

## RESEARCH COMMUNICATION

# Presence of Tumour-infiltrating FOXP3<sup>+</sup> Lymphocytes Correlates with Immature Tumour Angiogenesis in Renal Cell Carcinomas

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### Abstract

**Background:** FOXP3<sup>+</sup> regulatory T cells (Tregs) inhibit effector T cell functions and are implicated in tumour progression. However, together with microvessel density (MVD) they remain controversial prognostic predictors for renal cell carcinoma (RCC), and potential associations have yet to be determined. The objective of this study was to determine the prognostic significance of Tregs and MVD and their potential relationship in RCCs. **Design:** Paraffin-embedded tissues from 62 RCC patients were analysed using immunohistochemistry to detect FOXP3<sup>+</sup> lymphocytes, and double immunohistochemistry to detect different microvessel types in the tumour interior, rim and normal kidney tissue, and their correlation with clinicopathological characteristics. Survival analysis was also performed. **Results:** The presence of FOXP3<sup>+</sup> cells in the tumour interior or the rim showed no correlation with death from RCC and other pathological characteristics. Negative correlations were noted between the immature MVD in the tumour interior or the rim and tumour size, tumour stage and overall survival; however, there was no correlation with the nuclear grade or pathological type. A positive correlation between FOXP3<sup>+</sup> Tregs and immature MVD ( $r=0.363$ ,  $P=0.014$ ) and mature MVD ( $r=0.383$ ,  $P=0.009$ ) was confirmed in the tumour interior. However, there was no correlation between FOXP3<sup>+</sup> Tregs and mature MVD ( $r=0.281$ ,  $P=0.076$ ) or immature MVD ( $r=0.064$ ,  $P=0.692$ ) in the tumour rim. **Conclusions:** In this study, a positive correlation between the presence of FOXP3<sup>+</sup> Tregs and immature and mature MVD in RCC was confirmed, which suggests a link between suppression of immunity, tumour angiogenesis and poor prognosis.

**Keywords:** Renal cell carcinoma - FOXP3 - regulatory T cells - angiogenesis

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### Introduction

Regulatory T cells (Tregs), characterised by a CD4<sup>+</sup> CD25<sup>+</sup> phenotype, play an essential role in immune regulation by facilitating peripheral tolerance and precluding autoimmunity (Thornton and Shevach, 1998). CD4<sup>+</sup> CD25<sup>+</sup> Tregs express the transcriptional repressor forkhead box P3 (FOXP3) which is essential for their development (Hori and Sakaguchi, 2004; Fontenot and Rudensky, 2005). The capacity of Tregs to suppress effector T-cell proliferation and cytokine production can enable tumour cells to elude immunological surveillance, as evidenced by data showing increased levels of Tregs in the peripheral blood of some cancer patients (Griffiths et al., 2007). A study of previously untreated renal cell carcinoma (RCC) patient demonstrated an association between increased levels of Tregs and an adverse overall survival (Griffiths et al., 2007). Interestingly, another study of RCC patients who had undergone radical or partial nephrectomy showed that increased levels of CD4<sup>+</sup> CD25<sup>+</sup> FOXP3<sup>-</sup> T cells were associated with an increased risk of death from RCC (Siddiqui et al., 2007).

Vasculature is a critical process in the tumour growth and metastasis. For most of solid tumours, microvessel density (MVD) correlates with poor prognosis. However, MVD remains a controversial prognostic predictor for RCC (Kirkali et al., 2001; Sabo et al., 2001a; Suzuki et al., 2001; Dekel et al., 2002; Kinouchi et al., 2003; Yildiz et al., 2008; Qian et al., 2009), a significant contributory factor being that blood vessels in RCC may be regarded as being monolithic in structure and function, which might not accurately reflect the functional diversity based on microvessel differentiation and location. Mature blood vessels are usually covered by pericytes, and pericyte coverage is regarded as an indicator of vascular maturation (Gerhardt and Semb, 2008).

Since Tregs are associated with tumour progression and MVD is critical for tumour prognosis, it was hypothesized that the level of FOXP3 and MVD correlate with RCC pathology and be associated with disease prognosis. To this end, we determined the level of MVD and FOXP3<sup>+</sup> Treg in tissues resected from RCC patients by immunohistochemistry and analysis their correlation with the clinicopathological parameters and survival data.

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## Materials and Methods

### *Patients and tissue specimens*

The study protocol was approved by the Institutional Review Board of The Third Affiliated Hospital, Sun-Yat Sen University (Guangzhou, China). Patients were informed of the investigative nature of the study, and written consent in accordance with institutional regulations was obtained prior to study entry. Sixty two patients with unilateral or sporadic RCC treated with radical nephrectomy or nephron-sparing surgery were enrolled between January 1998 and January 2009. None of the enrolled patients had received preoperative immunotherapy or renal arterial embolization therapy. Paraffin blocks were selected based on the availability of complete clinicopathological and follow-up data for the patients. The selected tissue sections (4µm thickness) were taken from areas away from necrotic and haemorrhagic tissue. For each patient, three tissue samples, obtained from different areas with a 5 mm diameter scope in the centre of tumour nests, the nearest cancerous margin, and normal kidney tissue (designated as interior, rim and normal, respectively) were studied. Analysed pathological characteristics for each patient included tumour size, tumour TNM stage, nuclear grade and pathological type. Tumours were staged according to the 2002 WHO TNM staging system. Nuclear grade was assigned according to the criteria of Fuhrman et al. (1982). Specimens were evaluated by a pathologist blinded to the outcome.

Overall survival was evaluated. Patient follow-up was completed on 1<sup>st</sup> March 2009. The duration of follow-up was calculated from the date of nephrectomy to the date of cancer progression (i.e., distant metastases after nephrectomy for the primary tumour), death or last follow-up. All patients were prospectively monitored by serum creatinine, abdomen ultrasonography and chest X-ray every 1-6 months according to the postoperative time and individual factors. For suspicious cases, computed tomography and/or magnetic resonance imaging were used to verify the development of metastases.

### *FOXP3 immunohistochemistry*

Immunohistochemistry was performed as previously described (Bates et al., 2006). Briefly, paraffin sections were deparaffinized and hydrated. After microwave antigen retrieval, endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 minutes. Immunohistochemistry to label FOXP3<sup>+</sup> Treg was performed using diluted rabbit anti-human FOXP3 monoclonal antibody (Sigma, England, dilution 1:700) for 120 min in 37 °C. After incubation with primary antibodies, secondary antibody serially, the sections were developed in 3, 3'-diaminobenzidine solution under the microscope and counterstained with haematoxylin. The control tissues used for immunostaining of FOXP3 were lymph nodes. Negative control slides omitting the primary antibodies were included in all assays.

Each tissue sample in the tumour interior, tumour rim and normal tissue was respectively examined using × 100 optical fields (10 fields per location) and the number of FOXP3<sup>+</sup> lymphocytes was recorded in each field. The

mean value of these measurements was assigned as the score for each location.

### *Double immunohistochemistry for microvessels*

Paraffin sections (4 µm) of tumour tissue samples were deparaffinized and rehydrated, prior to heating in a microwave in 0.01mol/L citrate buffer (pH 7.8) for antigen retrieval. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol. The sections were incubated over night at 4°C with a combination of mouse anti-human CD31 monoclonal antibody diluted 1:10 (M0823; Dako, Copenhagen, Denmark) to stain endothelial cells and a rabbit anti-human a-SMA (smooth muscle actin) monoclonal antibody directly conjugated to alkaline phosphatase diluted 1:400 (ab32575; Abcam, Cambridge, UK) to detect pericytes. The immunoreaction for CD31 was detected by a horseradish peroxidase labelling goat anti-mouse IgG (DS-0002; Zhongshan Goldenbridge, Beijing, China), and the α-SMA immunoreaction was detected with the alkaline phosphatase goat anti-rabbit IgG (DS-0002; Zhongshan Goldenbridge, Beijing, China) at 37°C for 25min. Staining was carried out using the alkaline phosphatase substrate Vector Red and the chromogen 3, 3'-diaminobenzidine. The sections were counterstained with haematoxylin, dehydrated and mounted. Normal mouse IgG1 was used as a substitute for the primary antibody in the negative controls.

MVD was assessed according to previously described criteria (Yao et al., 2007). Briefly, eight most vascularized areas (hotspots) were selected from the centre of tumour nests or in the peripheral areas or in the normal kidney, images were taken at a magnification of ×400 with a Nikon microscope. All CD31-positive entities associated with a-SMA-positive cells were counted as mature, and CD31-positive entities associated with a-SMA-negative cells were counted as immature vessels. Stromal and necrotic areas were excluded from the analysis. The mean number of mature vessels or immature vessels of tumour rim or tumour interior was assigned as the score for each case.

### *Statistical analysis*

Recorded data were independently processed by a biostatistician. The cut-off value for definition of the immunohistochemical marker subgroups was the median value. Cox proportional hazard regression models were fitted for univariate analysis. Comparisons of pathological features by patient subgroups were analysed using Fisher's exact test. Continuous data were compared by Mann-Whitney test. Overall survival was determined using the Kaplan-Meier method and the difference between groups assessed using the log-rank test. The correlation between FOXP3<sup>+</sup> Tregs and MVD were evaluated by Spearman's correlation. Data were analyzed using SPSS 15.0 (SPSS, Inc., Chicago, IL, USA). A two-tailed P<0.05 was considered to indicate statistical significance.

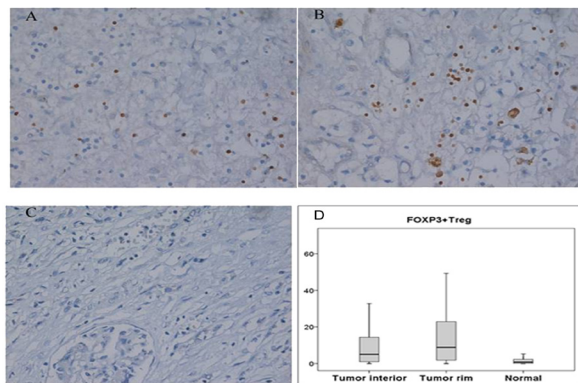
## Results

### *Patient population*

A total of 62 patients with RCC were enrolled into the

**Table 1. The Pathologic Characteristics of 62 Patients with RCC**

Characteristics	N (%) of patients	
Gender	Male	39 (62.9)
	Female	23 (37.1)
Tumour size	<7cm	46 (74.2)
	≥7cm	16 (25.8)
Tumour stage	I	39 (62.9)
	II	8 (12.9)
	III	4 (6.5)
	IV	11 (17.8)
Nuclear grade	1	23 (37.1)
	2	28 (45.2)
	3	7 (11.3)
Pathological type	absent	4 (6.5)
	Clear cell RCC	54 (87.1)
	Non- Clear cell RCC	8 (12.9)



**Figure 1. The Immunohistochemistry for the FOXP3+ Cells at 200× Magnification.** The number of FOXP3+ cells in the tumor interior (A) or the rim (B) was more than in the normal kidney tissue (C). Quantification of immunohistochemistry result detecting FOXP3+ Treg (D) in normal tissue, tumor interior or rim

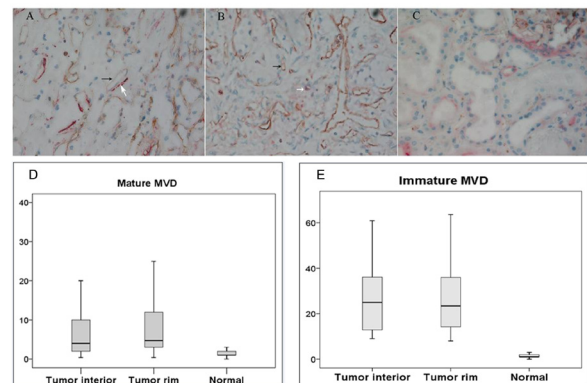
study and followed up for a median (range) duration of 57.2 (5.5-102) months. The patients' clinicopathological characteristics are summarised in Table 1. At the last follow-up 12 patients had died. Higher overall survival was significantly associated with early tumor stage, lower nuclear grade, and pathological type (clear cell RCC) (Log rank test;  $P < 0.001$ ,  $P = 0.033$ , and  $P = 0.007$ , respectively).

*FOXP3+ Tregs in normal kidney tissue and RCC microenvironment.*

In normal kidney tissue and RCC, FOXP3 was expressed exclusively by stroma infiltrating lymphocytes; tumour cells were negative for FOXP3 in all cases (Figure 1). The number of FOXP3+ cells in the tumour interior (5.06 (1.0-15)/high power field [HP]) or the rim (9.2 (2.0-25.0)/HP) was higher than in normal kidney tissue (0.8 (0-2.4)/HP), with statistically significant differences ( $P < 0.05$ ). Using a cut-off point of the median value, FOXP3+ cells in the tumour interior or rim were divided into 'high' and 'low' FOXP3+ infiltration groups. The association of FOXP3+ cells with pathological features of the tumour are summarised in Table 2. The presence of FOXP3+ cells in the tumour interior or the rim was not significantly associated with overall survival, as determined by univariate analysis ( $RR = 0.957$ ,  $P = 0.138$  and  $RR = 0.970$ ,  $P = 0.068$ , respectively; Figure 3A and B).

**Table 2. Comparison of Pathologic Characteristics by Presence of FOXP3+ Cells in 62 Patients with RCC**

Feature	FOXP3+ Treg in the tumor interior			FOXP3+ Treg in the tumor rim			
	<10.6	≥10.6	P	<14.5	≥14.5	P	
Tumour size	<7cm	21	25	0.246	23	23	1
	≥7cm	10	6		8	8	
Tumour stage	I	18	21	0.467	19	20	0.279
	II	3	5		2	6	
	III	3	1		3	1	
	IV	7	4		7	4	
Nuclear grade	1	10	13	0.595	18	12	0.387
	2	16	12		16	12	
	3	4	3		2	5	
Pathological type	Clear cell RCC	27	27	1	26	28	0.449
	Non- Clear cell RCC	4	4		5	3	
Metastasis	present	10	4	0.068	9	5	0.224
	absent	21	27		22	26	
Overall Survival	Yes	24	26	0.52	25	25	1
	No	7	5		6	6	

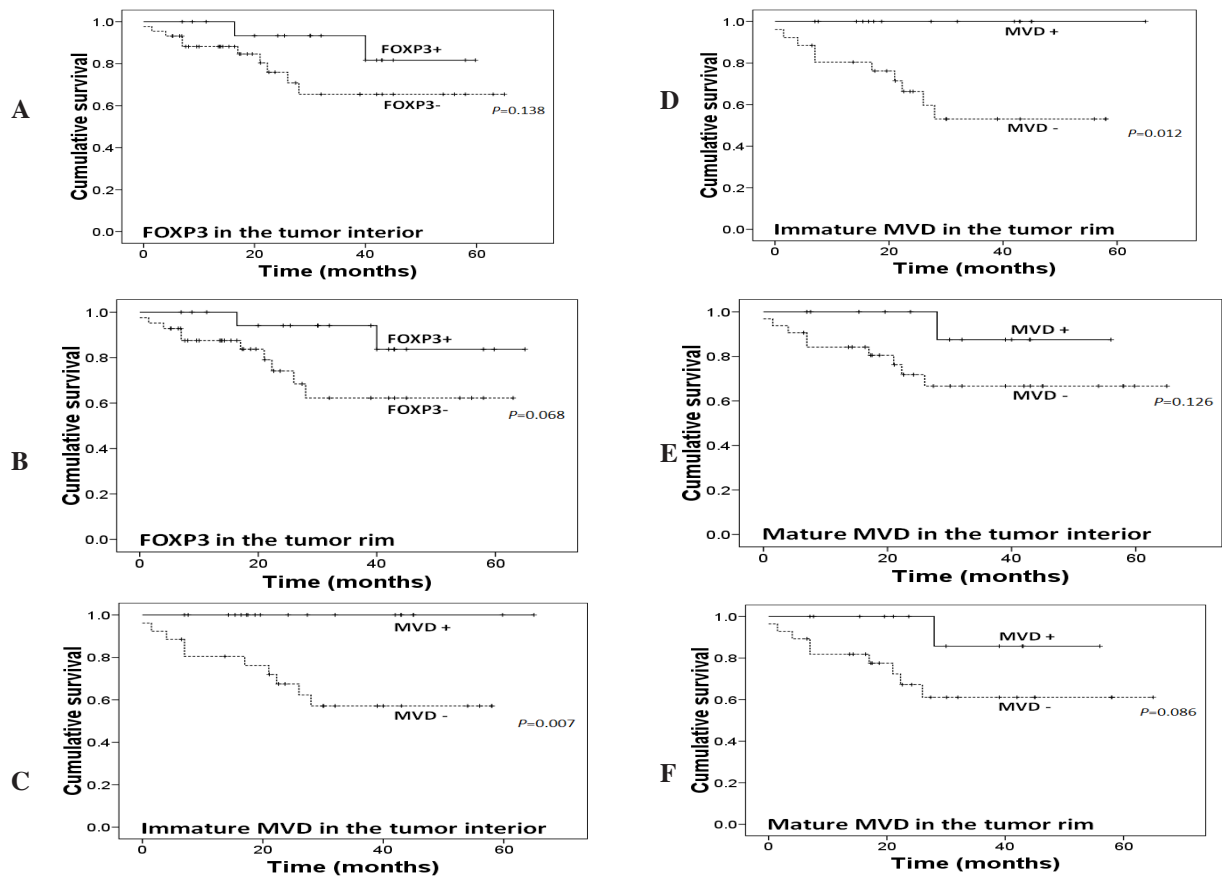


**Figure 2. Double Immunohistochemistry for the Detection of Endothelial Cells** (brown cytoplasm shown with arrows) **and Pericytes** (red cytoplasm with hollow arrows) respectively in the tumour interior (A), rim (B) and normal kidney tissue (C) at 400× magnification. All CD31-positive entities associated with  $\alpha$ -SMA-positive cells were counted as mature vessels, and CD31-positive entities associated with  $\alpha$ -SMA-negative cells were counted as immature vessels. Quantification of immunohistochemistry results detecting mature MVD (D) and immature MVD (E) in normal tissue, tumor interior or rim

*MVD and pathological characteristics in RCC.*

The number of mature microvessel in the tumour interior (4.0 (2-7.75)/HP) or the rim (4.7 (3.0-12.0)/HP) was higher than normal kidney tissue (1.0 (1.0-2.0)/HP), with a statistically significant difference ( $P < 0.05$ ). Furthermore, the number of immature microvessels in the tumour interior (25 (12.9-36.11)/HP) or the rim (23.4 (14.3-36.0)/HP) was also higher than normal tissue (1.0 (1.0-2.0)/HP), as illustrated in Figure 2 ( $P < 0.05$ )

The relationship between immature MVD in the tumour interior or rim and clinical pathological characteristics is summarised in Table 3. A negative



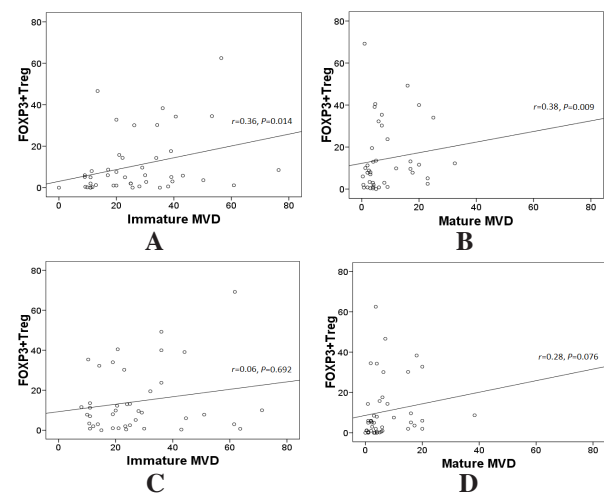
**Figure 3. Kaplan-Meier Curves for Patients with RCC.** A) FOXP3 in the tumour interior (log-rank test,  $P=0.138$ ); B) FOXP3 in the tumour rim (log-rank test,  $P=0.068$ ); C) the immature MVD in the tumour interior (log-rank test,  $P=0.007$ ); D) the immature MVD in the tumour rim (log-rank test,  $P=0.012$ ); E) the mature MVD in the tumour interior (log-rank test,  $P=0.126$ ); F) the mature MVD in the tumour rim (log-rank test,  $P=0.086$ ). The dotted line represents the negative group (below the cut-off point) and the solid line the positive group (above the cut-off). The cut-off point was the median value of FOXP3, immature MVD and mature MVD

correlation was confirmed between the immature MVD in the tumour interior or rim and tumour stage ( $r=-0.586$ ;  $P=0.007$  and  $r=-0.602$ ;  $P=0.037$ , respectively in Table

**Table 3. The Relationship Between Immature MVD in the Tumour Interior or Rim and Clinicopathological Characteristics**

	Tumour interior (n=48)		P	Tumour rim (n=46)		P
	$\leq 27.1$	$>27.1$		$\leq 27.0$	$>27.0$	
Tumour size						
<7cm	15	17	0.05	12	18	0.043
$\geq 7$ cm	12	4		11	5	
Tumour stage						
I	9	16	0.005	6	17	0.003
II	4	4		4	4	
III	5	0		5	0	
IV	9	1		8	2	
Nuclear grade						
1	9	9	0.588	7	9	0.744
2	11	7		10	8	
3	5	2		4	3	
Pathological type						
Clear cell RCC	22	19	0.381	18	21	0.218
Non- Clear cell RCC	5	2		5	2	
Metastasis						
present	10	2	0.01	8	4	0.045
absent	15	21		15	19	
Overall Survival						
Yes	16	21	0.001	15	20	0.044
No	11	0		8	3	

3) and overall survival ( $P=0.002$ ,  $P=0.007$ , as shown in Figure 3 C and D). However, there was no correlation with the nuclear grade or pathological type. There was a negative correlation between immature MVD with tumour stage and with overall survival ( $P=0.005$ ;  $P=0.001$ , respectively) (Table 3). There were no correlation between the mature MVD and tumour size, tumour stage, nuclear



**Figure 4. Correlation Between FOXP3+ Tregs and MVD.** A. FOXP3+ Tregs and immature MVD in the tumor interior ( $r=0.363$ ,  $P=0.014$ ); B. FOXP3+ Tregs and mature MVD in the tumor interior ( $r=0.383$ ,  $P=0.009$ ); C. FOXP3+ Tregs and immature MVD in the tumor rim ( $r=0.064$ ,  $P=0.692$ ); D. FOXP3+ Tregs and mature MVD in the tumor rim ( $r=0.281$ ,  $P=0.076$ )

grade, pathological types (Table 3) and overall survival in the tumour interior or the rim ( $P>0.05$ , respectively; Figure 3E and F).

### Correlations

In the tumour interior, there was a positive correlation between FOXP3<sup>+</sup>Tregs and immature MVD ( $r=0.363$ ,  $P=0.014$ ) and mature MVD ( $r=0.383$ ,  $P=0.009$ ). In the tumour rim, no correlations between FOXP3<sup>+</sup> Tregs and mature MVD ( $r=0.281$ ,  $P=0.076$ ) or immature MVD ( $r=0.064$ ,  $P=0.692$ ) were confirmed (Figure 4).

## Discussion

To our knowledge, this is the first report describing the relationship between tumour-infiltrating FOXP3<sup>+</sup> Tregs and MVD, and its effect on overall survival in patients with RCC.

CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> Tregs have been documented to be increased in the periphery of RCC patients compared with normal subjects, and may be associated with RCC pathology and prognosis (Cesana et al., 2006). Furthermore, the clinical significance of tumour-infiltrating FOXP3<sup>+</sup> Tregs in the cancer microenvironment, as a prognostic factor for poor survival, has been demonstrated in many studies with a variety of malignancies (Seo et al., 2001; Liyanage et al., 2002). As such, we expected the proportion of FOXP3<sup>+</sup> Tregs to increase in tumour infiltrating lymphocytes among patients with more aggressive forms of RCC. On the contrary, FOXP3<sup>+</sup> cells were not found to be significantly associated with RCC pathology or patient outcome, which is consistent with Siddiqui et al. (2007). In human malignancies, the role of infiltrating FOXP3<sup>+</sup>Tregs in disease biology remains a subject of debate. Alvaro et al. (2005) found that increased FOXP3 expression within reactive lymphocytes in Hodgkin's lymphoma patients was associated with improved survival. Siddiqui et al. (2007) further noted that CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> T cells, but not CD4<sup>+</sup> CD25<sup>+</sup> FOXP3<sup>+</sup> T cells, were associated with higher TNM staging, larger tumour size, presence of coagulative tumour necrosis, and poorer cancer-specific survival.

Increased MVD has been associated with early progression in a number of tumours, including breast, colon and prostate. However, the correlations between MVD and pathological factors in RCC remains controversial. In our study, we recorded the mature MVD and immature MVD in the different locations of the tumour. Our results showed negative correlations between the immature MVD in the tumour interior or rim and tumour size, tumour stage and overall survival, but no correlations with nuclear grade or pathological type. In addition, we found no correlation between the mature MVD and tumour size, tumour stage, nuclear grade, pathological type or overall survival. The data showed that distinct types of vasculature in RCC correlated with a distinct prognosis, and the immature MVD was more important to the prognosis of RCC than mature MVD.

Tumour angiogenesis can lead to immunosuppression, as a result of defective lymphocyte recruitment through a complex process involving impairment of immune

cell adhesion to endothelial cells in vessels. Vascular endothelial growth factor (VEGF) is secreted at high levels in many tumours, including RCC, and it is a major regulator of tumour growth and metastasis with a key role in tumour angiogenesis (Folkman, 1995). VEGF has also been linked to immunosuppression and shown to inhibit the development of dendritic cells (Gabrilovich et al., 1998) and enhance the number of FOXP3<sup>+</sup> Tregs within the tumour microenvironment (Giatromanolaki et al., 2008). Recently, the correlation between the presence of FOXP3<sup>+</sup> Tregs and high vessel density were confirmed in endometrial adenocarcinomas (Gupta et al., 2007) and breast carcinoma (Li et al., 2006). The increased Treg cells in metastasis in RCC patients were reduced after treatment with angiogenesis inhibitors such as sunitinib (Finke et al., 2008; Ko et al., 2009)

In RCC, the presence of FOXP3<sup>+</sup> lymphocytes was significantly associated with high immature angiogenesis in the tumour interior, scored as vessels expressing the endothelial marker CD31, but not expressing the pericyte marker  $\alpha$ -SMA. The numbers of Tregs in RCC may be an indirect affect due to their promotion of tumour vasculature. As previously studied, Tregs play the immunosuppressive function through the secretion of some cytokines such as IL-10, IL-4, TGF- $\beta$ , while the TGF- $\beta$  may also act on tumour vascular pericytes and promote tumour angiogenesis (Gaengel et al., 2009). Recently, Dace et al. (2008) found that IL-10 also promote pathological angiogenesis by regulating macrophage responses to hypoxia.

For many cancers, abundant tumor vascularity is associated with a greater risk for tumor progression (Sabo et al., 2001a). It is generally accepted that increased vascularization is associated with tumor growth and greater invasive potential. Multiple studies have found that in contrast to other neoplasms, lower MVD in RCC is associated with poor prognosis (Yoshino et al., 1995; Köhler et al., 1996; Delahunt et al., 1997; Herbst et al., 1998; Sabo et al., 2001a, 2001b). It is unclear why poorly vascularized RCC exhibits a worse prognosis. It is possible that the decreased MVD in high grade RCC reflects an inability of the vascularization to keep pace with tumor growth and increased tumor vasculature permeability may compensate for the lower MVC (Sabo et al., 2001b). Another possibility is that tumor progression may results in larger vascular channels within a RCC tumor and compensates for the lower MVD in providing sufficient blood flow (Delahunt et al., 1997). This difference in vascularisation activities between RCC and many other cancers may also result in reduced metabolism and extensive necrosis in late stage RCC. As the tumor becomes necrotic, viable tumor cells may gain access to the vascular system resulting in tumor metastasis (Sabo et al., 2001b).

In conclusion, immune suppression and angiogenesis in the tumour microenvironment simultaneously exist in major tumours, including RCC. In our study, the correlation between the presence of FOXP3<sup>+</sup> Tregs and immature MVD in RCC were confirmed, which may be one factor contributing to poor treatment outcome and survival after metastasis of RCC. The mechanism

responsible for the interaction FOXP3<sup>+</sup> Tregs and immature MVD warrants further study. The association of MVD and FOXP3 with overall survival may also suggest that they be useful prognosis markers. Combined treatment with immunotherapy and anti-angiogenic therapies would be of benefit to patients with RCC.

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