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Immunohistochemical staining of mTOR, vascular endothelial growth factor, CD34 and microvascular density in clear cell renal cell carcinoma and its correlation with clinicopathological profile of the tumour

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Abstract
Background: The study analyses immunoexpression of vascular endothelial growth factor (VEGF) and mammalian target of rapamycin (mTOR) based pathways which are mainstay of drug therapy in advanced and metastatic clear cell renal cell carcinoma (ccRCC) and to delineate their role as prognostic markers in a cohort of primary ccRCC.
Methods: Descriptive study wherein immunohistochemical profiling of 100 cases of primary ccRCC was carried out using primary antibodies against mTOR, VEGF and CD34. Mean vascular density (MVD) and immunoreactivity was correlated with grading, staging, risk stratification and survival data.
Results: mTOR and VEGF immunostaining was positive in 54% and 70% cases respectively with strong immunoreactivity correlating with high grade, high stage and high risk tumours with low survival. Inverse association was observed with MVD wherein low grade, low stage and low risk ccRCCs were associated with higher MVD.
Conclusion: mTOR and VEGF expression in ccRCCs is associated with unfavourable prognosis. High MVD correlates with better outcome though concordance is limited by tumour heterogeneity.

Keywords: Clear Cell Renal cell carcinoma, Prognostic and Predictive markers

1. Introduction
Clear cell RCCs (ccRCC) are closely associated with VHL gene mutations that lead to stabilization of hypoxia inducible factors (HIF-1α and HIF-2α) and seen in approximately 80% of the ccRCCs [1]. Mutation or inactivation of *VHL* impairs the cell's ability to degrade HIF resulting in an overabundance of vascular endothelial growth factor (VEGF) and other pro-angiogenic factors [2]. In addition, there is an mammalian target of rapamycin (mTORC1) pathway, deregulated by mutations in *mTOR, TSC1, PIK3CA* and *PTEN* in approximately 20% of ccRCCs and may predict sensitivity to mTOR inhibitors [3]. The agents for subspecific therapies constitute two classes: Firstly angiogenesis inhibitors targeting the VEGF ligand (bevacizumab) or VEGF receptors (sunitinib, sorafenib); and secondly inhibitors of the mTOR signalling (temsirolimus, everolimus) [4, 5].
The tumour expression of VHL, the endothelial marker CD34, PDGFRα and VEGF are important markers for prognosis and outcome of patients with advanced ccRCC. The applicability of these molecular markers for prediction of sunitinib response has been demonstrated by recent studies [6]. Moreover these markers also provide prognostic information which is vital for monitoring disease progression. However presently molecular characterization is not routinely carried out and hence prediction of response to therapy is not established at the outset. Hence this study was undertaken to analyse immunoexpression of biomarkers incorporating VEGF and mTOR based pathways using immunohistochemical techniques and delineate their role as prognostic markers by correlating them with the clinicopathological profile in a cohort of primary ccRCC managed at a tertiary care centre

2. Materials and Methods

Our study cohort consisted of 100 patients who underwent radical or partial nephrectomy for ccRCC at a tertiary care super speciality centre in Delhi between 2016 and 2018. Approval of institutional ethical committee was taken. Written informed consent was obtained. All tumours with morphology other than that of ccRCC, all clear cell variants (clear cell papillary RCC, translocation carcinomas), tissue suboptimal for ancillary studies and inadequate follow up details were excluded from the study. Patient's demographic data along with clinical details (age, gender, Eastern Cooperative Oncology Group performance status for risk assessment, metastasis and mortality) was noted. For risk assessment UCLA staging system was used and patients were categorised into low risk, intermediate risk and high risk [7].

2.1 Histopathology: 100 Nephrectomy specimen obtained as part of clinical management were processed as per standard guidelines issued in college of American pathologist (CAP) protocol. 5µm thick hematoxylin and eosin (H&E) stained section were examined and tumour graded as per conventional Fuhrman grading system.

2.2 Immunohistochemical staining and evaluation: IHC was carried out on formalin fixed paraffin embedded tissues using the following antibodies: VEGF (mouse monoclonal, clone VG1, isotype IgG1, Prediluted, Diagnostic Biosystem, Catalogue no PDM165), PhosphomTOR (Phospho-mTOR Ser2448 (49F9) Rabbit monoclononal #2976, concentrated, 1:100 dilution Cell signalling technology) and CD34 (Rabbit monoclonal, clone EP 88, isotype IgG, Prediluted, BioSB catalogue no 6486). Secondary detection system used was single step polymer based detection system (Envision detection system, peroxidise/DAB, rabbit/mouse). Cytoplasmic staining for mTOR, membranous staining for CD34 and cytoplasmic membranous staining for VEGF was considered positive.

2.2.1 Interpretation of staining: For VEGF and mTOR immunostaining greater than 10% of tumor cells was scored as a positive. The interpretation score was as follows: 0 or negative = ≤10% tumor cell positivity; +1 or weak = 11–25% tumor cell positivity; +2 or moderate = 26–50% tumor cell positivity; and +3 or strong = >50% tumor cell positivity.

2.2.2 CD 34 staining and calculation of mean vascular density: CD34 staining was performed to highlight the vascular channels for evaluation of mean vascular density (MVD). For assessment of the vascular density within a tumour section, one hotspot (most vascularised microscopic field) was selected and four areas were chosen randomly. Microvessels in sclerotic areas and immediately adjacent to the tumour and normal renal tissue interface were not considered in the vessel counts Photomicrographs were taken and vessel counts were carried out at × 200 magnification using an optical grid. For vessel count Dewinter Biowizard image analysis software was used. The photomicrograph was placed in an optical grid and grid area was determined on the software. The vessels which were highlighted by CD 34 staining were further highlighted in the software along with its negative images and automated count within the optical grid area was carried out. The total density was then expressed as number of vessels per mm^2. An average MVD of five sections was taken.

2.3 Statistical analysis: IHC results were tested for their association with the histological parametres and survival data (alive vs. dead) using appropriate descriptive statistics. All statistical analyses were performed using the SPSS 21.0 software. Statistical significance was considered when P value ≤ 0.05.

3. Results

3.1 Demographic data: Age of patients (n=100) ranged between 40 years and 79 years with a mean age of 55 years (SD-9.84). Male patients were 64% while females were 36%.

3.2 Clinical characteristics: Patients underwent radical nephrectomy (87%) and partial nephrectomy (13%) depending upon the clinical characteristics, tumour size and tumour location. Right kidney was affected in 52/100(52%) cases whereas in 48/100(48%) cases tumour was present in the left kidney. The tumours were grouped into the following T stage based on their maximum dimension; T_1 = 74/100, T_2 = 18/100 and T3= 8/100. Regional lymph node metastasis (N_1) was observed in 12 cases while 86 cases were negative for nodal metastasis (N_0). 24/100 cases were positive for tumour metastasis to different sites. 8/100 showed metastasis to bone, 12/100 cases showed metastasis to lung and 4/100 cases metastasized to liver. The remaining 76/100 cases had no evidence of metastasis. The distribution of TNM (Tumour, Node and Metastasis) stage grouping is as follows: Stage 1=62%, Stage 2=16%, Stage 3 =2% and Stage 4=20%.

3.3 Follow up: Mean follow up period of patients was 71 months +- SD 32.19 months with median of 75 months and range of 12 months to 170 months. During follow 20 /100 patients died due to disease progression (disease recurrence, progressive disease, metastasis) while 80 patients were alive.

3.4 Histopathological examination: All the cases were graded as per Fuhrman nuclear grading system as Grade 1 = 21/100, Grade 2=37/100, Grade 3= 30/100 and Grade 4=12/100.

3.5 Immunohistochemical examination and correlation with tumour characteristics

3.5.1 mTOR immunostaining: mTOR positivity was observed in 54/100 cases as diffuse cytoplasmic staining. 24/100 cases showed 1+ positivity, 18/100 exhibited 2+ positivity and 12/100 cases were 3+ positive staining. (Figure 1A&B). mTOR staining of the tumour was correlated with its Fuhrman nuclear grade, nodal status, metastasis and stage grouping and results are exhibited Table 1&2. Positive statistical correlation was observed between these variables and status of mTOR immunostaining. Higher immunoreactivity scores correlated with higher grade and stage of the tumour (Pearson chi-square - x^2 =27.515[a], p =0.001 and x^2 =34.431[a] p=0.000). Similarly high intensity of mTOR staining also correlated

with positive nodal status (x^2 = 16.330[a] and P value = 0.001) and metastasis (x^2 = 28.865[a] and P value = 0.001) though T status did not show statistical significance (x^2 = 7.107[a] and P value = 0.064). Table 1&2.

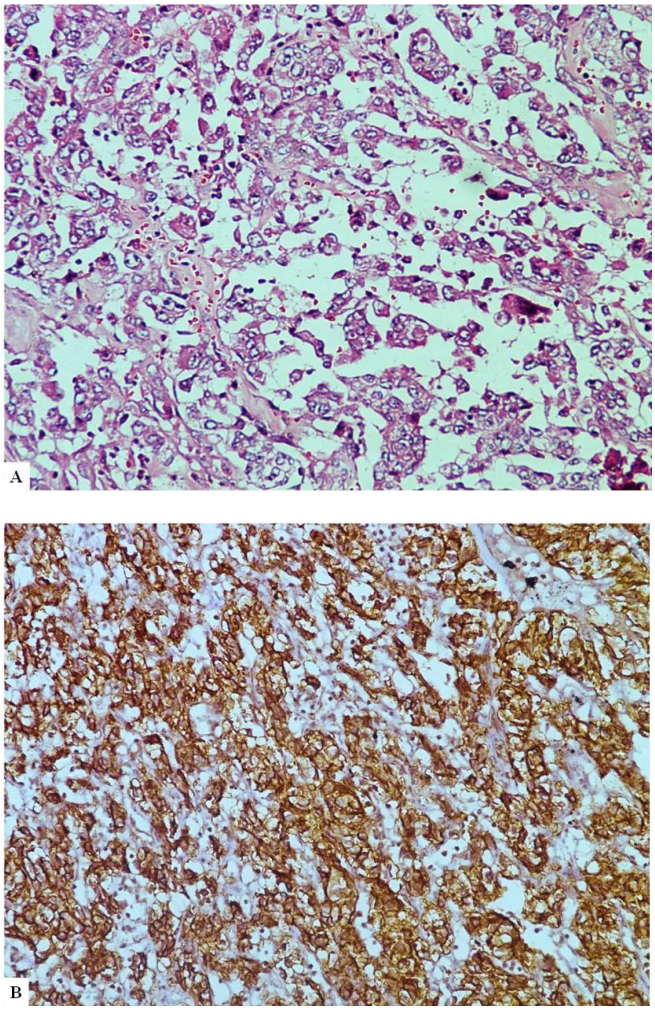


Figure 1: Clear cell RCC Furhman grade IV (A) Haematoxylin & eosin, magnification 200X. (B) mTOR staining showing diffuse cytoplasmic pattern, Immunoperoxidase x diaminobenzaldehyde stain, immunoreactivity score 4, magnification 200X.

Table 1: Distribution of mTOR immunopositivity with respect to Furhman grade and stage of tumour.

N=100

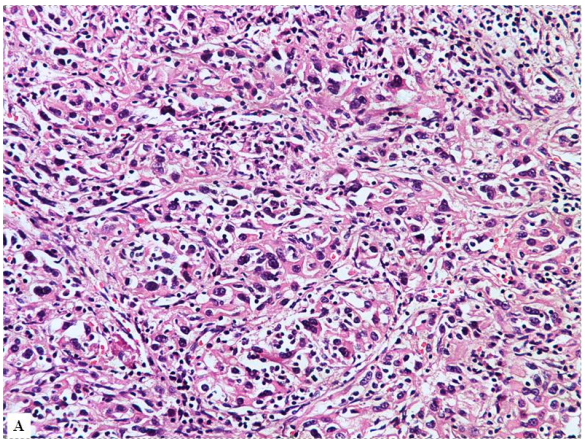
mTOR IHC scores	Furhman grade				Total
	cc	2	3	4	
0	16	24	6	0	46
1	6	6	12	0	24
2	0	2	8	8	18
3	0	4	4	4	12
Total	22	36	30	12	100
	Stage grouping				Total
	1	2	3	4	
0	34	8	0	4	46
1	20	4	0	0	24
2	8	4	2	4	18
3	0	0	0	12	12
Total	62	16	2	20	100

Table 2: Distribution of mTOR immunostaining status with respect to tumour, nodal status and metastasis.

N=100

mTOR IHC scores	Tumour status			Total
	T_1	T_2	T_3	
0	36	8	2	46
1	20	4	0	24
2	12	4	2	18
3	6	2	4	12
Total	74	18	8	100
	Nodal status			Total
	Negative		Positive	
0	46		0	46
1	24		0	24
2	10		8	18
3	8		4	12
Total	88		12	100
	Metastasis			Total
	Negative		Positive	
0	42		4	46
1	24		0	24
2	14		4	18
3	0		12	12
Total	80		20	100

3.5.2 Vascular endothelial growth factor immunostaining: Immunohistochemical staining for VEGF exhibited granular homogenous cytoplasmic positivity with 70/100 cases (70%) positive for VEGF; 32/100 cases (32%) stained 1+ positive, 30/100 cases (30%) stained 2+ positive and 8/100 cases (8%) stained 3+. (Figure 2 A&B). Furhman grade and tumour stage when correlated with VEGF staining exhibited positive statistical association between higher nuclear grade (x^2 = 51.514[a] and P value = 0.000) and high tumour stage (x^2 = 28.865[a] and P value = 0.001) with higher intensity of the VEGF staining. The intensity of VEGF staining also correlated positively with the nodal status (x^2 = 33.58[a] and P value = 0.000) and metastasis(x^2 = 12.78[a] and P value = 0.002) as stronger VEGF expression was associated with positive lymph node status and metastasis. No correlation was found between Tumour size and VEGF immunostaining status (x^2 = 9.606[a] and P value = 0.06). (Table 3&4).



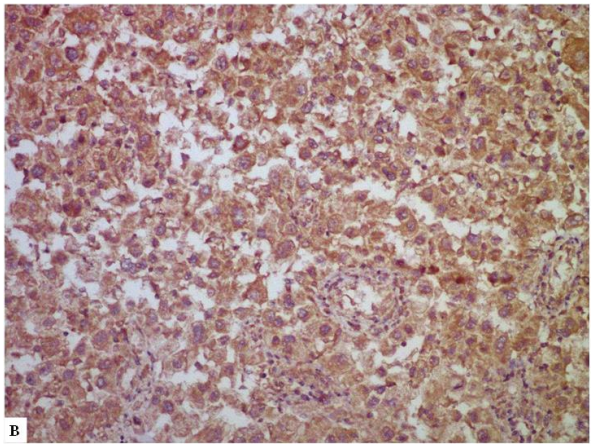


Fig 2: Clear cell RCC Furhman grade IV (A) Haematoxylin & eosin, magnification 200X. (B) Vascular endothelial growth factor staining showing diffuse cytoplasmic immunostaining, immunoperoxidase x diaminobenzaldehyde stain, immunoreactivity score 4, magnification 200X

Table 3: Distribution of vascular endothelial growth factor immunopositivity with respect to Furhman grade and stage of tumour.

N=100

VEGF IHC scores	Furhman grade				Total
	1	2	3	4	
0	14	16	0	0	30
1	8	12	12	0	32
2	0	8	18	4	30
3	0	0	0	8	8
Total	22	36	30	12	100
	Stage grouping				Total
	1	2	3	4	
0	24	4	0	2	30
1	26	4	0	2	32
2	10	8	2	10	30
3	2	0	0	6	8
Total	62	16	2	20	100

Table 4: Distribution of Vascular endothelial growth factor immunostaining status with respect to Tumour, nodal status and metastasis.

N=100

VEGF IHC scores	Tumour status			Total
	T_1	T_2	T_3	
0	12	3	0	15
1	14	2	0	16
2	8	4	3	15
3	3	0	1	4
Total	37	9	4	50
	Nodal status			Total
	Negative		Positive	
0	15		0	15
1	16		0	16
2	13		4	15
3	4		2	4
Total	44		6	50
	Metastasis			Total
	Negative		Positive	
0	14		1	15
1	15		1	16
2	10		5	15
3	1		3	4
Total	40		10	50

3.5.3 CD 34 immunostaining and mean vascular density: MVD obtained in 100 ccRCC cases was 25.25+- 10.35 SD with median and mode of 29.26 and 30.90 respectively. The tumours were classified into low grade (grade 1 and II) and high grade (grade III and IV) for simplification of results and their MVD was correlated with standard parameters using one way ANOVA. Table 5, figure 3A&B, figure 4C&D.

Table 5: Distribution of mean vascular density with respect to tumour characteristics

n=100

Parameters	MVD	No of cases	SD	F value	P value
Furhman grade					
Low grade	32.7831	29	3.90811	140.355	0.000
High grade	14.8610	21	6.74648		
Stage grouping					
Low stage	27.3076	41	9.09948	10.725	0.002
High stage	15.9089	9	11.06409		
Tumour status					
T_1	26.6481	37	10.04429	1.93	0.15
T_2	23.3544	9	10.05441		
T_3	16.6550	4	11.68139		
Node status					
N_0	27.5818	44	8.68837	29.12	0.001
N_1	8.1983	6	2.02198		
Metastasis					
M_0	26.6940	40	9.60452	4.1	0.04
M_1	19.5030	10	11.73269		

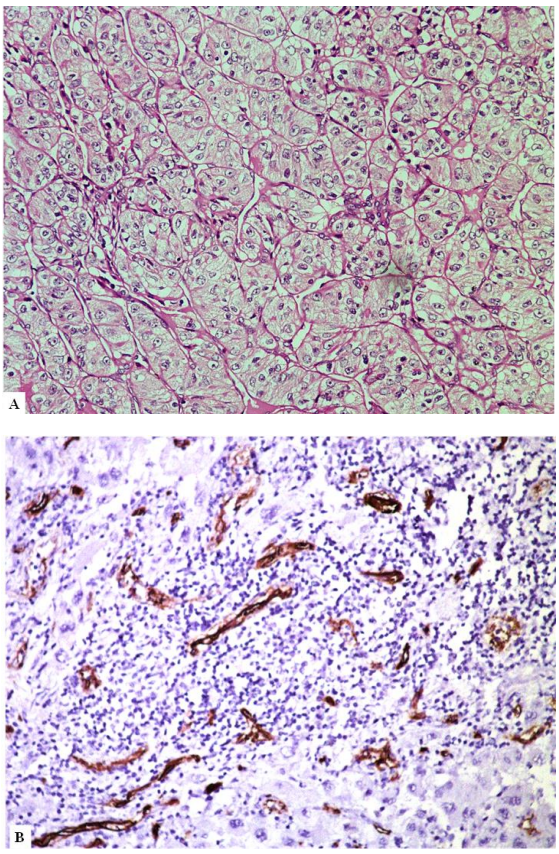


Fig 4: Clear cell RCC Furhman grade 4, Haematoxylin & eosin, magnification 200X. (B) CD34 immunostaining highlighting sparse vascular network, immunoperoxidase x diaminobenzaldehyde, magnification 200X.

3.6 Risk stratification and survival data

All 100 cases of ccRCC were stratified into low risk, intermediate risk and high risk cases according to UCLA guidelines. Low risk cases were 38%, intermediate 48% and 14% were high risk cases. Intense mTOR and VEGF expression was associated with high risk cases while high MVD was associated with low risk tumours. $x^2 = 22.10^a$ and P value = 0.001, $x^2 = 41.99^a$ and P value = 0.000 and $x^2 = 31.78^a$ and F value = 0.000 for mTOR, VEGF and MVD respectively. All cases who died on follow up (n=20) had tumours exhibiting strong mTOR and VEGF expression with low MVD. Table 6

Table 6: Distribution of survival data with respect to tumour immunohistochemical characteristics.

N=100

Parameters	Survival		x^2	P
	Dead	Alive		
mTOR				
0	1	45	40.587[a]	0.000
1	2	22		
2	8	10		
3	9	3		
Vascular endothelial growth factor				
0	2	28	19.049[a]	0.000
1	5	27		
2	7	23		
3	8	2		
Mean vascular density				
16.945	20	80	19.284	0.000
27.333				

3.7 Immunohistochemical expression of markers in controls

06 cases of normal renal tissue obtained from autopsies were also immunostained. mTOR positivity of low intensity was noted in the renal tubules of normal renal tissue. VEGF expression was also noted in the tubular epithelium and trace to weak expression in the glomeruli.

4. Discussion

The heterogeneity of the ccRCCs in the term of molecular signatures led various studies to evaluate the prognostic and predictive markers in current scenario of personalized medicine. Precise histopathological diagnosis as well as molecular characterization is essential for therapeutic response. Moreover management of advanced and metastatic RCC has evolved rapidly with FDA approval of *six* targeted agents directed against VEGF and mTOR pathways. However, these treatments also pose challenges in terms of selecting the best therapy for a given patient. Hence this study was undertaken for characterization of our cohort of clear cell RCCs into two major categories of VEGF and mTOR based pathways using immunohistochemical techniques and correlating them with the clinicopathological profile.

4.1 mTOR

mTOR pathway is up-regulated in many tumours and therapeutic agents targeting the mTOR pathway are in various stages of clinical trials. At present, the mTOR inhibitors temsirolimus (CCI-779), AP23573 and everolimus (RAD001) have been part of the clinical regimen for advanced and metastatic disease. However not all RCCs are equally amenable to mTOR targeted therapy and therefore evaluation of mTOR expression or expression of downstream markers like PTEN, pAkt, p27, and pS6 which constitute phosphatidylinositol 3-kinase/mammalian target of Rapamycin (PI3K/mTOR) pathways may serve as surrogate parameters for patient selection and predicting prognosis [8].

Current literature on mTOR immunostaining, which is not much, is in consonance with our findings. Pantuk AJ *et al*. in 375 cases of ccRCC found nuclear pAkt, PTEN, and pS6 immunostaining as independent prognostic factors and hence suggested that the mTOR pathway is more significantly altered in high-grade ccRCC and tumors with poor prognostic features [8]. Stephan Kruck *et al*. analysed mTOR and p-mTOR immunostaining and found that the mTOR to p-mTOR ratio might serve as a sensitive surrogate parameter to predict survival or response to mTOR targeted therapy. This ratio was not attempted in our study. Their results suggested that immunohistochemical evaluation of mTOR might be useful in predicting the outcome of patients with primary ccRCC in association with conventional prognostic parameters [9]. Luciana Schultz *et al*. in their multivariate model demonstrated that mTOR expression is the single most important marker for survival and tumour progression, and when included, Fuhrman grade and stage lost significance [10].

Yousiff TA *et al*. in their cohort found that cytoplasmic mTOR expression in RCC metastases was the only marker of mTOR pathway that was found to be independently associated with poorer cancer-specific survival [11]. Furthermore, cytoplasmic p-mTOR expression in primary RCC was also correlated with metastases, suggesting its potential use to predict mTOR activation status in metastatic tumors. The authors also suggested that tumour tissues should be collected and immunostained before and after treatment to assess true response versus off-target effects. However similar correlation were not reported by Cho *et al*. in their analyses of primary and metastatic RCC with mTOR inhibitor temsirolimus therapy [12]. Some of our cases of metastatic RCCs on follow up were biopsied however such correlation was not attempted in our study.

4.2 VEGF

VEGF expression has been studied extensively in various studies. Our study revealed high VEGF expression in high stage, high grade and high risk patients. In a study conducted by Jacobsen J *et al*., VEGF expression correlated with higher tumour stage in both conventional and papillary RCCs including the tumour size [13]. In our study higher VEGF expression was seen in larger tumours though the numbers were not statistically significant.

Liontos M *et al*. studied 79 patients of metastatic ccRCC treated with sunitinib and indicated that immunohistochemical expression for VEGF and mTOR proteins may be able to differentiate patients refractory to first-line sunitinib treatment with poorer prognosis [14]. Minardi D *et al*. in their cohort of ccRCC showed that expression of VEGF was directly related higher tumor stage and Fuhrman grade. Expression also predicted patient outcome, suggesting a potential use in identifying prognostically different patients of ccRCC [15].

4.3 CD 34 staining and mean vascular density

Clear cell RCCs are highly vascularised tumours and hence angiogenesis is integral to its pathogenesis. The current study evaluated tumour angiogenesis by CD 34 staining and by calculating the mean vascular density. In a study conducted by Amparo Ruiz-Saurí *et al*., CD 31 was used to stain the immature endothelial cells and CD 34 to stain mature ones in the tumour and it was found that ccRCC exhibited greater positivity for CD 34 showing that mature endothelial cells formed the major chunk of the tumour vasculature [16]. Hence CD 34 immunostaining was utilised in our study.

Our study revealed inverse association between MVD and tumour characteristics wherein high nuclear grade, high stage and high risk ccRCCs were associated with low MVD. Similarly inverse was true for low grade, low stage RCCs which exhibited high vascularity and high MVD. Our findings are concordant with study conducted Baldewinjs MM *et al*. who compared angiogenesis parameters in low-grade *vs* high-grade ccRCC and found that high-grade ccRCC exhibited low MVD than the low-grade subgroup [17]. Besides they also demonstrated that low grade ccRCC with high MVD are characterised by a lower VEGF protein expression which similar to our findings in our study. The authors hypothesised that tumour progression is associated with a reduction in the intrinsic propensity of cancer cells to undergo apoptosis. Moreover they postulated that tolerance of hypoxic conditions by high grade ccRCC cancer cells can afford an increased intercapillary distance with lower MVD in comparison with low-grade RCC.

However the results between vascularity as measured by MVD and prognosis are conflicting. An association between high MVD and poor prognosis was reported by Joo *et al*. [18]. Other groups have observed an association between high MVD and better prognosis, whereas some groups failed to confirm the association between MVD and prognosis [19, 20, 21]. These discrepancies may be attributable to the fact that MVD alone is not the sole predictor of survival in patients of ccRCC. The reason why poorly vascularized ccRCC tumors are more often associated with worse clinical prognosis is not clear which is in contrast to the growth patterns of other tumors where MVD is strongly associated with aggressive behaviour. Kohler *et al*. suggested that the decreased MVD in high-grade RCCs reflects the inability of tumor neovascularization to keep pace with the proliferation of the high-grade tumor cells, and increased tumor vessel permeability may compensate for their reduced amount [22]. Herbst *et al*. considered the RCC microvasculature as a potential parameter of tumor differentiation. Hence a well differentiated, low-grade RCC has an abundant vascular stroma [23]. On the contrary a high-grade solid RCC enlarges more rapidly, overcoming its vascular network and decreasing its architectural complexity. Delahunt *et al*. hypothesized that the development of large diameter vascular channels in larger tumours could be the reason for decreasing MVD in such tumors. Though our results are in consonance with the published literature there is significant heterogeneity amongst the studies which can hamper establishing mean vascular density as a prognostic marker for clear cell renal cell carcinoma. However the differences in angiogenesis biology might have impact on the effects of anti-angiogenic or anti-VEGF treatment of ccRCC [24].

5. Conclusion

To conclude high mTOR and VEGF expression in ccRCCs was significantly associated high grade, high stage tumours with metastatic disease and high risk stratification. Similar association as well as correlation with therapeutic response to mTOR /VEGF inhibitor therapy was observed in extensive review of literature. Studies recommend tissue evaluation for mTOR and VEGF expression for primary and metastatic disease to determine for future response to MTOR inhibitors and anti VEGF therapy. Concordance for MVD as prognostic and predictive marker is not very favourable due to significant variations in literature.[24]The above findings need validation with increased sample size and follow up. Besides it will be worthwhile to evaluate cases with the above markers specifically with advanced and metastatic disease before and after institution of therapy with follow up for better interpretation and elucidation of results in the form of survival curves and survival data

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