Original Article

Immunohistological Diagnosis of Primary and Metastatic Renal Cell Carcinoma Using Panel of Immunohistochemical Markers: A Single Centre Study

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ABSTRACT

Background: Tumour heterogeneity and lack of markers with high specificity makes diagnosis of renal cell carcinoma (RCC) challenging. The study was undertaken to evaluate panel of IHC markers to enable diagnosis and reproducible classification in primary and metastatic renal tumors.

Methods: Descriptive Study wherein 100 cases of RCC and 25 trucut biopsies (20 metastatic and 5 primary renal tumors) were evaluated for morphology and immunostained by panel of immunohistochemical (IHC) markers consisting of CA-9, CD10, CK-7, AMACAR and TFE-3 with additional markers as required.

Result: Morphologically tumors were grouped as clear cell and nonclear cell (eosinophilic and poorly differentiated). Clear cell RCCs (CCRCC), clear cell papillary RCC (CCPRCC) and multilocular cystic RCC (MCRNLMP) displayed strong statistical association of CA-9 immunostaining (p=50.00, x2-0.000). Inverse correlation was found between the intensity of the staining of CA-9 and tumor grade. (p=32.97, x2=0.000). CA-9 and CK-7 co-expression was evident in all cases of CCPRCC and MCRNLMP. Papillary RCC exhibited positive statistical correlation with CK-7 and AMACAR. E-cadherin and CD117 were required additionally to differentiate between oncocytoma and chromophobe RCC. CD10 and Pax 8 were most helpful in diagnosing metastatic RCCs

Conclusion: IHC panel consisting of CA-9, CD10, CK7, AMACR and TFE3 helps triage RCCs with clear cell/eosinophilic cell / papillary/ poorly differentiated pattern. In a setting of metastatic RCC, use of CD10 and Pax 8 together facilitate primary diagnosis of RCC when tissue available is limited.

Keywords: Immunohistochemistry Renal Cell Carcinoma Carbonic anhydrase Cytokeratin -7

Introduction

Renal cell carcinomas (RCCs) comprise of various clinicopathological entities, each displaying distinct morphological, immunohistochemical and molecular characteristics. These subtypes have different clinical outcomes and show different response to therapy. The emerging therapeutic possibilities have made accurate classification mandatory. [1]

RCC is notorious for its metastatic potential, presenting as a metastatic disease in 30% patients or recurrence in 50% after a radical surgery. [2,3] Usually, it is easy to diagnose RCC and to subclassify by routine histological examination in a nephrectomy specimen but diagnosis can be complicated by diverse histological variations. Besides, diagnosis and accurately classification of RCCs in trucut biopsy of a metastatic lesion without having access to the histology of the primary lesion is challenging. Though morphological assessment still remains the mainstay of RCC classification, there are immunohistochemical (IHC) and molecular markers that serve as adjuncts for precise histological classification

Hence this study was undertaken to evaluate the immunohistochemical characteristics of our cohort of primary and metastatic renal tumors. We proposed to re-look into the retrospective cases, evaluate the prospective cases and tried to subclassify them as described in WHO 2004 classification of renal tumours/ISUP classification. [4,5] using panel of IHC markers- Carbonic anhydrase-9 (Ca-9), Cytokeratin-7 (CK-7), α-Methylacyl coenzyme A racemase (AMACAR), CD 10 and TFE-3 followed by additional markers if and when required.

Materials and Methods

Study Design: Descriptive study with duration of four years, conducted from Dec 2014 to Jun 2018. The study
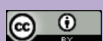
was carried out at Department of Laboratory Sciences and Molecular Medicine along with Department of Urology of a tertiary care multispeciality hospital in New Delhi. Clearance from institutional ethical committee was obtained. Patients of all age groups who were operated and on follow up were included in the study. Written informed consent was taken. The exclusion criteria were biopsy tissue suboptimal for ancillary studies. Patient's demographic data along with clinical and radiological features were obtained

Histopathology: 100 Nephrectomy specimen and 25 trucut biopsies obtained as part of clinical management of patient 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. Paraffin blocks and H&E stained sections for retrospective cases were retrieved from database and were re-assessed for morphology. In both retrospective and prospective cases provisional morphological diagnosis was made and classified based on ISUP/Vancouver Classification of Renal Neoplasia and graded as per conventional Fuhrman grading system.

Immunohistochemistry: IHC was carried out on formalin fixed paraffin embedded tissues using the following antibodies: CK7 (Bio SB, Clone- OV-TL12/30, Catalogue no BSB 5407, Ready to use, AMACR (Bio SB Clone -13H4, Catalogue no BSB 5057, Ready to use), CD10 (Bio SB Clone 56C6, Catalogue no BSB 5176, Ready to use), TFE-3 (Bio SB Clone-EP285, Catalogue no-BSB 3225, concentrated, dilution 1:80) CA-9 (Sigma Aldrich Clone EP161, Catalogue no 379R-1, concentrated, dilution 1:100). Additional antibodies used were Pax 8, Oct 3/4, CD117 and kidney specific Cadherin wherever required. Secondary detection system used was single step polymer-based detection system (Envision detection system, peroxidise/DAB, rabbit/mouse)

Immunostaining of 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. Cytoplasmic and/or membranous expression of CA-9, CK7 and AMACAR was considered positive. Only distinct nuclear staining for TFE was considered positive.

Statistical analysis: IHC results were tested for their association with the histological subtype using appropriate descriptive statistics. All statistical analyses were performed using the SPSS 21.0 software. Statistical significance was considered when P value ≤ 0.05

Results
Mean age of study population was 57± 10.994 years ranging from 25 to 75 years with male predominance (male -74% and females 26%). 20 cases presented first with metastasis and later renal mass was detected on imaging studies. In five cases imaging revealed cystic masses and diagnosis was conferred on trucut biopsy. Radical nephrectomy was carried out in 84(84%) cases while partial nephrectomy was done in 16(16%).

Histopathological Examination:
Nephrectomy Specimen: Tumours were categorized as composed of clear cells, cells with eosinophilic cytoplasm and poorly differentiated tumours. Patterns exhibited were alveolar pattern in 66/100(66%), solid pattern 20/100(20%) and true papillae 6/100 (6%). 4/100(4%) cases showed alveolar and true papillae while 2/100(2%) cases exhibited broad papillae with solid sheets. 2/100(2%) showed nested pattern. 10/100 (10%) cases exhibited focal sarcomatous areas. (Table 1)

Trucut biopsies: Of 25 trucut biopsies (20 metastasis and 05 renal masses), 18 were clear cell tumours while the rest 07 tumours exhibited high grade poorly differentiated morphology difficult to categorize on light microscopy alone. 02 tumours in addition showed sarcomatous areas and one exhibited papillary architecture.

Distribution of patients as per Fuhrman grade: 26 (26%) were grade I, 36(36%) cases were grade II, 26(26%) cases were grade III and 12(12%) cases were grade IV tumours.

CA-9
75(75%) cases showed immunopositivity for CA-9 of varying intensities. (Figure 1) (Table 2). All cases of clear cell RCC including multilocular cystic renal cell neoplasm of low malignant potential (MCRNLMP) and clear cell papillary RCC were positive for CA-9 displaying strong statistical association of CA-9 immunostaining (p=50.00, x2-0.000). There was inverse correlation between the intensity of the staining and grade of the tumour with high grade clear cell RCCs exhibiting weak and patchy staining and low grade clear cell RCCs exhibiting strong membranous staining. (p=32.97, x2=0.000). Spindle cell component of clear cell RCCs was either negative or exhibited weak patchy staining. (Table 2) Similar trend was observed in trucut biopsies. (Table 3)

CK 7
CK-7 immunopositivity was noted in 25/100 with distribution as Table 2. All cases of clear cell RCC were negative (P=34.119, x2-0.000) while all cases of papillary RCC, chromophobe RCC and clear cell papillary RCC and MCRNLMP were positive. (p=61.09, x2=0.000). (Figure

2) All six cases of type 1 papillary RCC exhibited strong CK-7 positivity while Type 2 papillary RCC including the solid variant exhibited weak and patchy CK7 staining. (Figure 3) The results of trucut biopsies were similar to surgical specimen (Table 2 & 3)

AMACAR, CD10 and TFE 3

AMACAR immunostaining exhibited negative statistical correlation with clear cell tumours, oncocytoma and chromophobe RCC. (p=34.47, x2=0.003). AMACAR positivity of moderate to high intensity was noted in all cases of papillary RCC. Immunopositivity of CD10 was noted in all cases of clear cell RCC, MCRNLMP, Papillary RCC

and collecting duct carcinoma irrespective of the grade of the tumour. 6 (6%) cases showed weak immunopositivity for TFE which was considered nonspecific. (Table 2) The results of immunostaining in trucut biopsies is shown in Table 3.

Details of additional immunohistochemistry with details is depicted in Table 4. In trucut biopsies all cases were assessed for Pax 8 staining. 20/24 (83%) cases of clear cell RCC while single case of papillary RCC were positive for Pax 8. The immunostaining of clear cell RCC was relatively weaker and patchy as compared to papillary wherein the staining was sharp and diffuse. (Table 3)

Table 1: Provisional Morphological Diagnosis based on cytological features and pattern. N=100

S no	Morphological Categories	Provisional morphological diagnosis
1	Clear cell morphology (72%)	Clear cell Renal Cell carcinoma Clear cell papillary carcinoma Multilocular cystic renal cell neoplasm of low malignant potential
2	Tumours with eosinophilic cytoplasm (21%)	Papillary renal cell carcinoma Chromophobe renal cell carcinoma Oncocytoma Conventional renal cell carcinomas (high grade) Collecting Duct Carcinoma
3	Poorly differentiated carcinoma (7%)	High grade Spindle cell tumour Poorly differentiated tumour dispersed in nested and trabacular pattern Poorly differentiated tumour with foci of spindle areas

Table 2: Histopathological Diagnosis following immunohistochemical analysis. (Nephrectomy Specimen) n=100

S no	Diagnosis	Cases N=100	CA-9	CK7	CD10	AMACAR	TFE-3
1	Clear cell RCC	68	68	0	68	1	6
2	Clear cell Papillary RCC	2	2	2	0	0	0
3	MCRNLMP	2	2	2	2	0	0
4	Papillary RCC	10	0	10	10	10	0
5	Chromophobe RCC	6	0	6	0	1	0
6	Oncocytoma	4	0	1	0	0	0
7	Collecting duct Carcinoma	1	1	1	1	0	0
8	Urothelial Carcinoma	3	2	3	0	0	0
9	Neuroendocrine Carcinoma	2	0	0	0	0	0
10	Epithelioid Angiomyolipoma	2	0	0	0	0	0

Legend to Table

MCRNLMP - Multilocular cystic renal cell neoplasm of low malignant potential

RCC - *Renal Cell Carcinoma*

* One case of high-grade spindle cell tumour on further sections revealed small focus of clear cells immunopositive weakly for CA-9 and CD 10.

**Papillary RCC Type 1 – 06 cases, Papillary RCC Type 2 – Four cases with one exhibiting oncocytic/solid variant

*** Chromophobe RCC eosinophilic variant – 01 case

03 cases of high-grade urothelial carcinoma were erroneously classified as Clear Cell RCCs

Table 3: Histopathological Diagnosis following immunohistochemical analysis. (Trucut biopsies) n=25

S no	Trucut Biopsy Morphology	Cases N=25	Immunohistochemistry	Diagnosis Trucut biopsy	Diagnosis Nephrectomy
1	Clear cell RCC	18*	CA-9, CD 10 Positive	Clear cell RCC	Clear Cell RCC (Grade 1& 2)
2	Poorly differentiated carcinomas / malignancy	4*	CA-9, CD10 Patchy weak positive CK7 Negative	High grade RCC	Clear Cell RCC (Grade 3&4)
		2**	CA-9, CD10, CK7, AMACAR – Negative	High grade Sarcoma Sarcomatoid RCC	Clear cell RCC with sarcomatous areas
		1***	CK-7, CD 10, AMACAR Patchy weak positive	Papillary RCC	Papillary RCC Type 2

Legend to Table

 RCC: Renal Cell Carcinoma

* Co expression of Cytokeratin and Vimentin

** Strong vimentin and very patchy weak equivocal positivity of Cytokeratin

*** Additional positivity for Pax 8 and negative for WT-1, CA-125, Estrogen and Progesterone receptors

Table 4: Additional immunohistochemistry and Final Diagnosis, N=100.

S. NO	Morphological Categories	Provisional morphological diagnosis	Primary immunohistochemistry Panel	Additional IHC	Final Diagnosis
1	**Tumours with Clear Cells**	Clear cell RCC	CA-9, CD10 + CK7 AMACAR TFE 3 –	NR	Clear cell RCC
		Clear cell papillary RCC	CA-9, CK7, CD10 + AMACAR, TFE 3 –	NR	Clear cell papillary RCC
		MCRNLMP	CA-9, CK7, CD10 + AMACAR, TFE 3 –	NR	MCRNLMP
2	**Tumours with eosinophilic cytoplasm**	Papillary RCC	CK 7, CD10, AMACAR + CA9 – CK7 weak in Type 2 Papillary RCC	NR	Papillary RCC
		Chromophobe RCC	CK7 + CD10,CA9, TFE 3, AMACAR -	CD 117+	Chromophobe RCC
		Oncocytoma	CK7 -/weakly + CD10,CA9, TFE 3, AMACAR -	CD117, E Cadherin +	Oncocytoma
		Conventional renal cell carcinoma	CD10, CD10 + (weak) CK7 AMACAR TFE 3 –	NR	Clear Cell RCC High grade
		Collecting duct carcinoma	CK7, CD10 + CA9, AMACAR, TFE -	Oct 3/4 +	Collecting duct carcinoma
3	**Poorly differentiated Tumour**	High grade Spindle cell tumour	CA-9, CD10 weakly + CK7, AMACAR -	CK weak + Vimentin +	Sarcamatoid RCC
		Poorly differentiated carcinoma	CA-9, CD10, CK7, TFE 3 AMACAR -	CD53, synaptophysin, Chromogranin +	Neuroendocrine carcinoma
			CK 7 and CA9 + CD10, AMACAR, TFE 3 -	Uroplakin, p63+	Urothelial carcinoma
			CK 7 Positive CD10, CA9, AMACAR, TFE 3 –	Napsin , TTF-1 +	Metastasis *of lung adenocarcinoma

S. NO	Morphological Categories	Provisional morphological diagnosis	Primary immunohistochemistry Panel	Additional IHC	Final Diagnosis
		Poorly differentiated tumour with foci of spindle areas	CA-9, CD10, CK7, TFE 3 AMACAR -	HMB45 +	Epithelioid Angiomyolipoma

Legend to Table
MCRNLMP: Multilocular cystic renal cell neoplasm of low malignant potential
RCC : Renal Cell Carcinoma, NR: Additional IHC not required, + : positive, - : Negative
* Case of dual malignancy. Clear cell RCC with metastasis of lung adenocarcinoma in kidney
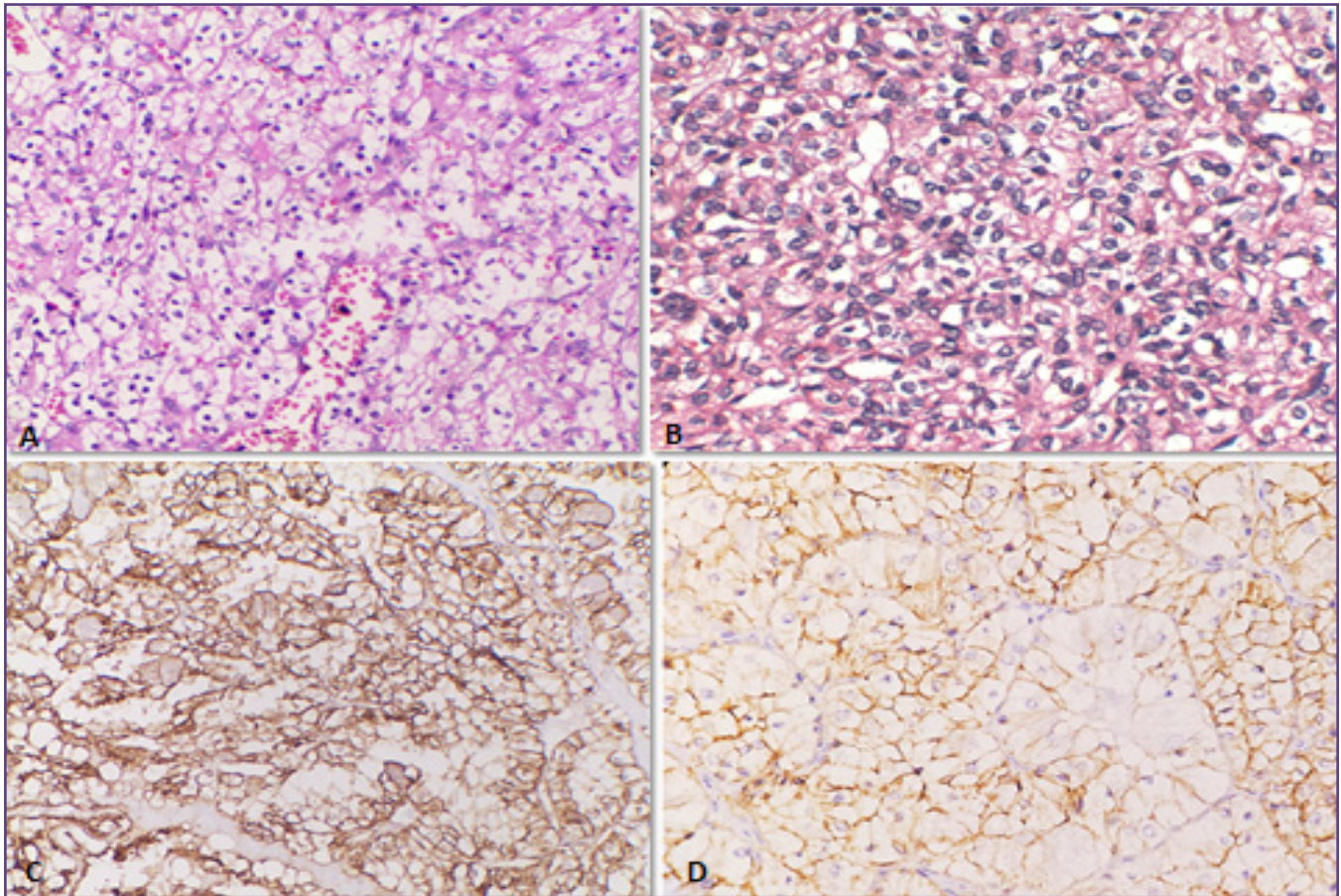
Fig. 1: Clear cell RCC (A) Furhman Grade 1 (B) Furhman Grade III, haematoxylin & eosin, magnification: 200x (C) Furhman Grade 1 with carboxy anhydrase-9 expression showing membranous box like pattern (D) Furhman Grade III with patchy carboxy anhydrase-9 membranous pattern, magnification 400x. Immunoperoxidase x diaminobenzaldehyde..
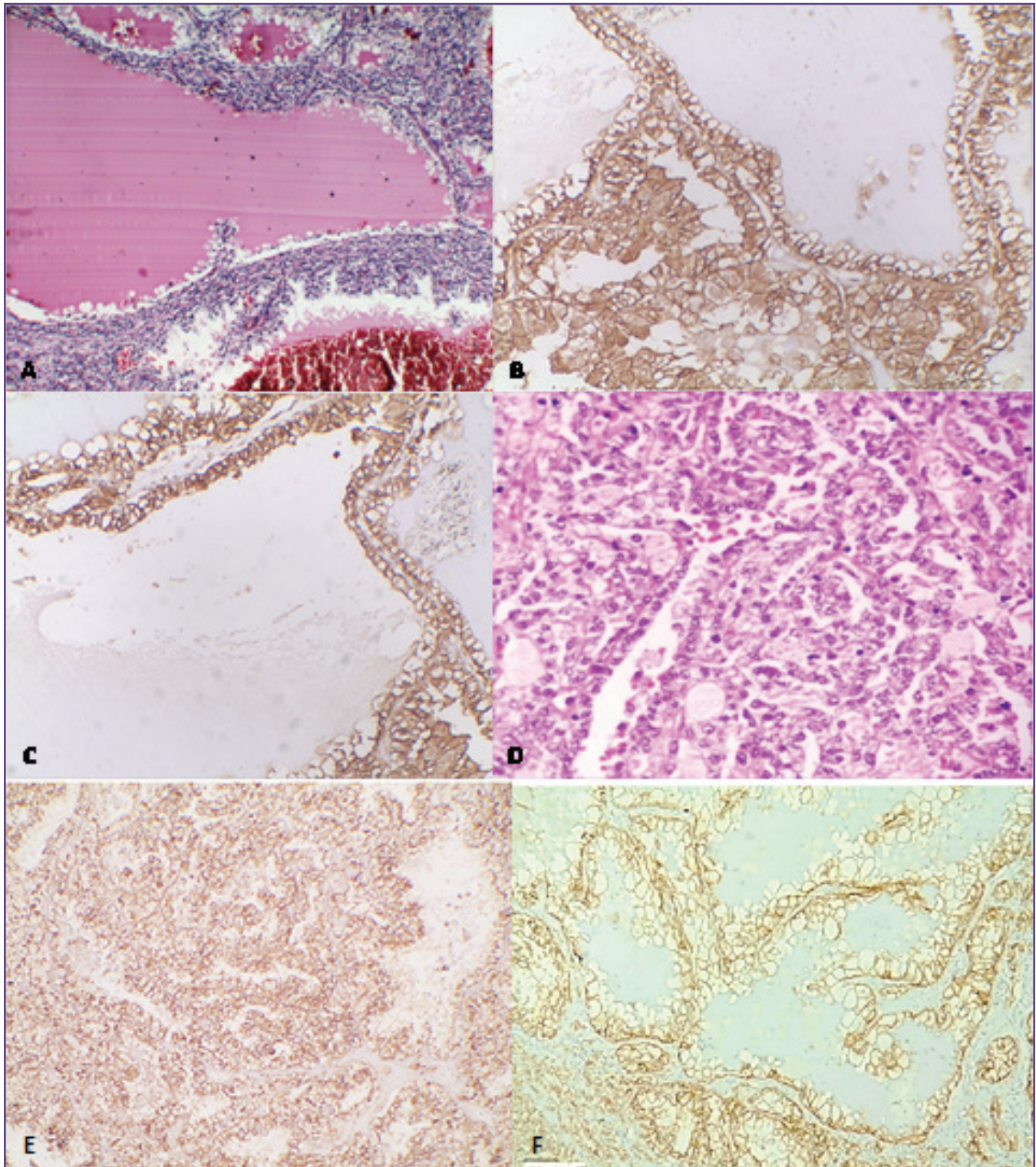
Fig. 2: (A) Multilocular cystic renal cell neoplasm of low malignant potential, haematoxylin & eosin, magnification-100x with (B) carboxy anhydrase-9 and (C) cytokeratin 7 co- expression. Immunoperoxidase x diaminobenzaldehyde; magnification 400x. (D) Clear cell papillary RCC, haematoxylin & eosin; magnification: 200x with (E) cytokeratin 7 and (E) basal carboxy anhydrase-9 co-expression. Immunoperoxidase x diaminobenzaldehyde; magnification 400x.
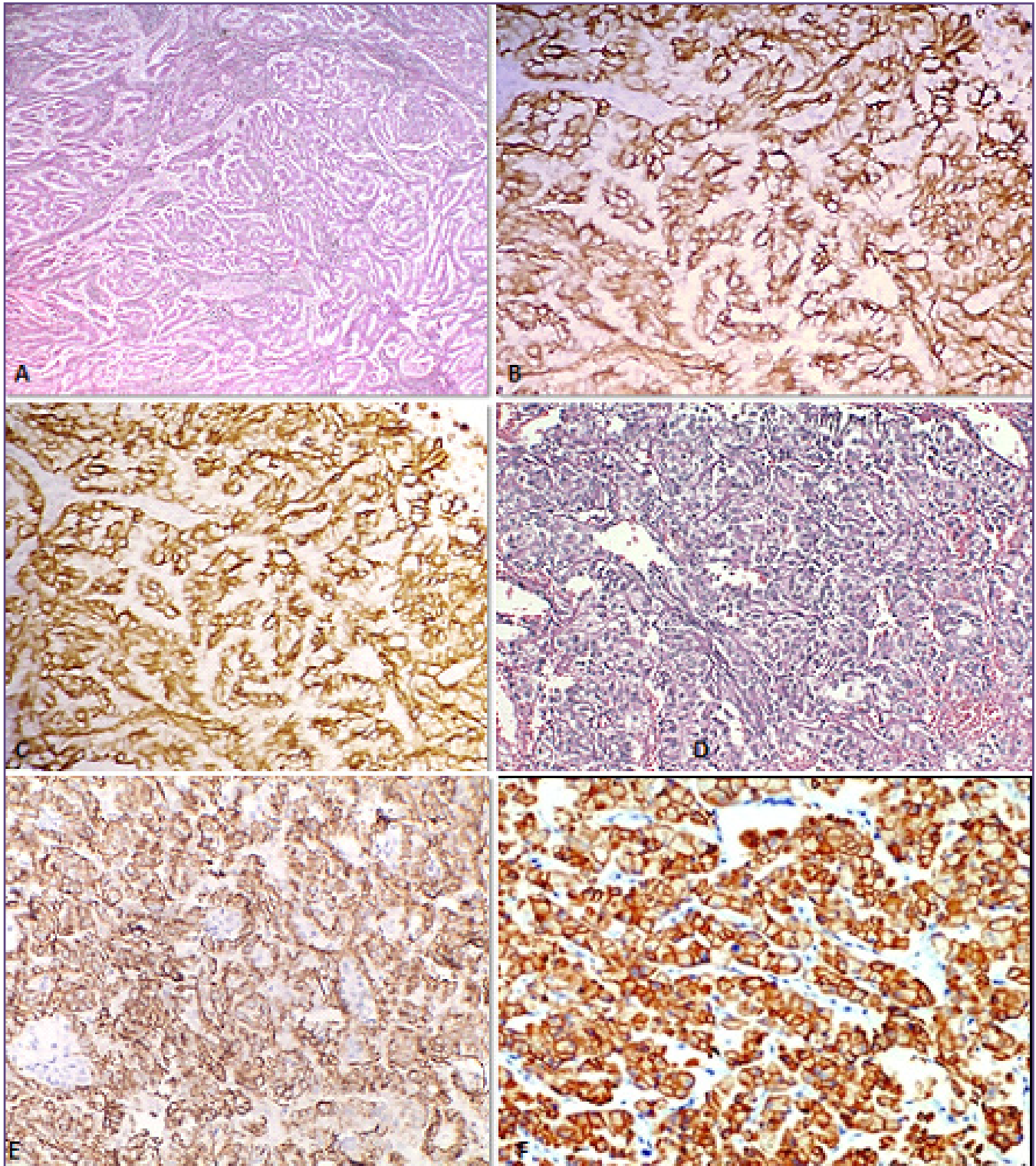
Fig. 3: (A) Papillary RCC,Type 1, haematoxylin & eosin; magnification: 200x with diffuse (B) cytokeratin 7 and (C) AMACAR immunostaining. Immunoperoxidase x diaminobenzaldehyde; magnification 200x. (D) Type 2 papillary RCC showing (E) relatively weaker and patchy cytokeratin 7 immunopositivity and (F) strong diffuse AMACAR immunostaining. Immunoperoxidase x diaminobenzaldehyde; magnification 200x.
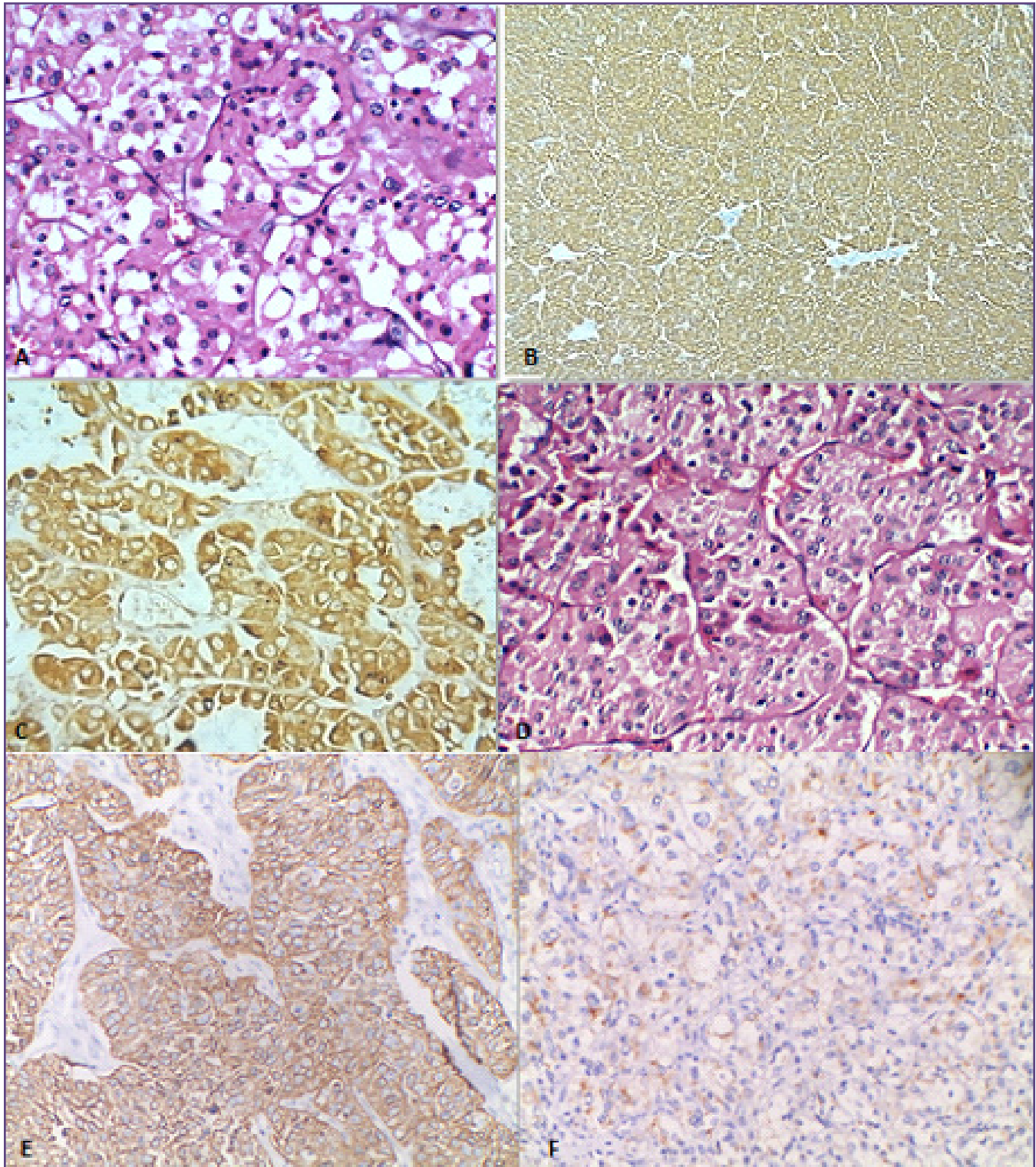
Fig. 4: (A) Chromophobe RCC, haematoxylin & eosin; magnification 200x with (B) CD117 and (C) diffuse cytokeratin 7 immunopositivity. Immunoperoxidase x diaminobenzaldehyde; magnification 200x respectively (D) Oncocytoma eosinophilic variant, haematoxylin & eosin; magnification 200x with (E) E-Cadherin and (F) very weak patchy cytokeratin 7 immunostaining. Immunoperoxidase x diaminobenzaldehyde; magnification 200x respectively.

Discussion

RCC is heterogeneous group lacking IHC markers with high specificity for primary diagnosis and subcategorization. A panel of IHC markers was used to define and evaluate different subtypes of renal cell carcinoma based on specific cytological appearance and architectural variations.

Clear cell RCC, when present with a typical morphology, diagnosis can be made upfront as evident in most of our cases. Ancillary techniques are required if morphology is unusual, or usual morphology overlaps with different variants. Clear cell papillary and Xp11 translocation RCC characteristically feature both papillary architecture and clear cells, a pattern which is rare in clear cell RCC. [6] Similarly RCC with eosinophilic cytoplasm has overlapping diagnosis of papillary RCC type 2, oncocytoma, chromophobe RCC and epithelioid angiomyolipoma. Oncocytic variant of papillary RCC, eosinophilic variant of chromophobe RCC and high-grade clear cell RCC where cytoplasmic eosinophilia increases are problematic situations as are high-grade tumours with spindle cell differentiation, high grade urothelial carcinoma, adrenocortical carcinoma and metastatic carcinoma. [7,8]

CA-9

CA-9 is a hypoxia-induced protein and its expression in clear cell RCC was first reported by Laio et al. [9] Our study displayed strong statistical correlation of CA-9 with clear cell RCC which makes it very useful marker for differenting clear cell RCC from other subcategories. Moreover basal pattern of CA-9 expression in clear cell papillary RCC helps segregate it from clear cell RCC. [10,11] Genega et al and Gupta et al evaluated 366 cases of RCC for CA-9 expression and found similar association of CA-9 staining with clear cell RCC.[3,12,13] However in variance to our findings focal weak expression was also noted in few cases of papillary and one case of chromophobe RCC. However, when clear versus nonclear cell RCCs were considered the statistical association was strong. Furthermore, we found that CA-9 expression was associated with the grade of clear cell RCC with intensity of staining reducing with increasing grade of tumour. Similar results were exhibited by Genega et al and Bui et al who found that with expression of CA-9 reduces as the grade of the tumour increases and that decreased levels of expression were independently associated with poor outcome. [12,14,15]

CA-9 is uniformly negative in chromophobe RCC and oncocytoma which is in sync with our findings. However, CA-9 is also expressed in other tissues as endometrium, stomach, cervix, breast, lung, and liver, brain and neuroendocrine tumors, and hence the marker may be not useful, as a solitary marker, for distinguishing RCC from other tumours at the metastatic sites. [9]

Cytokeratin -7

Renal oncocytoma, conventional RCC and chromophobe RCC share overlapping morphologic and immunohistochemical features. Differentiating them may be very challenging on pure morphology. The problem gets compounded when one needs to distinguish between an oncocytoma from an eosinophilic variant chromophobe RCC. In our study all cases of chromophobe RCCs were positive for CK-7 while only one case of oncocytoma exhibited weak cytoplasmic positivity. All cases of clear cell RCCs were negative. However, CA-9 coexpression with CK-7 was noted in clear cell papillary RCC and MCRNLMP in consonance with literature. Strong and diffuse CK7 staining favours a diagnosis of chromophobe RCC which later can be confirmed by CD117/E- Cadherin staining. Geramizadeh B, et al in their 76 cases of RCCs showed that 100 % CK7 in chromophobe RCCs, 8% in clear cell RCC and was negative in all oncocytomas.[16,17]

Type 1 and type 2 papillary RCC with typical histology are straightforward diagnosis. However, one case in our study displayed solid architecture with oncocytic features and exhibited CK-7 positivity. Extended panel was negative for e-cadherin and positive for AMACAR and CD10. Case was labelled as oncocytic variant of papillary RCC. Similarly, in cases of high-grade urothelial carcinoma misinterpreted as RCC and case of synchronous metastatic lung carcinoma and RCC, CK-7 positivity directed secondary panel. Hence overall CK-7 staining in association with other antibodies helped to triage and plan the extended panel.

AMACAR and CD10

All cases of papillary carcinoma exhibited strong correlation with AMACAR staining. Molinie V et al in a series of 110 renal tumours found AMACAR immunostaining in 96.4% of papillary RCC.[18] Only 5 of 25 clear cell RCCs and 1 of 9 oncocytomas were focally reactive while rest of the tumours were negative. Our numbers though very small reflect the result of this study. AMACAR is largely useful for distinguishing papillary carcinomas especially the solid variant/oncocytic variant from chromophobe/oncocytoma but it is worthwhile to include CD117/E-cadherin into the diagnostic panel when confronted with the problem of eosinophilic granular cytoplasm.[8,19] Additional markers like S100 A 1and CD82 can help differentiate the two entities. The immunohistochemical profile of eosinophilic variant of chomophobe RCC is quite variable, but most are positive for CK7 and AMACAR as seen in our study as well. [7] Figure 4.

All cases of clear cell RCC, MCRNLMP, papillary carcinoma and collecting duct carcinoma were positive for CD10. Hence CD10 is a useful marker for general diagnosis of RCC.[8] No case of translocation carcinoma was present in our series and no tumour exhibited conclusive positivity for TFE3 protein.[20]

Small Biopsy Specimen
In context of RCC, studies have demonstrated relatively high diagnostic accuracy of needle biopsies based on the H&E section alone. In our series trucut biopsies from primary renal masses were straightforward diagnosis of clear cell RCC on light microscopy. Al-Ahmadie et al and Hanan Al et al showed that standard morphologic evaluation, in combination with the judicious use of 5 markers (CAIX, CD117, AMACR, CK7, and CD10), can produce an accurate diagnosis in greater than 90% of cases in needle biopsy of renal tumours. [15,21]

Situation in metastatic disease is more complicated and in spite of the availability of several renal markers, the diagnosis of metastatic RCC is a difficult proposition. In these situations, the amount of tumor tissue available is often quite limited and ancillary studies, are a must for confirmatory diagnosis. Metastasis may follow or precede primary diagnosis of RCC with long latent period, are known to metastasize to virtually any body site, are great histological mimickers with unusual metastatic sites. Besides metastasis may occur in patient with dual malignancies and thus lesion may represent a new primary tumor, metastatic RCC, or metastasis of the other tumour.

Metastatic low-grade clear cell RCCs, diagnosis was clinched on morphology coupled with standard IHC results (Table 3; Co expression of Cytokeratin and Vimentin, Pax 8, CD10, CA-9 +++) with strong and clear IHC staining. The high grade poorly differentiated morphology of the lesions presented a diagnostic dilemma. The positivity for standard panel of markers for RCC, were largely retained in their metastases, but the staining was weak and patchy presenting a significant problem in interpretation. CD10, CA-9 and Pax 8 were useful markers for the diagnosis of metastatic RCC and were positive in all ccRCCs.[2,7,21,22] Sarcomatoid RCC however were either completely negative or exhibited very focal weak positivity for these markers and final diagnosis was largely amalgamation of clinical profile, imaging and extensive sampling of the nephrectomy specimen received after preliminary diagnosis on trucut biopsy. Single case of type 2 Papillary RCC required IHC to be differentiated from other papillary adenocarcinomas

RCC markers though helpful are expressed in many other primary or metastatic carcinomas. Renal carcinoma antigen which was not used in our study also has limitation of weak/absent expression in high grade RCCs. The available review of literature suggests that the panel for evaluating potential metastatic RCC should include PAX8, CD10, CA-9 supplemented by other markers depending on morphology and differential diagnosis

Conclusion
To conclude, immunohistochemical staining for CA-9, CD10, CK7, AMACR and TFE3 for RCCs composed of clear cells and CD117/E-Cadherin in addition to above panel comprises a concise panel for distinguishing RCC with clear cell/eosinophilic cell /granular cell/ papillary pattern. In a setting of metastatic RCC, CD10 and Pax 8 as primary RCC markers followed by use of markers according to tumour morphology in different diagnostic situations will facilitate confirmatory diagnoses, particularly when diagnostic tissue samples are limited.

Acknowledgements

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