radical or partial nephrectomy) at our department between 1995 and 2006. The mean age of the patients was 62.4 years (ranging from 36 to 84). The follow-up examination performed at 3-month intervals in the first year included a general examination, urinalysis, serum creatinine, and abdominal ultrasound. During the following 3 years, abdominal ultrasound was performed every 6 months, and chest and abdominal CT were performed every 12 months for the first 5 years. Patients were divided into 3 groups depending on postoperative follow-up: I – patients without metastases, II – patients with metastases during follow-up, and III – patients with metastases recognized before the operation.

Staging was assessed using the 2002 TNM classification. The nuclear grade was determined using the criteria of Fuhrman.

**Table 1.** Patient characteristics.

	Group I n=77	Group II n=20	Group III n=36	All n=133
Sex n (%)				
Male	40 (57)	15 (80)	27 (75)	82 (62)
Female	37 (43)	5 (20)	9 (25)	51 (38)
Follow up period months (range)	82.7 (12–156)	55 (13–140)	25.4 (1–146)	63.3 (0.5–156)
Mean age (years)	61.3	62.7	65.2	62.4
Side n (%)				
Right	41 (53)	8 (40)	20 (56)	69 (52)
Left	36 (47)	12 (60)	16 (44)	64 (48)
Grade n (%)				
G1	19 (25)	1 (5)	1 (3)	21 (16)
G2	44 (57)	11 (55)	12 (33)	67 (50)
G3	13 (17)	7 (35)	18 (50)	38 (29)
G4	1 (1)	1 (5)	5 (14)	7 (5)
T path. stage n (%)				
T1a	14 (18)	3 (15)	0 (0)	17 (13)
T1b	31 (41)	5 (25)	5 (14)	41 (31)
T2	11 (14)	3 (15)	3 (8)	17 (13)
T3a	15 (20)	6 (30)	11 (31)	32 (24)
T3b	5 (6)	1 (5)	9 (25)	15 (11)
T4	1 (1)	2 (10)	8 (22)	11 (8)
Mean maximal tumor diameter cm	6.0	6.7	8.4	6.7



**Figure 1.** Immunostaining with antibody D2-40 revealed intratumoral lymphatic vessels (ITL) in clear cell renal cell carcinoma (magnification 100 $\times$ ).

The main clinical and pathologic characteristics of the patients are shown in Table 1.

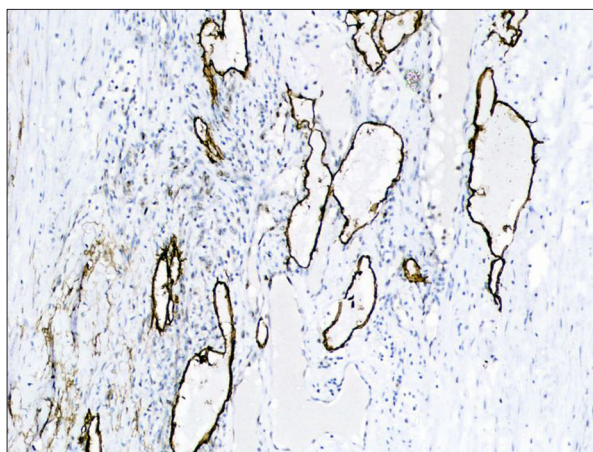
### Immunohistochemistry

The expression of peritumoral and intratumoral lymphatics was studied by immunohistochemical methods in paraffin-embedded tumor specimens. Paraffin-embedded tissue sections (5  $\mu$ m) were deparaffinized in xylene and rehydrated. The sections were treated with 0.01 M citrate buffer, pH 6.0, at 96°C (using a microwave oven set at 350 W) for 8 minutes to unmask antigenic sites. Endogenous peroxidase was blocked with peroxidase-blocking reagent (Dako). Peritumoral lymphatics (PTL) and intratumoral lymphatics (ITL) were immunostained with a 1:100 dilution of monoclonal antibody D2-40 (clone D2-40, isotype IgG1 kappa, Dako), which reacts with lymphatic endothelium and is unreactive with vascular endothelium (Figures 1 and 2).

A positive reaction was revealed using the streptavidin-biotin-peroxidase technique (Universal LSAB<sup>®</sup> Dako) and using chromogen DAB. The sections were then counterstained with hematoxylin. Positive controls for the reaction were performed with specific paraffin-embedded sections, and negative controls were made by substituting the primary antibody with non-immune serum. Normal kidney tissue from 20 specimens without renal cancer was used for the internal control.

### Evaluation

One pathologist with no prior knowledge of our pathologic findings independently interpreted the stained sections. Evaluation of lymphangiogenesis was determined by using D2-40 antibody, which reacts with lymphatic endothelium and is unreactive with vascular endothelium. Lymphatic



**Figure 2.** Immunostaining with antibody D2-40 revealed peritumoral lymphatic vessels (PTL) in clear cell renal cell carcinoma (magnification 100 $\times$ ).

vessels were assessed at 100 $\times$  magnification in every specimen in 2 compartments: intratumoral (ITL) and peritumoral (PTL). Mean number of lymphatics was calculated from 5 randomly selected fields that contained the greatest fraction of positively stained lymph vessels.

### Statistical analysis

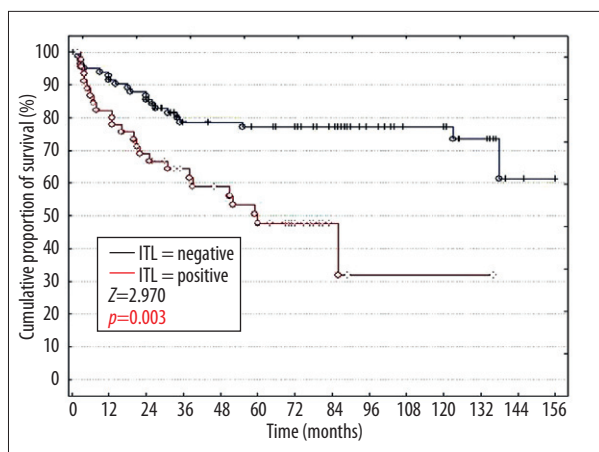
Mann-Whitney U and Kruskal-Wallis tests were used to compare nonparametric data, and the chi-squared and Spearman tests were used to assess the association between the clinicopathologic parameters and proteins expression. The threshold levels of measurable parameters have been determined using ROC curves. To conduct quantity evaluation of the test diagnostic accuracy, the area under the ROC curve was calculated, and the results were between 0.5 (lacks ability to differentiate 2 groups of objects) and 1.0 (perfect discrimination ability).

Kaplan-Meier curves were used to compare survival parameters, and the difference between groups was tested using the log-rank test.  $P < 0.05$  was considered to indicate statistical significance in all tests.

### Results

Overall median follow-up was 63.3 months (range, 1–156 months). Five- and 10-year survival proportions were 96.4% and 86.5% in group I, 34.1% and 16.8% in group II, and 11.9% and 1.6% in group III.

In the entire group of patients, the mean number of peritumoral lymphatics (PTL = 13) was 10 times higher than intratumoral lymphatics (ITL=1.3). Mean number ( $\bar{x}$ ) of peritumoral lymphatics and standard variation ( $s$ ) were of the following



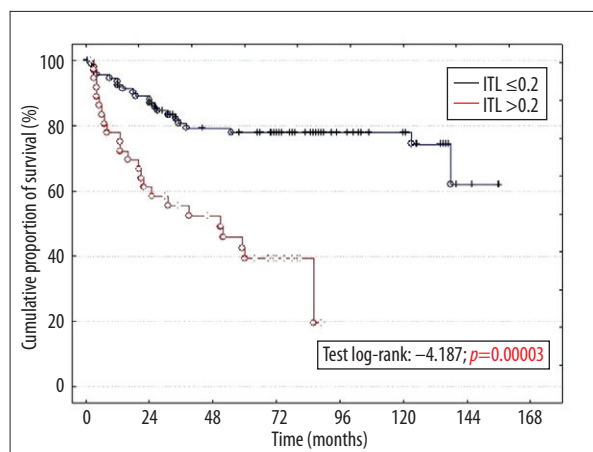
**Figure 3.** Cancer specific survival rates according to presence of ITL ( $p=0.003$ ).

levels in groups: I –  $\bar{x}=14.1$  ( $s=8.5$ ), II –  $\bar{x}=10.6$  ( $s=4.2$ ), III –  $\bar{x}=12.1$  ( $s=8.4$ ). Mean number ( $\bar{x}$ ) of intratumoral lymphatics and standard variation ( $s$ ) were of the following levels in group I –  $\bar{x}=0.7$  ( $s=2.6$ ), in group II –  $\bar{x}=2.3$  ( $s=5.5$ ), in group III –  $\bar{x}=2.3$  ( $s=4.0$ ). A statistically significant difference was found in ITL ( $p=0.002$ ).

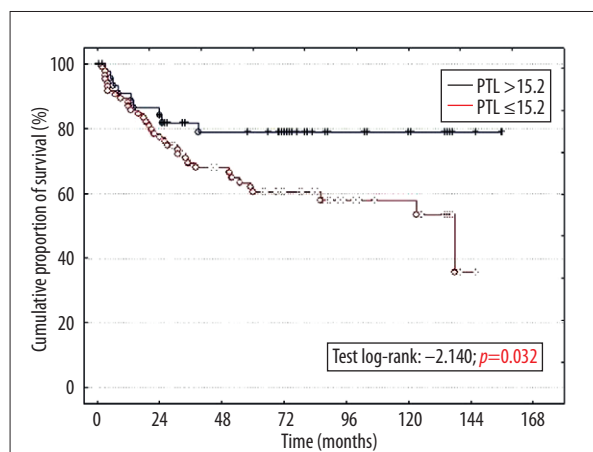
Analysis revealed significant positive associations ( $p<0.05$ ) for ITL with cancer stage according to AJCC (correlation coefficient  $r_s=0.212$ ), pathological degree of spread to local lymph nodes – pN ( $r_s=0.369$ ), disease progress after surgery ( $r_s=0.240$ ), degree of differentiation – G ( $r_s=0.178$ ), histological evaluation of tumor necrosis ( $r_s=0.236$ ), platelet count ( $r_s=0.208$ ), and erythrocyte sedimentation rate – ESR ( $r_s=0.369$ ). On the contrary, in the case of PTL, we found only negative associations ( $p<0.05$ ) with pathologic stage – pT ( $r_s=-0.180$ ), degree of differentiation – G ( $r_s=-0.243$ ), renal capsular infiltration ( $r_s=-0.213$ ), platelet count ( $r_s=-0.195$ ), and erythrocyte sedimentation rate – ESR ( $r_s=-0.395$ ).

Expression of intratumoral lymphatics was associated with clinical outcome in evaluated groups. Mean number of ITL  $\geq 1$  correlated with increased risk of developing lymph node ( $p=0.0001$ ) and distant metastases ( $p=0.022$ ). In the group with ITL  $\geq 1$ , mean percentage of positive lymph nodes and distant metastases after surgery was 26% and 51%, respectively, whereas in the group with ITL  $< 1$  it was 5% and 30.5%, respectively.

In the matter of ROC (receiver operating characteristic) curves analysis, the values of researched markers of the highest predictive meaning in the aspect of prediction, the occurrence of metastases after surgical treatment have been established. Threshold values have been established for examined factors: ITL  $> 0.2$  (sensitivity 48.2%, specificity 86.7%), PTL  $\leq 15.2$  (sensitivity 78.6%, specificity 45.3%). We also determined threshold



**Figure 4.** Cancer specific survival rates according to mean number of ITL ( $p=0.00003$ ).



**Figure 5.** Cancer specific survival rates according to mean number of PTL ( $p=0.032$ ).

values for classical prognostic factors: degree of differentiation G  $> 2$  (sensitivity 40%, specificity 81.8%), and pathologic stage pT  $> T1b$  (sensitivity 60%, specificity 58.4%). Sensitivity and specificity values of examined markers are comparable with values of ordinal predictive factors.

There was no statistically significant difference in comparison of curves for time to disease progression for 2 groups that differ with the value of analyzed markers (above and below the cut-off value determined by ROC curves). In the analysis of cancer-specific survival curves in the groups of patients with positive and negative expression of the researched markers, the statistically significant difference has been noticed ( $p=0.003$ ) only in the case of ITL parameter (Figure 3). The comparison also took place within the matters of survival curves for cancer in the groups of patients differing with the cut-off value determined through ROC curves. Patients with mean number ITL  $> 0.2$  and PTL  $\leq 15.2$  had significant shorter cancer-specific survival ( $p=0.00003$ ,  $p=0.32$ ) (Figures 4 and 5).

## Discussion

Compared to angiogenesis, little is known about lymphangiogenesis in renal cancer. One of the reasons is the lack of credible indicators enabling the differentiation of lymphatic and blood capillaries within tissue of the tumor. However, there have been several indicators identified (podoplanin [14], LYVE-1 [17], VEGFR-3 [18], VEGF-C [19,20], D2-40 [11,12]), for which specific antibodies have been created. Among them, D2-40 seems to be the most promising antibody in the matter of lymphangiogenesis diagnosis. Horiguchi et al. used antibody D2-40 to mark lymphatic vessels within clear cell carcinoma (ITL) and its surroundings (PTL). In most of the patients (94%), the presence of PTL has been revealed, and only in 18.8% of the patients has ITL been found. Correlation between ITL number and a degree of severity, metastases in lymph nodes, and shorter patient survival for renal cancer has been shown. Similar observations have been conducted in the case of other tumors (colorectal, urothelial [7–9]). The research proves the thesis that vessels are localized with the tumor, not within its neighborhood, and that they are the main way clear cell cancer cells can transfer to local lymph nodes [14].

Different results were presented by Iwata et al., who did not find out any associations between the presence of lymphatic vessels within renal cancer and its neighborhood, and clinical as well as pathological factors. They also did not find any differences between the amount of vessels in the direct neighborhood of the tumor and distant areas. Iwata et al. suggested that the process of lymphangiogenesis in RCC is of minor importance for the progress of the disease [15]. Similarly, Baldewijns et al. did not find any associations between a degree of lymphangiogenesis in renal cancer and TNM and G parameters. They showed insignificant amounts of lymphatic vessels in a tumor in comparison with the regular renal tissue [21]. The insignificant amount of lymphatic vessels within the tumor was also noticed by Voss et al., but they also found a significant amount within the pseudocapsule of a tumor [22]. Differences in the evaluation of lymphangiogenesis and its predictive value in the above-mentioned works may come from the fact that the authors used different methods of marking and interpreting the immunohistochemical tests and that the researched groups were too small.

In our own material we observed 10 times more lymph vessels in the surroundings of the tumor in comparison with the area inside the renal cancer. In the researched group, the average number of vessels ITL was 1.3, and PTL was 13. The above data prove the previous observations that lymphangiogenesis is present in renal cancer in a lower degree than in angiogenesis. This may explain why RCC metastases occur more often by bloodstream than by the lymphatic system. In the case of ITL vessels number, we found statistically important

differences in groups of patients differing in clinical course of disease. The number of ITL vessels in the group without metastases was more than 3 times lower than in the group of patients with metastases after surgeries and during the diagnosis of a renal tumor. The analysis regarding the dichotomic division of preparations with the average ITL value of  $\geq 1$  and  $< 1$  showed that the ITL number  $\geq 1$  is associated with higher risk of metastases to local lymph nodes and with higher risk of metastases after radical surgery because of the renal cancer.

In the researched group, we were able to find positive ITL associations between many factors of clinical and pathological character with degree of pathologic condition of lymph nodes (pN), degree of differentiation according to Fuhrman (G), degree of severity according to AJCC, and with progress of cancer after the surgery. In the matter of PTL, we observed counter-correlations between, among others, pathological degree of severity (pT) and degree of differentiation according to Fuhrman (G).

Threshold value of the number of lymphatic vessels within a tumor (ITL  $> 0.2$ ) and outside it (PTL  $\geq 15.2$ ) that was determined in ROC analysis, enabled the prediction of clear cell cancer after radical treatment with sensitivity of 48.2% and 78.6% and specificity of 86.7% and 45.3%. Analogically determined threshold values for ordinary predictive factors, ie, the grade (G  $> 2$ ) and local severity (pT  $> 1b$ ), showed comparable test sensitivity and specificity.

There were no significant differences found when comparing the curves of time and occurrence of the disease in 2 groups above and below ITL and PTL values determined through ROC curves. In the matter of cancer-specific survival in the groups of patients differing with the cut-off value determined through ROC curves, we found significantly lower survival in the groups of ITL  $> 0.2$  and PTL  $\geq 15.2$ . During the analysis of the cancer-specific survival curves, in a group of patients with intratumoral lymphatics (ITL), we observed significantly shorter survival in comparison with the group without ITL.

## Conclusions

Our results prove a promising predictive value of examining lymphangiogenesis in the case of renal cancer. The number of lymphatic vessels in clear cell renal cell cancer (ccRCC) is significantly lower than in the regular renal tissue. Lymphangiogenesis shows an association with researched features of clinical and pathological character in ccRCC. An increased number of lymphatic vessels within a tumor (ITL) is connected with more severe forms of the cancer and with risk of metastases. ITL and PTL parameters show association with the cancer-specific survival. They also enable the identification of the patients who are at the highest risk of having the metastases.

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