Original Article

Prostate specific membrane antigen (PSMA) expression in vena cava tumour thrombi of clear cell renal cell carcinoma suggests a role for PSMA-driven tumour neoangiogenesis

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Background: Clear cell renal cell carcinoma (ccRCC) is a malignant renal neoplasm with a peculiar propensity to propagate as a contiguous tumor extension via the renal vein and inferior vena cava, occasionally reaching the right atrium. This intravascular tumor extension, often referred to as a tumor thrombus, represents the active growing front of the cancer. Prostate specific membrane antigen (PSMA), a glycoprotein that is extensively used in prostate cancer diagnostics, is a useful vascular marker for a variety of solid tumors. It is expressed in renal carcinomas. The aim of the current investigation was to analyse and compare the expression of PSMA at the growing front of the vena cava tumor extension with that found in the primary renal lesion.

Methods: Immunohistochemical (IHC) analysis of PSMA and CD34 was performed on archived paraffin embedded vena cava tumour thrombus tissue and matching renal tumours. These specimens were collected from radical nephrectomies of 10 patients with vena cava invasive (pT3b) ccRCC in a large tertiary hospital in Australia. Quantitative and qualitative morphometric analysis of PSMA IHC expression was performed with Aperio ImageScope morphometry using intensity and positive pixel counts of CD34 and PSMA from the IVC tumour slides and the corresponding renal tumour mass.

Results: PSMA and CD34 immunostaining were noted in the neovasculature of IVC tumour and renal tumour tissue. There was a higher PSMA/CD34 positive pixel count ratio noted in IVC tumour tissue when compared to renal tumour tissue. PSMA showed consistently increased expression in vena cava tumour, in comparison with the renal tumour mass.

Conclusions: Intravascular venous tumour extension expresses PSMA more intensely compared to intrarenal tumour tissue neovasculature. Our data suggest a possible mechanism for PSMA in neoangiogenesis and local progression of ccRCC and therefore its usefulness as a biomarker of neoangiogenesis for future diagnostic and therapeutic advancements.

Keywords: Prostate specific membrane antigen (PSMA); renal cell carcinoma; tumour thrombi; neoangiogenesis

Submitted Apr 23, 2019. Accepted for publication Apr 26, 2019.
doi: 10.21037/tau.2019.04.10
View this article at: http://dx.doi.org/10.21037/tau.2019.04.10

Introduction

Kidney cancer accounts for approximately 2% of all malignant neoplasms (1). Most malignant kidney tumours are renal cell carcinoma (RCC). Of these neoplasms, clear cell RCC (ccRCC) accounts for 60–70% of all RCC and represents the most common subtype (2). Nearly 50% of all ccRCC cases are of clinical stage T3, or greater at diagnosis, making prognosis of this group of malignant neoplasms unfavorable (3). Clinical stage T3b, a stage that consists of contiguous extension of tumour into the infradiaphragmatic portion of the inferior vena cava (IVC), represents 4–10% of cases (4). Presence of intravascular tumour extension may represent a useful model for studying contributors to the aggressive invasive front of neoangiogenesis in locally advanced ccRCC.

For this investigation, prostate-specific membrane antigen (PSMA) and CD34 were selected as immunohistochemical (IHC) biomarkers. CD34 is a vascular marker of widespread usage in surgical pathology. It is present in endothelial cells of vessels of all sizes, including capillaries. CD34 positivity highlights well-differentiated endothelial cells of most normal tissues and various vascular neoplasms (5-7). PSMA is a glycoprotein that was first discovered in normal and neoplastic prostatic epithelium (8-12). It has been utilized extensively in prostate cancer for diagnostic and prognostic ancillary techniques (13). For example, ^{68}Gallium bound HBED-CC PSMA binding ligand (14), ^{18}Fluorine-DCFPyL PSMA binding ligand (15,16) or ^{111}indium-labelled 7E11 conjugate (17) are currently used as radiotracers for prostate cancer imaging with positron emission tomography (PET).

Despite its name referring to a prostatic origin, PSMA has been identified in many other tumour types, including transitional cell carcinoma, malignant melanoma, lung cancer soft tissue sarcomas, and renal tumours (18-20). Previous studies have demonstrated that there is PSMA expression in normal kidney proximal tubular epithelial cells and in RCC neovasculature (21-23). There is no recorded expression of PSMA in vessels of normal kidney tissue. This discrimination supports its role as a marker of neoangiogenesis in different types of kidney epithelial neoplasms (22,23). The hypothesis of the current study was that PSMA expression, a marker of neoangiogenesis, is expressed differently in ccRCC vena cava neoplastic thrombi (invasive front) when compared to the paired renal tumour mass.

Methods

Patients

Between 2013 and 2015, 202 patients diagnosed with RCC were surgically treated at Princess Alexandra Hospital (Queensland, Australia). Of these cases, 131 consisted of ccRCC, of which ten presented with IVC tumour extension. Normal paired kidney tissue, renal tumour and leading edge of the neoplastic thrombus (invasive front) were collected from these patients. Approval for this research was obtained from the human research ethics committees of Princess Alexandra Hospital and Greenslopes Private Hospital (HREC/05/QPAH/95 and protocol 13/23) and informed patient consent was obtained. The surgical specimens were fixed in formalin, paraffin embedded, step-sectioned at 3 to 4 µm intervals and mounted on treated glass slides. Hematoxylin and eosin (H&E) staining was routinely performed and assessed microscopically.

Immunohistochemistry

CD34 was selected as the preferred pan endothelial IHC marker. It highlights a higher percentage of endothelial cells compared to other endothelial markers such as CD31, CD105 or factor VIII-related antigen (6,7). Primary antibody for CD34 was mouse anti-human CD34 MAb, clone QBend-10 Cat No. Vector VP-C345 or Dako M7165, diluted 1:150 in Tris-buffered saline (TBS). Primary antibody for PSMA was Dako Flex pre-diluted mouse anti-human PSMA, clone: 3E6 cat. No. IR089. Positive and negative control sections were used for IHC and batch staining of the slides was utilized. IHC for CD34 and PSMA was performed using standard protocols. Briefly, the slides were dewaxed and rehydrated through descending graded alcohols to TBS. Endogenous peroxidase activity was then blocked by incubating the sections in 1.0% hydrogen peroxide (H_2O_2) in TBS for 10 minutes. For antigen retrieval (AR), the samples were transferred to Dako Target Retrieval Solution pH 9.0 and submitted to heat (30 minutes at 100 ℃ for CD34 and 20 minutes at 97 ℃ for PSMA) using the Biocare Medical Decloaking Chamber. After AR was completed, the slides were removed and allowed to cool for a further 20 minutes before transferring back to TBS for washing. Biocare Medical Background Sniper with 1.0% bovine serum albumin/BSA for CD34 and 2.0% BSA for PSMA was used for 20 minutes to block non-specific antibody binding. The slides were then incubated

Table 1 Clinical data of the patients and histopathological information of the lesions

Cases	Age	Sex	Grade	TVA (%)	TTVA (%)	TN (%)	TTN (%)	TH	TTH
1	61	M	2	13	10	–	–	–	–
2	80	M	4	2	5	–	100	Mild	Intense
3	56	M	3	8	10	10	5	Mild	–
4	55	M	3	11	2	5	80	–	Mild
5	45	F	4	7	3	10	30	Mild	Intermediate
6	75	M	3	8	6	–	–	–	–
7	55	F	2	4	1	–	60	–	Intense
8	43	M	2	4	14	10	–	Mild	Mild
9	66	M	3	2	5	–	–	–	–
10	40	M	4	3	2	–	90	Mild	Intermediate

Grade, WHO/ISUP grade; TVA, tumor vascular area; TTVA, tumor thrombus vascular area; TN, tumor necrosis; TTN, tumor thrombus necrosis; TH, tumor hemosiderin; TTH, tumor thrombus hemosiderin.

with the primary antibodies for CD34 or PSMA for 60 minutes. Signals were developed in 3,3'-diaminobenzidine hydrochloride (DAB) with H_2O_2 as substrate for 5 minutes. Additional copper sulphate enhancement for 5 minutes was used for PSMA. The slides were washed in distilled water, counterstained with Mayer's hematoxylin, dehydrated, and mounted with Depex mounting medium.

Morphometric analysis with Aperio ImageScope

There is much controversy around the subject of intratumoural microvascular density (iMVD) measurements (23-26). While some groups, like Weidner *et al.*, proposed specific effective methods for measuring iMVD in some neoplasms (7), other researchers, such as Nico *et al.* (24), carried out a systematic review detailing several different possible methods for iMVD assessment in various tumours, either manually, using vessel number and vascular density classification such as grades 0 to 3, or by computerized image, using pan endothelial cell markers. The present study adopted specific methods for morphometric analysis of iMVD, which included subjective (qualitative) methods and objective (quantitative) methods. The qualitative method considered PSMA and CD34 vascular expression. Strong (diffuse strong) staining was scored 3, moderate (diffuse weak or focal strong) staining was scored 2, weak staining (weak staining in less than 5% of vessels) was scored 1, and the absence of staining was scored 0. Positive PSMA in tumour-associated neovasculature was defined as a staining score of 2 or greater. Objective tumoural neovasculature morphological patterns were assessed by counting vessel number in 10 high power microscope fields (HPF) and measuring total vascular area in each field (sum of the measurements of each vessel area). The averages of all values obtained in 10 HPF were then obtained, as well as the measurements in the highest vascular density areas (vascular "hot spots"). Positive pixel analysis was used both for CD34 and PSMA, comparing the whole sections, highest positivity areas (hot spots) and selected areas of 10 paired HPF between tumour mass and thrombus, excluding areas of necrosis and hemosiderin deposition. The results were analyzed by paired Student's *t*-test, with a $P<0.05$ taken as significant.

Results

Clinical and histopathological data

Clinical and histopathological data are shown in *Table 1*. The mean age of the patients was 59 years with eight of the ten patients being male. WHO/ISUP grade 3 and tumour diameter varied from 65 to 95 mm in the majority of cases. All of the patients were staged as pT3 or higher and had unilateral tumours with no signs of lymph node metastasis (pN0) or distant metastasis (pM0). When comparing adjacent normal kidney tissue, only one patient showed signs of chronic kidney fibrosis, and no alterations in adjacent kidney parenchyma correlated with any histological patterns in the tumour mass or thrombus.

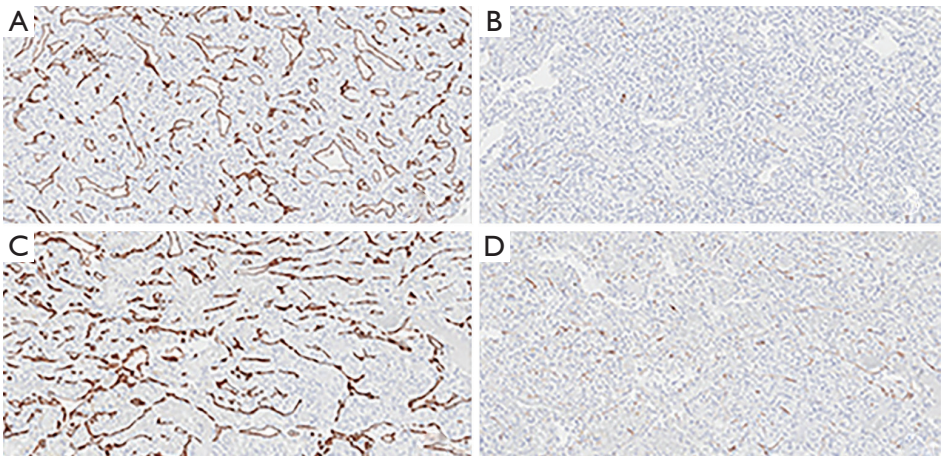

Figure 1 Examples of slides stained using immunohistochemistry, and scanned at ×20 objective (×200 final magnification), immunohistochemical expression of: (A) CD34 in tumor; (B) PSMA in tumor; (C) CD34 in tumor thrombus; (D) PSMA in tumor thrombus. While CD34 shows a somewhat constant staining intensity, there is an increase in PSMA expression in tumor thrombi when compared with tumor tissue. PSMA, prostate specific membrane antigen.

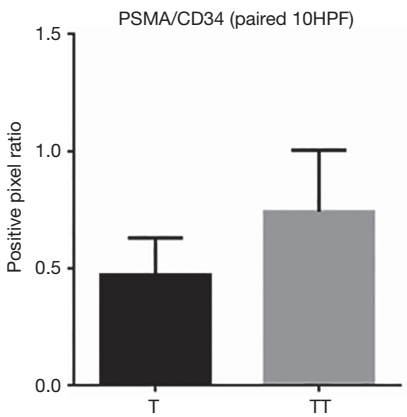

Figure 2 Comparative digital analysis, PSMA/CD34 positive pixel ratio of 10 paired high powered microscopy fields, showing higher ratio in tumor thrombus (0.74) compared to tumor (0.47), with a 57% increase in relative expression of PSMA (P<0.01). T, tumor; TT, tumor thrombus; PSMA, prostate specific membrane antigen.

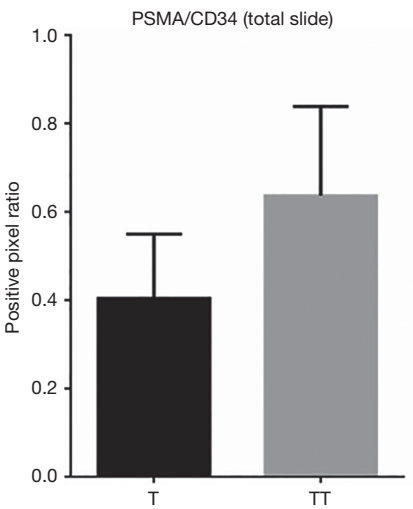

Figure 3 Comparative digital analysis, PSMA/CD34 positive pixel ratio evaluation of whole histological sections, showing increased ratio in tumor thrombus (0.64) compared to tumor tissue (0.40) with a 60% increase in relative expression of PSMA (P<0.01). T, tumor; TT, tumor thrombus; PSMA, prostate specific membrane antigen.

Immunohistochemistry and quantification

When comparing the morphological patterns of the neoplasm and neoplastic thrombus, there was no clear distinction between vascular patterns, vascular density, and tumour necrosis or hemosiderin deposition. CD34 IHC showed a diffuse strong staining in all vessels, both in tumour and tumour thrombi samples. PSMA IHC showed a predominantly weak to moderate diffuse staining in most samples, with a subjective impression of increased expression in the vena cava tumour thrombi when compared to tumour tissue (*Figure 1*). This impression is proven objectively using a PSMA/CD34 positive pixel expression ratio, both in 10 HPF average, excluding areas of necrosis and hemosiderin deposition (*Figure 2*) and in full slide digital analysis (*Figure 3*). This ratio was used to rule out the great variation in iMVD observed due to intertumoural

Table 2 Morphological and positive pixel analysis, using three methods of analysis

Variables	Group	Average	St Dev	Median	Est diff	Confid Int (95%)		P*
Vessel number (HotSpot)	Thrombus	74.3	47.38	52.5	−45.9	−102.9	11.114	0.1019
	Tumour	120.2	46.36	117.5				
Vascular area (HotSpot)	Thrombus	52,086.62	32,302	49,759.15	3,308.4	−19,564	26,181	0.751
	Tumour	48,778.21	20,511	46,933.07				
PSMA (HotSpot)	Thrombus	0.47	0.15	0.5	−0.025	−0.161	0.1113	0.6888
	Tumour	0.49	0.07	0.5				
CD34 (HotSpot)	Thrombus	0.29	0.11	0.28	0.0395	−0.057	0.136	0.3784
	Tumour	0.25	0.07	0.25				
PSMA/CD34 (HotSpot)	Thrombus	1.66	1.29	1.22	0.9469	0.1295	1.7644	0.0278*
	Tumour	0.71	0.25	0.69				
PSMA (10 HPF Avg)	Thrombus	0.32	0.16	0.33	−0.049	−0.185	0.086	0.4309
	Tumour	0.37	0.08	0.39				
CD34 (10 HPF Avg)	Thrombus	0.19	0.11	0.16	0.0236	−0.064	0.111	0.556
	Tumour	0.16	0.06	0.18				
PSMA/CD34 (10 HPF Avg)	Thrombus	0.74	0.26	0.71	0.2704	0.0884	0.4523	<0.01*
	Tumour	0.47	0.16	0.46				
CD34 (total)	Thrombus	0.3	0.17	0.28	−0.067	−0.202	0.0687	0.2937
	Tumour	0.37	0.07	0.37				
PSMA (total)	Thrombus	0.18	0.11	0.18	0.0325	−0.063	0.1276	0.4593
	Tumour	0.15	0.06	0.14				
PSMA/CD34 (total)	Thrombus	0.64	0.2	0.64	0.2323	0.0718	0.3927	<0.01*
	Tumour	0.4	0.15	0.39				

(I) HotSpot, the most vascular field analyzed; (II) average values obtained from 10 high powered fields (10 HPF Avg); (III) total slide section positive pixel analysis. St Dev, standard deviation; Est Diff, estimated difference; Confid Int, confidence interval. *, P value with <0.05 significance.

and intratumoural heterogeneity. There was a 57% increase in the PSMA/CD34 ratio using 10 HPF average and a 60% increase using total slide digital analysis. The P value for both comparisons was <0.01 (*Table 2*).

Of the 10 cases, one case showed peculiar preoperative radiological and tumoural IHC characteristics. The preoperative contrasted computerized tomography (CT) revealed a right kidney neoplasm (*Figure 4*) extensive IVC tumour which appeared to infiltrate the IVC both superiorly (*Figure 4B*) and inferiorly (*Figure 4C*). ^{68}Gallium PSMA-PET-CT Scan study showed PSMA avidity in the right renal neoplasm associated with intense PSMA avidity in the IVC (*Figure 4D,E,F and G*). This PSMA avidity was more obvious in the suprarenal portion of the IVC (*Figure 4G*) than the infra renal portion (*Figure 4H*). Following surgery, the superior part of the thrombus was composed exclusively by viable tumour thrombus (*Figure 4I* and *Figure 5*), while the inferior part was entirely bland thrombus, without any viable neoplastic cells (*Figure 4J* and *Figure 5F*). On IHC staining, there were CD34 positive vessels in both tumour thrombus (superior leading front) and also in the bland thrombus (inferior portion of IVC thrombus) (*Figure 5B,C,G* and *H*). However, there were significant numbers of PSMA positive vessels in the superior neoplastic thrombus portion (*Figure 5D,E*) and focal weak PSMA staining noted in the inferior IVC bland thrombus

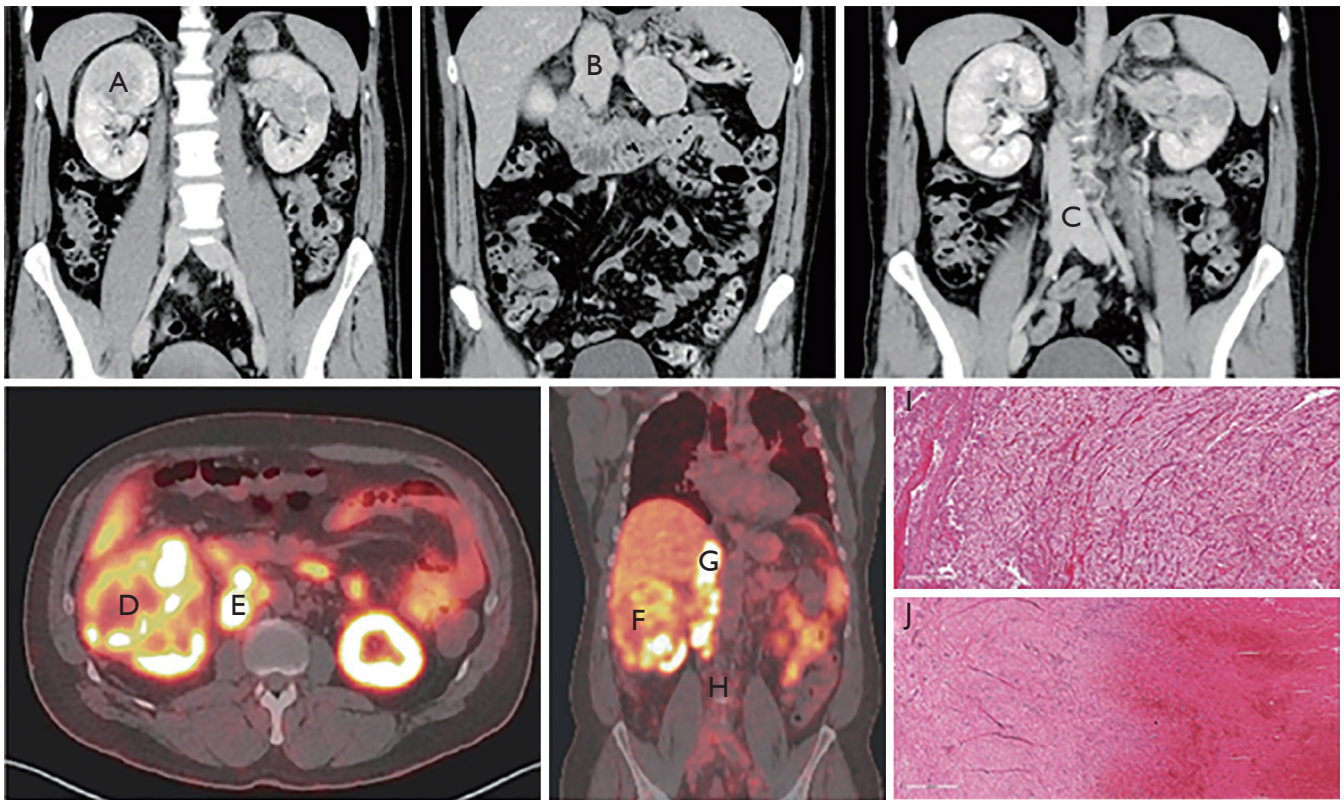

Figure 4 Upper row: Coronal section contrasted CT scan (A,B,C); (A) Right RCC; (B) IVC tumor thrombus invasion upwards; (C) IVC tumor thrombus invasion downwards. Bottom row, to the left: PSMA PET CT scan Axial (D,E) and coronal (F,G,H); (D,F) PSMA avid right RCC; (E,G) IVC tumor thrombus with PSMA positivity in superior ramification; (H) IVC tumor thrombus with no PSMA avidity in inferior ramification. Bottom row, to the right: Histopathological slides for IVC thrombus (I,J); (I) H&E slide of superior ramification of IVC tumor thrombus (composed entirely of viable ccRCC); (J) H&E slide of inferior ramification of IVC bland thrombus. (I) and (J) scanned at ×20 objective (×40 final magnification). IVC, inferior vena cava; PSMA, prostate specific membrane antigen; RCC, renal cell carcinoma; ccRCC, clear cell RCC; PET, positron emission tomography.

portion (*Figure 5I,J*).

Discussion

The current study investigated whether PSMA was expressed differently in ccRCC vena cava tumour extensions compared with the paired intrarenal tumour mass. The results showed significantly increased PSMA/CD34 ratio in the intravascular tumour extension versus intrarenal tumour tissue. We propose that PSMA activity in ccRCC tumour thrombi may be a useful marker for neoangiogenesis and help provide better understanding of the invasive nature of the kidney tumour. Despite no clear morphological difference of microvascular characteristics of tumour thrombi evaluated by light microscopy using H&E stained sections, PSMA expression paired with CD34+ vessels revealed increased expression in the invasive front. This phenomenon strengthens the hypothesis that PSMA is involved in tumour neoangiogenesis, a fact that may lead to future clinical implications such as targeting PSMA for treatment of cancers (27). We have also confirmed that there is a peripheral pattern of neovascularization in the tumour that is evident with the increased PSMA intensity of neovascularization in the invasive front in the vena cava tumour extension.

The one clinical case that demonstrated increased diffuse PSMA IHC staining in the neovessels of the IVC tumour extension compared with focal weak PSMA IHC staining in the bland thrombus portion of the IVC, provides further evidence that PSMA is involved with tumour neovascularization. This was also highlighted with the

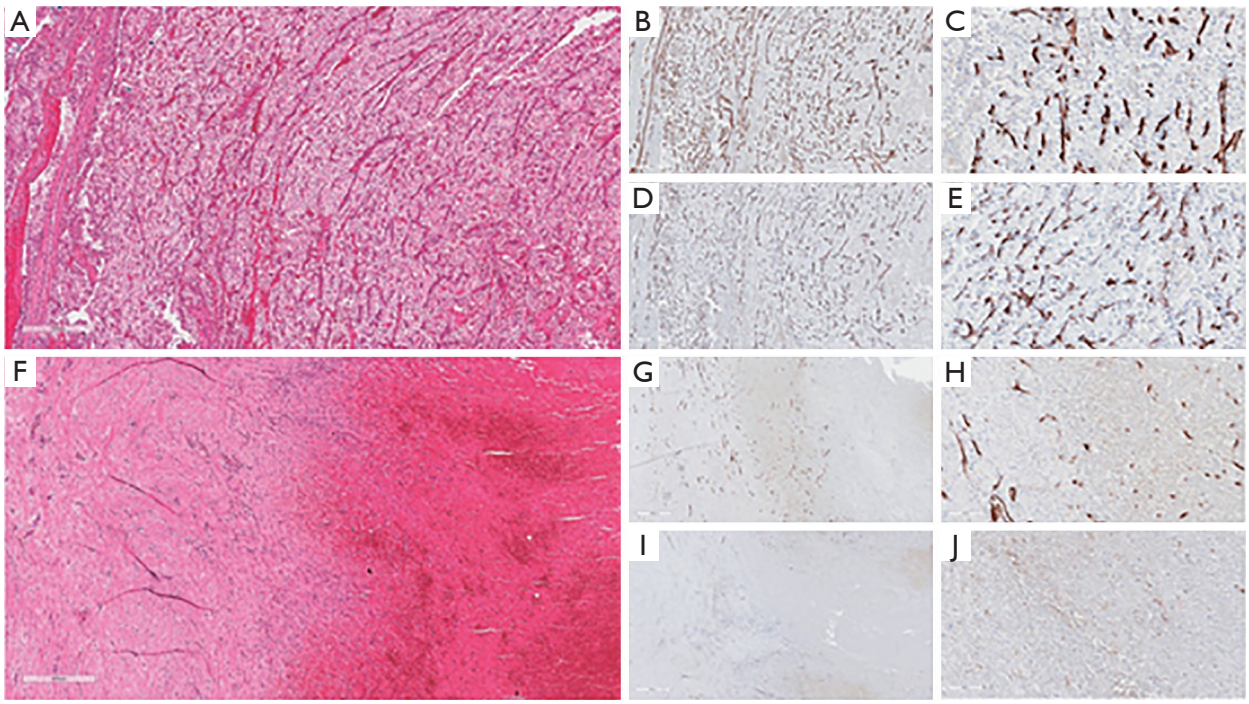

Figure 5 Histological section of the tumor thrombus, superior ramification (A,B,C,D,E) and inferior ramification (F,G,H,I,J): (A) viable tumor, with strong and diffuse expression of CD34 (B and C) and PSMA (D and E); (F) Bland thrombus, with no viable neoplastic cells, showing moderate staining for CD34 (G and H) and no staining for PSMA, even in CD34 positive vessels (I and J). (A,B,C,D,E,F,G,H,I,J) scanned at ×20 objective and presented at ×100 magnification. (A and F) Hematoxylin and eosin staining. (B,C,D,E) and (G,H,I,J), immunohistochemical staining, with antibodies as indicated. PSMA, prostate specific membrane antigen.

preoperative PSMA PET scan that showed PSMA avidity in the portion of the IVC thrombus that was confirmed histologically as contiguous tumour extension. The PSMA PET image of this case was published by Rhee *et al.* (20). The present study provides further evidence showing the corresponding PSMA PET avidity with PSMA positivity on histology of the superior portion of IVC tumour thrombus.

The findings have important implications for future research in areas of tumour neoangiogenesis and invasiveness (24). First, the PSMA IHC demonstrated its usefulness in highlighting neovascularization (23), a differentiation that cannot be made by CD34 alone. This may have implications for further characterizing neoplasms histologically. Second, in understanding the molecular mechanisms of angiogenesis, PSMA expression is gaining interest as an important enzyme related to neovascularization. Conway *et al.* have shown previously that enzymatic activity of PSMA regulates angiogenesis by modulating integrin signal transduction, resulting in endothelial cell migration and invasion (25). Nguyen *et al.* demonstrated that PSMA was not expressed in human umbilical endothelial cells (HUVEC), a commonly used cell line for in vitro models of angiogenesis. However, in culturing the cells with conditioned media from PSMA-expressing cells, the HUVEC cells expressed PSMA, as well as achieving a tubular morphology. The authors concluded that tumour-related factors were responsible for neovasculature formation and PSMA expression (26). Recent adoption of PSMA imaging in a variety of cancers, increased interest in molecular cancer research, and PSMA-targeted therapies (27,28) make this present study timely. Further increase in knowledge in these areas is likely to help deliver PSMA targeted therapies for a variety of cancers.

This is the first study to highlight the increased expression of PSMA in vena cava tumour thrombi originating from ccRCC. This novel result suggests the role of PSMA-driven neoangiogenesis in ccRCC leading to their progression and metastasis. Future research into the mechanism of PSMA in neoangiogenesis can, hopefully, lead to improved diagnostic and therapeutic pathways.

Acknowledgments

None.

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: Approval for this research was obtained from the ethics committees of Princess Alexandra Hospital and Greenslopes Private Hospital (HREC/05/QPAH/95 and protocol 13/23) and patient consent obtained.

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Cite this article as: Morgantetti G, Ng KL, Samaratunga H, Rhee H, Gobe GC, Wood ST. Prostate specific membrane antigen (PSMA) expression in vena cava tumor thrombi of clear cell renal cell carcinoma suggests a role for PSMA-driven tumor neoangiogenesis. Transl Androl Urol 2019;8(Suppl 2):S147-S155. doi: 10.21037/tau.2019.04.10

