Prognostic significance of periodic acid-Schiff-positive patterns in clear cell renal cell carcinoma

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Background: *The ability of aggressive tumors to form nonendothelial tumor cell-lined microvascular channels is known as "vasculogenic mimicry" (VM). VM channels are revealed as periodic acid-Schiff (PAS)-positive patterns, and in some tumors their presence predicts clinical outcomes.*

Objective: *We aimed to study VM channels in clear cell renal cell carcinoma (cRCC) tumors and explore their prognostic significance and relationship to other suggested prognostic factors such as thymidine phosphorylase (TP) and vascular endothelial growth factor (VEGF) expression.*

Methods: *We retrospectively studied 45 patients who had undergone radical nephrectomy for clinically confined cRCC (stage T2-T3NOMO) at the Russian Cancer Research Center. The tumor sections were reviewed for disease stage, nuclear grade, perirenal fat invasion, and lymph node involvement, and we performed immunohistochemical staining for VEGF and TP expression, and PAS staining. Disease-free survival probabilities were determined by Kaplan-Meier estimates and prognostic factors were evaluated by univariate analysis.*

Results: *PAS-positive patterns observed in the cRCC tumor included back-to-back closed loops, networks, arcs, and parallel patterns. There was a significant decrease in disease-free survival among patients with PAS-positive networks ($p = 0.005$), but not among patients with other PAS-positive patterns. TP expression was also a significant predictor of disease-free survival ($p = 0.035$), but this factor did not correlate with the presence of PAS-positive networks. Notably, in our small sample, the six patients whose tumors were positive for both factors had the highest risk of cancer recurrence.*

Conclusions: *The presence of PAS-positive networks is an independent and relevant prognostic parameter for disease-free survival in patients with cRCC. Our data suggest that the combination of PAS-positive networks and TP expression may identify patients with the highest risk of cancer recurrence.*

Key Words: renal cell carcinoma, PAS-positive patterns, prognostic significance

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Introduction

Growth, proliferation, and metastasis of tumors are thought to be angiogenesis-dependent processes.[1] However, some tumors acquire microcirculation in an alternative, angiogenesis-independent manner.[2,3] Highly aggressive primary and metastatic melanomas, unlike nonaggressive melanomas, have been shown to form a pattern of matrix-rich, tumor cell-lined circulation channels, similar to embryonic vasculogenic patterned networks.[4] Reconstitution of matrix patterns from melanoma tumor cells is termed "vasculogenic mimicry" (VM), because as in vasculogenesis, the

channels are generated de novo. VM has been described in several other aggressive tumor models, including breast, prostate, and ovarian carcinoma.[5-7] Based on histological observations showing anastomoses between VM channels and normal blood vessels, and given that *in vivo* confocal angiograms partially match the VM patterns, it has been suggested that tumor cell-lined channels contribute to a functional microcirculation in tumors.[8]

In tissue sections, the basement membrane of VM channels stains positive with periodic acid-Schiff reagent (PAS) and this can take the form of several morphological patterns: straight channels, parallel straight channels, cross-linked straight channels, arcs (incompletely closed loops), closed loops, and networks (that can be defined as at least three, back-to-back, closed PAS-positive loops).[9] Investigators have reported a strong statistical association between the presence of PAS-positive patterns and clinical outcomes in primary uveal and cutaneous melanomas.[9] In that study, the histological presence of networks or cross-linked, parallel PAS-positive structures was strongly associated with death from metastatic uveal melanoma. Subsequent independent studies also identified these patterns and confirmed their prognostic significance.[10,11]

Kidney cancer is often diagnosed at an advanced stage when widespread metastases have already occurred. Even when a kidney tumor is diagnosed early, in many cases micrometastases and distant metastases are already present at the time of diagnosis.[12,13] In adults, 80% to 90% of all primary cancers originating in the kidney are renal cell carcinomas (RCCs) and are associated with a defect in the von Hippel-Lindau (VHL) tumor suppressor gene.[14] Recent advances in tumor biology have led to the development of various treatment options in RCC. With the advent of promising drugs, predicting the outcomes in patients with RCC has gained immense importance, since viable options for further treatment can now be explored in high-risk patient groups.

Until recently, tumor stage at the time of nephrectomy was considered the most important prognostic factor for RCC.[15] With increasing evidence that this parameter does not accurately predict outcome, several investigators have attempted to identify other prognostic factors. Many authors have focused their efforts on studying the relationship between angiogenesis and disease recurrence in RCC,[16-18] but to date, results have been inconclusive. One study demonstrated that compared to papillary RCC tumors, cRCC tumors were able to up-regulate angiogenic growth factor receptors more efficiently.[19] This study suggested that another pathway in addition to VHL might be involved in the regulation of angiogenesis in cRCC, and this combined regulation might account for the more aggressive phenotype seen in cRCC. Based on the observation that VM channels appear in aggressive tumors, we hypothesized that in a similar manner, cRCC may generate matrix-rich, embryonic-like patterned networks, which may somehow be involved with angiogenic pathways in disease progression. To investigate this, we explored VM in cRCC and tested its relationship with known angiogenic regulators such as TP and VEGF.

Materials and methods

Patients

The Russian Cancer Research Center maintains a database of patients who were seen at the Russian Cancer Research Center, which contains clinical and pathological records, treatment methods, and outcomes. The current study included patients in the database for whom unstained slides of cRCC were available. Node involvement was determined by intraoperative and preoperative findings from ultrasonography and lymphodissection. Metastasis category was based on results from computed tomography scans, chest x-rays, and bone scans.

Materials

PAS-staining kits were purchased from the Sigma Chemical Company (St. Louis, Missouri, United States). Anti-VEGF (monoclonal, clone VG1), anti-laminin (polyclonal, rabbit), anti-CD31 (clone JV70A) and anti-CD34 (clone QBEnd-10) primary antibodies were obtained from DakoCytomation (Glostrup, Denmark). Anti-thymidine phosphorylase (clone P-GF.44C) primary antibody was obtained from Oncogene Research Products (San Diego, United States).

Light microscopy

Histochemical staining of nonvascular structures was performed as described elsewhere.[4] Briefly, we prepared slides from serial sections of paraffin-embedded, formaldehyde-fixed tissues that had a thickness of 5 µm. To highlight matrix-rich networks, the slides were deparaffinized, hydrated in water, and oxidized in 0.5% periodic acid solution for 5 minutes. The slides were rinsed in distilled water, placed in Schiff reagent for 15 minutes, and washed in lukewarm water for 5 minutes. PAS-positive patterns were identified by light microscopy using a Nikon Eclipse TE2000 microscope (Nikon Instech, Japan).

Immunohistochemical staining

Immunostaining for laminin in the tissue sections was performed using a monoclonal anti-laminin antibody. Briefly, the samples were treated with proteinase K for 15 minutes, washed with deionized water, and rinsed with Tris-EDTA (pH 9.0) for 10 minutes. Subsequent steps (3% H_2O_2 for 5 minutes, avidin for 15 minutes, biotin for 15 minutes, DAKO protein block serum-free for 7 minutes, and laminin antibody for 45 minutes) were performed using EnVision+kits (DakoCytomation, Glostrup, Denmark). Cellular expression of TP, VEGF, CD31, and CD34 proteins was determined by immunohistochemical staining using monoclonal antibodies to TP, VEGF, CD31, and CD34. LSAB+kits (DakoCytomation) were used as a visualization system. Antigen retrieval was done in 0.01 mM citrate buffer (pH = 6.0) for 30 minutes. Tumors were designated as being TP-positive if more than 20% of the tumor cells were stained with a 2+ or 3+ intensity.

Statistical analysis

Statistical evaluations were performed using Statistical Package for Social Sciences, version 10 (SPSS).[20] A p value of less than 0.05 was considered significant for all tests. Survival curves were developed using the Kaplan-Meier method and were analyzed using the log-rank test. The presence or absence of each PAS-positive pattern was tested against disease-free survival, as an outcome measure.

Results

The study population comprised 45 patients (27 men and 18 women) with cRCC who were seen in the Department of Urology at the Russian Cancer Research Center, and who underwent radical nephrectomy to have their tumors removed. None of the patients had received prior chemotherapy or radiation therapy. The patients ranged in age from 35 to 75 years. Their mean age ± SD was 54.6 ± 9.7 years). Most patients, 42 (93.3 %) were diagnosed with stage T2 disease, and three patients (6.7 %) had stage T3 disease. Of the 45 patients, 3 (6.7 %), 22 (48.9%), and 20 (44.4 %) had grade 1, grade 2, and grade 3 disease, respectively. Perirenal fat invasion was microscopically diagnosed in two patients, resulting in upstaging the disease to stage T3a. None of the patients had lymph node involvement. Tumor recurrence or metastasis was observed in 14 patients (31.1%). The median time to recurrence was 23 months. Disease-free survivors were followed for 46 to 160 months. By study end, seven patients had died from cRCC, five patients had died from other diseases, and 33 patients remained alive.

The clinical and pathological characteristics of the patients and tumors, for cases with and without tumor recurrence, are summarized in Table 1. Immunohistochemical staining was performed to determine if expression of VEGF and TP angiogenic factors correlated with disease-free survival. The expression of VEGF in a tumor had no statistically significant impact on disease-free outcome (p = 0.176). However, TP expression did have a statistically significant impact on disease-free outcome (p = 0.035).

To evaluate the putative existence of tumor cell-lined channels in kidney cancer, the cRCC tumors were examined histologically using PAS staining. The tumors contained the following PAS-positive patterns: back-to-back loops in 8 (17.7 %) tumors, networks (at least three loops) in 11 (24.4 %) tumors, parallel patterns in 3 (6.6 %) tumors, arc patterns in 9 (20%) tumors, and straight patterns in 14 (31.1%) tumors, Figure 1a -1c. Loops and networks tended to be localized at the peripheries of the tumors. In tumors with evidence of VM channels, ten high-power fields were examined

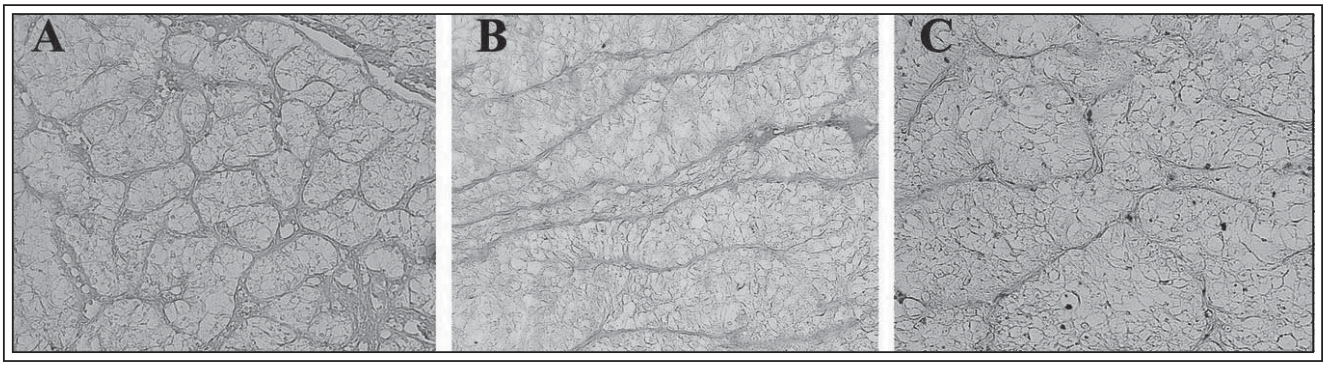

Figure 1. PAS-positive patterns in cRCC. (a) PAS-positive material from back-to-back closed loops. Networks were defined by the presence of at least 3 loops. (b)Parallel PAS-positive patterns. (c) PAS-positive arc with branching.

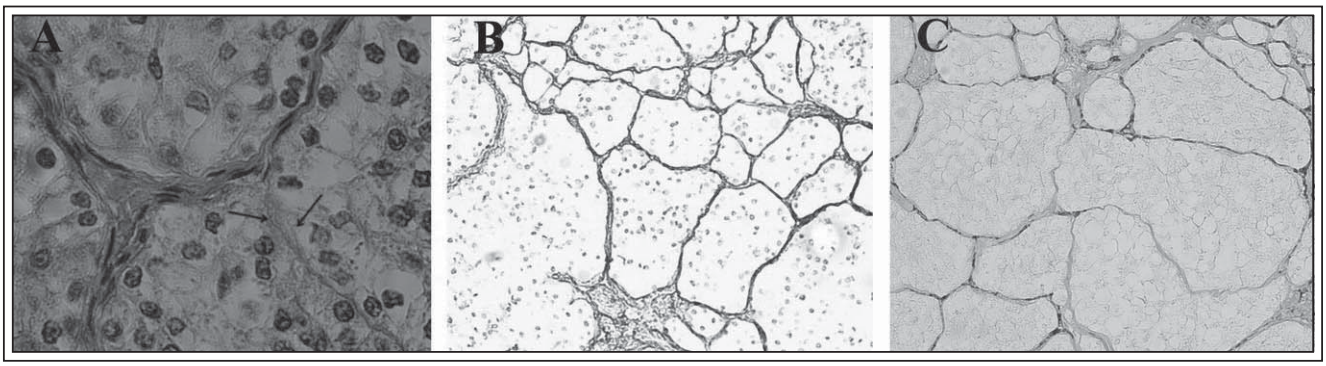

Figure 2. Histology of the cRCC microcirculation. (a) Representative light microscopy pictures of high-stage cRCC from an H&E stained histological section. The structures corresponding to the tumor cell-lined channels consist of solid cords (arrow) that connect to round channels lined by endothelium (arrowhead). Note that these channels are lined by tumor cells and no endothelial cells are identified. (b) Immunohistochemical peroxidase staining of a high-stage cRCC histological section showing laminin. (c) Tissue sections were stained by anti-(CD31 + CD34) antibodies and by PAS. The connection between tumor cell-lined channels and endothelial cell-lined vessels are visible. Original magnification x 60 (a), x 40 (b, c).

to estimate the proportion of channels that were not lined by endothelium; this ranged from 30% to 40% in the three most aggressive tumors that exhibited this phenomenon.

Conventional H&E staining revealed the PAS-positive loops contained a solid component. The VM channels also connected to somewhat larger vascular spaces, Figure 2a. The expression of the extracellular matrix molecule laminin, a component of looping patterns in human melanoma, ovarian, and breast cancers, confirmed the presence of tumor cell-lined channels, Figure 2b. Double staining of tissue sections of cRCC with both anti CD31 and anti CD34 antibodies as well as PAS reagent confirmed that the structures corresponding to the PAS-positive components of networks connected to round channels lined by endothelium, Figure 2c.

Therefore, by conventional light microscopy and immunohistochemical analysis, the looping patterned, matrix-associated vascular channels of cRCC were not found to be lined by vascular endothelium.

Kaplan-Meier survival statistics were used to test for disease-free outcomes with each PAS-positive

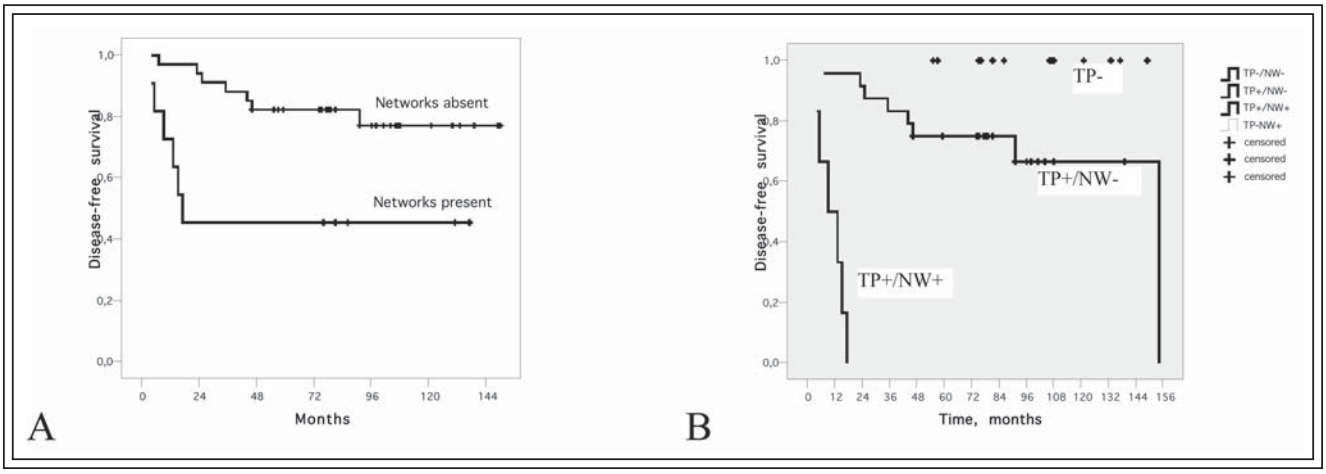

Figure 3. Kaplan-Meier curves for cause-specific survival. (a) cRCC without networks versus cRCC with networks. (b) TP-negative cRCC versus TP-positive cRCC; TP-positive cRCC with networks versus all other TP-positive cases; and TP-positive cRCC with networks versus all other cRCC with networks. The differences between the curves were evaluated using the log-rank test. $p = 0.036$ between TP (-) and NW (-) TP (+). $p < 0.001$ between TP (-) and NW (+) TP (+).

pattern, Table 1. The only VM pattern that showed an independent statistically significant impact on disease-free outcomes was a PAS-positive network pattern ($\chi 2 = 7.813$; $p = 0.005$). Figure 3a indicates that the group of patients whose tumors contained PAS-positive network patterns had a significantly lower probability of disease-free survival than the group of patients whose tumors did not contain these patterns. Notably, the three patients with stage T3 tumors did not have PAS-positive networks. As a prognostic factor, the presence of PAS-positive networks did not correlate with TP expression in tumors ($p = 1.00$). Moreover, as the cancer in TP (-) patients in our sample never progressed, combining the two factors brought about a dramatic division in the network-positive group: the cancer in each of the six NW (+) TP (+) patients progressed within 15 months, whereas it did not progress in any of the 5 NW (+) TP (-) patients within a 5 year or longer follow up ($p < 0.001$).

TABLE 1. **Patient and tumor characteristics in patients with versus without tumor recurrence**

Characteristic	Patients	Recurrence (14 patients)	No recurrence (31 patients)	$\chi 2$ (log rank test)	p (log rank test)
Patient					
Age (years)					
Mean ± SD	54.6 ± 9.7	56.2 ± 11.7	54.0 ± 9.0		ns
Median (range)	56.5 (35-75)	57 (36-75)	56 (35-66)		
Sex				1.248	0.264
Male	27	9 (33.3%)	18 (66.7%)		
Female	18	5 (27.7%)	13 (72.3%)		
Tumor					
Granular component				0.616	0.433
Absent	38	13 (34.2%)	25 (65.8%)		
Present	7	1 (14.2%)	6 (85.8%)		
Stage (TNM 1997)				1.120	0.29
T2	42	11 (26.2%)	31 (73.8%)		
T3	3	3 (100%)	0		
Grade				1.054	0.59
1	3	0	3		
2	22	8 (36.3%)	14 (63.7%)		
3	20	6 (30.0%)	14 (70.0%)		
Tumor size (cm)				1.592	0.451
< 4	8	2	6		
4.1-7	15	4	11		
> 7	22	9	13		
PAS-positive patterns present					
Straight	14	3 (21.4%)	11 (78.6%)	0.457	0.499
Parallel	3	1 (33.3%)	2 (66.7%)	0.1	0.751
Loops	8	0 (0%)	8 (100%)	3.465	0.063
Arc	9	4 (44.4%)	5 (55.6%)	0.005	0.942
Networks	11	6 (54.5%)	5 (45.5%)	7.813	0.005
TP expression	8 (-)	0	8		
	37 (+)	14	23	4.436	0.035
		networks 6			
		straight 3			
		arc 5			
VEGF expression	26 (-)	12	14		
	19 (+)	2	17	1.834	0.176

PAS = periodic acid-Schiff; TP = thymidine phosphorylase; VEGF = vascular endothelial growth factor

Thus, in our small sample, patients with a TP (-) phenotype had a very small chance of cancer progression after radical nephrectomy; patients in the NW (+)TP (+) group were at highest risk and had a good chance of cancer recurrence within a year and a half after the surgery; and patients in the NW (-) TP (+) group had an approximate 50% probability of cancer recurrence within 2 to 11 years after the surgery.

Discussion

A major problem in management of patients with RCC is predicting tumor behavior. Although stage, grade, and histologic subtype are well known prognostic factors,[13] patients with histologically comparable tumors can have completely different outcomes. Furthermore, despite the increasing rate of incidental detection of RCC, the rate of mortality in these patients has remained the same and is increasing parallel to the incidence rate.[16] This has led to an extensive search for new tumor characteristics that might influence patient outcomes. In this study, we tested whether the formation of VM channels could be such a factor.

We observed various PAS-positive VM patterns in all 45 patients. The identification of PAS-positive VM patterns was reproducible. Light microscopy revealed that the interior of these hollow channels was not lined by endothelium. We also examined the expression of laminin, a critical glycoprotein and a major component of VM looping patterns in human cutaneous melanoma, ovarian cancer, and inflammatory breast cancer.[21] As expected, the clear cell tumors were able to deposit laminin in looping patterns. Immunohistochemical double staining with anti-(CD31+CD34) antibodies and PAS also clearly showed that VM channels existed in cRCC and that these channels were connected with vessels lined by endothelium.

We investigated a somewhat small, but relatively homogeneous group of patients with cRCC and no metastases at the time of radical nephrectomy who received no treatment until recurrence of renal cancer, if any. As two-thirds of the patients did not show disease recurrence within a follow up of 46 months or longer, and as disease recurrence in the remaining patients ranged across a broad time interval (4 to 154 months), we focused on the ability of factors to predict disease-free survival. We used Kaplan-Meier statistics to test for disease-free survival associated with each PAS-positive pattern. This analysis indicated that patients whose tumors contained PAS-positive VM network patterns had significantly lower disease-free survival probabilities than patients whose tumors contained only looping, arcing, straight, or parallel PAS-positive VM patterns. Tumors that contained network patterns also tended to be thicker. As in similar previous studies, in addition to PAS patterns, TP expression also carried prognostic significance in patients with RCC,[22] whereas VEGF expression did not.[16,23]

Our data strongly suggest that a combination of PAS-positive networks and elevated TP expression may be a good predictor of RCC recurrence. It should be noted that the tendency of longer disease-free survival in patients with RCC who have low levels of TP expression is well documented in much larger patient populations. For example, in one large study,[22] 5-year cause-specific survival among patients with low TP expression was 93.6%, and a high level of TP expression was a significantly unfavorable prognostic factor in patients with stage pT1 and stage pT2 cancers.

In many studies, although disease-free survival was not directly analyzed by the authors, the study's statistics definitely support a longer disease-free survival in patients with RCC who have low levels of TP expression. In one such study, the risk of death in patients with RCC that expressed high levels of TP was 3.95-fold higher than that in patients with carcinomas that expressed no or low levels of TP (95% confidence interval; $p = 0.039$).[21] In our somewhat small sample, this tendency was striking. There was no RCC recurrence in patients in the TP (-) group (8 cases); all the cases of early RCC recurrence were in patients in the NW (+) TP (+) group (6 cases; recurrence times 4, 5, 9, 12, 13 and 15 months) and all the late recurrence cases were in patients in the NW(-) TP (+) group (8 cases; recurrence times 25, 28, 35, 44, 46, 78, 94, and 154 months). Therefore, our study suggests that VM channels are a significant independent prognostic factor in cRCC, and this warrants a large-scale study of NW/TP phenotype as a disease-free predictor in cRCC.

To date, little functional data exist that show that tumor cell-lined channels contribute to the overall survival of the tumor. However, even among individuals who have questioned the concept of VM, there is generally a consensus that the prognostic significance of PAS-positive patterns is valid. The presence of looping patterns in histological sections of primary tumors has been repeatedly shown to have an exceptionally strong association with death from metastatic cancer. The findings of our study are entirely in agreement with the literature suggesting that prominent VM patterns are a feature of more aggressive metastatic tumors. We believe that this correlation could become much stronger if the TP phenotype is also taken into account. Perhaps the simultaneous presence of these factors may be required

to turn on a "malignant switch." If this is confirmed in larger studies, the prognostic significance of the NW/TP phenotype may suggest the possibility of real cooperation between a VM network and angiogenesis in the metastatic process, which in turn suggests that a combination of VM-inhibiting substances and angiogenesis-inhibiting drugs (such as TP inhibitors) might offer therapeutic benefit.

Conclusion

Our results demonstrated various PAS-positive patterns in cRCC and showed that detection of PAS-positive networks was associated with poor clinical outcomes. TP expression, which has been previously shown to be important in RCC[22] appeared in our study to be an independent prognostic factor not correlated with PAS-positive networks. Notably, in our small sample, the combination of these two factors effectively identified the highest risk group. □

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