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IDENTIFICATION OF IMMUNOHISTOCHEMICAL BIOMARKERS THAT DIFFERENTIATE CHROMOPHOBE RENAL CELL CARCINOMA FROM RENAL ONCOCYTOMA THROUGH MOLECULAR PROFILING OF RENAL TUMOURS

Keng Lim Ng

Bachelor Science (Med) (Univ of New South Wales)

Bachelor of Medicine, Bachelor of Surgery (Univ of New South Wales)

Masters of Surgery (University Malaya)

Certified Board of Urology (Malaysia)

FRCS (Urol) (Royal College of Surgeons Glasgow)

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School of Medicine

Abstract

There is increasing detection of renal tumours, especially small renal masses largely due to widespread availability of radiological imaging modalities. However, imaging techniques and renal lesion biopsies cannot accurately define malignant from benign renal tumours. Therefore such indeterminate renal tumours undergo surgical resection. Unfortunately a significant proportion of resected renal tumours turn out to be benign on histopathological diagnosis. The impact of this scenario is the morbidities of unnecessary surgery and loss of valuable nephrons with the associated increased risk of chronic kidney disease and cardiovascular complications. Reduction of unnecessary surgeries would also translate into decreased costs, and allow for more efficient utilisation of health budgets.

Another diagnostic dilemma faced by pathologists is the occasional difficulty in distinguishing some renal tumour subtypes, due to overlapping morphological and histological features. Differentiating malignant chromophobe renal cell carcinoma (chRCC) from benign renal oncocytoma (RO) is one such dilemma. Their correct diagnosis is crucial as prognosis, management and surveillance protocols differ between the two tumour subtypes. Therefore effective and reproducible immunohistochemistry (IHC) biomarkers need to be identified, together with novel biomarkers, through molecular profiling of these tumour subtypes.

In **Chapter 1**, the relevant background, significance of the research topic, literature review, hypothesis and aims of this PhD research were presented. Contents of this chapter have also been published as a review article. The review article provided the clinical presentation,

explained the diagnostic dilemma, and described the value of current molecular markers to assist in differentiation between chRCC and RO.

The aims of this research project were to: 1) identify a panel of IHC biomarkers which can effectively differentiate chRCC from RO through a comprehensive literature search and meta-analysis approach, as well as using some of the research results from my laboratory; 2) analyse the molecular profiles of renal cancers via IHC and morphometry techniques using selected biomarkers on renal tumour and normal renal tissue samples; 3) specifically, to investigate IHC biomarkers that are useful in differentiating chRCC from RO via IHC and morphometry; and 4) initiate and develop a comprehensive Renal Tumour Biobank (clinical data, urine, sera, renal tumour and normal tissue) from patients with renal tumours undergoing nephrectomy.

In **Chapter 2**, the general materials and methods were presented, including the use of prospective RCC patient samples, IHC of archived RCC tissue blocks, morphometry and statistical analyses. The methodology of IHC of the biomarkers on the tissue slides and morphometric analyses were discussed. Prospective RCC patient samples which included renal tumour, normal renal tissue, sera and urine were collected, processed and stored in the Centre for Kidney Disease Research (CKDR) leading to the creation of the Renal Tumour Biobank. This work involved a significant amount of time and resources throughout the PhD research project, achieved Aim 4 of the thesis, and will be a legacy of this PhD research.

Chapter 3 examined the existing IHC biomarkers that have been reported as useful in differentiating chRCC from RO. A meta-analysis and systematic review was conducted in

this chapter to assess the most effective IHC biomarkers, and it has been published. In summary, we recommended a selection from a panel of IHC biomarkers, namely, amylase α1A, Wnt-5a, FXYD2, ARPP, CD63, TGFβ1, CK7, S100A1, caveolin-1 and claudin-7 to aid in the differentiation of chRCC and RO.

In **Chapter 4**, we investigated the molecular profiles of nuclear factor-κB (NF-κB) subunits in RCC disease. Our results represent the first and largest study to report on the IHC expression of NF-κB subunits (p65, p50, p52, cRel) and their associated prognostic cancer specific survival outcome in RCC patients. Although there were no associations with RCC subtypes, overexpression of p65 and decreased expression of other subunits were noted in renal tumours compared with normal renal tissue. Moreover, p65 overexpression was correlated to a poorer cancer survival outcome.

In **Chapter 5**, the IHC of various established and novel biomarkers: CK7, Cav-1, S100A1, leptin and its receptor (Ob and ObR) and kidney injury molecule-1 (KIM-1) were investigated in archived renal tumour specimens and paired normal kidney. The utility of selected IHC biomarkers to differentiate clear cell RCC, chRCC and RO was analysed using morphometry. We demonstrated that chRCC had higher CK7 overall expression intensity compared to RO, presented the difference in Cav-1 staining patterns between the two subtypes, and demonstrated that higher Ob nuclear expression and higher KIM-1 overall expression were seen in RO compared with chRCC.

In **Chapter 6**, the summary of major results and future directions gained from this research were presented. The results will spur further research into the possible diagnostic roles of

these biomarkers through non-invasive methods of sera and urine analyses. The results from this PhD will no doubt add to the better understanding of the molecular signatures of renal tumours and hopefully be translated into clinical practice to improve the quality of life of renal tumour patients.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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PUBLICATIONS DURING CANDIDATURE

Peer reviewed publications directly related to thesis

Ng KL, Morais C, Bernard A, Saunders N, Samaratunga H, Gobe G, Wood S. 2016. A systematic review and meta-analysis of immunohistochemical biomarkers that differentiate chromophobe renal cell carcinoma from renal oncocytoma. *Journal of Clinical Pathology* **0**: 1-11. DOI:10.1136/jclinpath-2015-203585

Ng KL, Rajandram R, Morais C, Yap NY, Samaratunga H, Gobe GC, Wood ST. 2014. Differentiation of oncocytoma from chromophobe renal cell carcinoma (RCC):- can novel biomarkers help solve an old problem? *Journal of Clinical Pathology* 67:97-104 DOI:10.1136/jclinpath-2013-201895

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Peer reviewed publications indirectly related to thesis

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Ng KL, Rajandram R, Morais C, Yap NY, Samaratunga H, Gobe GC, Wood ST. 2014. Differentiation of oncocytoma from chromophobe renal cell carcinoma (RCC):- can novel biomarkers help solve an old problem? *Journal of Clinical Pathology* **67**:97-104
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- incorporated as Chapter 1

Contributor	Statement of contribution
Keng Lim Ng (Candidate)	Wrote the paper (80%) Edited the paper (20%)
Retnagowri Rajandram	Wrote the paper (10%)
Christudas Morais	Edited the paper (15%)
Ning Yi Yap	Wrote the paper (10%)
Hemamali Samaratunga	Edited the paper (15%)
Glenda C Gobe	Edited the paper (25%)
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- incorporated as Chapter 3

Contributor	Statement of contribution
Keng Lim Ng (Candidate)	Literature search (80%) Data extraction and analyses (100%) Statistical analysis (40%)
Chritudas Morais	Literature search (20%) Edited the paper (10%)

Anne Bernard	Statistical analysis (60%) Edited the paper (10%)
Nicholas Saunders	Concept of paper (50%) Edited the paper (10%)
Hemamali Samaratunga	Edited the paper (10%)
Glenda C Gobe	Concept of paper (50%) Edited the paper (30%)
Simon T Wood	Edited the paper (30%)

CONTRIBUTIONS BY OTHERS TO THE THESIS

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STATEMENT OF PARTS OF THE THESIS SUBMITTED TO QUALIFY FOR AWARD OF ANOTHER DEGREE

None.

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KEYWORDS

Renal tumours, renal cell carcinoma, biomarkers, immunohistochemistry, chromophobe, oncocytoma, differentiation.

AUSTRALIAN AND NEW ZELAND STANDARD RESEARCH CLASSIFICATIONS (ANZSRC)

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LIST OF ABBREVIATIONS

%	Percent
°C	Degrees Celsius
µm	Micrometre
Ab	Antibody
ACAT	Acyl-coenzyme A:cholesterol acyl transferase
ALK	Anaplastic lymphoma kinase
AMACR	Alpha-methylacyl-coenzyme A racemase
AMPK	5' Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
AR	Ankyrin repeats
ARPP	Ankyrin-repeated protein with a proline-rich region
ATPase	Adenosine triphosphatase
BCA2	Breast cancer-associated gene 2
BSA	Bovine serum albumin
CAIX	Carbonic anhydrase 9
Cav-1	Caveolin-1
ccpRCC	Clear cell tubulopapillary renal cell carcinoma
ccRCC	Clear cell renal cell carcinoma

CD	Cluster of differentiation
cdRCC	Collecting duct renal cell carcinoma
chRCC	Chromophobe renal cell carcinoma
CI	Confidence interval
CK	Cytokeratin
CKDR	Centre for Kidney Disease Research
c-KIT	Transmembrane tyrosine kinase receptor
cm	Centimetre
CT	Computed tomography
CTA	Cancer-testis antigen
DAB	Diaminobenzidine hydrochloride
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
EABA	Endogenous avidin-binding activity
E-cadherin	Epithelial-cadherin
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
eGFR	Estimated glomerular filtration rate
EMA	Epithelial membrane antigen

EMT	Epithelial to mesenchymal transition
EpCAM	Epithelial cell adhesion molecule
ERA	Epithelial-related antigen
ESA	Epithelial specific antigen
FDG	Fluorodeoxyglucose
FISH	Fluorescence in situ hybridization
g	Gram
GST-α	Glutathione S-transferase alpha
H&E	Haematoxylin and eosin
H_2O_2	Hydrogen peroxide
HIFα	Hypoxia inducible factor alpha
HMCK	High molecular weight cytokeratin
HP-1α/β	Heterochromatin-associated protein - 1 alpha/beta
HRP	Horseradish peroxidase
IAP	Inhibitor of apoptosis
IgG	Immunoglobulin G
IHC	Immunohistochemical/immunohistochemistry
IKK	Inhibitor of kappa B kinase
IL	Interleukin
IRS	Insulin receptor substrate

ISUP	International Society of Urological Pathology
IκB	Inhibitor of kappa B
JAK/STAT	Janus kinase/signal transducer and activator of transcription
kDa	Kilodalton
KIM-1	Kidney injury molecule-1
Ksp	Kidney specific
LMCK	Low molecular weight cytokeratin
M	Molar
M0	No metastases
M1	Metastases
m^2	Metre square
MAGE-A3/4	Melanoma-associated antigen A3/4
mcRCC	Multilocular cystic renal cell carcinoma
MDR	Multidrug resistance
min	Minute
MiT	Microphthalmia-associated transcription
mL	Millilitre
mM	Millimolar
MOC31	Monoclonal antibody that recognises epithelial glycoprotein 2
MRI	Magnetic resonance imaging

MT1-MMP	Membrane type 1 metalloproteinase
mTOR	Mammalian target of rapamycin
MUC-1	Mucin-1
N-cadherin	Neural-cadherin
NEMO	Nuclear factor-kappa B essential modulator
NF-κB	Nuclear factor–kappa B
NLS	Nuclear localisation signal
NPM	Nucleophosmin
Ob	Leptin
ObR	Leptin receptor
OR	Odds ratio
PAX8	Paired box gene 8
PBS	Phosphate buffered saline
PET	Positron emission tomography
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PICF	Patient Information and Consent Form
PLCε	Phospholipase Cε
pRCC	Papillary renal cell carcinoma
PRISMA	Preferred reporting items for systematic reviews and meta-analyses
QIMR	Queensland Institute of Medical Research

RCC	Renal cell carcinoma
RCCma	Renal cell carcinoma marker
Rel	Reticuloendotheliosis
RHD	Reticuloendotheliosis homology domain
RNA	Ribonucleic acid
RO	Renal oncocytoma
RON	*Recepteur d'Origine Nantais*
rpm	Rotations per minute
S100A1	S100 calcium binding family of proteins alpha 1
sens	Sensitivity
spec	Specificity
TBS	Tris buffered saline
TGF-β1	Transforming growth factor beta 1
TMA	Tissue microarray
TNF-α	Tumour necrosis factor - alpha
TNM	Tumour Node Metastasis
TRI	Translational Research Institute
UEA-1	Ulex europaeus agglutinin-1
UMMC	University Malaya Medical Centre
VEGF	Vascular endothelial growth factor

VHL	von Hippel Lindau

CHAPTER 1

LITERATURE REVIEW, HYPOTHESIS AND AIMS

CHAPTER 1

LITERATURE REVIEW, HYPOTHESIS AND AIMS

1.1 GENERAL INTRODUCTION

The incidence of renal tumours has been increasing steadily in Europe, United States and Australia for the past three decades (Ljungberg et al. 2011). The widespread use of cross-sectional imaging has increased the detection of incidental smaller tumours (Duchene et al. 2003), while the 20-30% incidence of advanced metastatic tumours has remained relatively constant (Gupta et al. 2008). Small renal masses are usually defined as less than 4 cm in diameter (Jayson and Sanders 1998). Despite current imaging techniques and the availability of renal lesion biopsy, the current clinical paradigm is to regard all solid renal lesions suspicious for renal cell carcinoma as malignant and most contemporary surgical series continue to report significant rates (approximately 26%) of benign lesions amongst resected small renal masses (Duchene et al. 2003; Schachter et al. 2007). Preoperative biopsy of small lesions is not widely utilized. A key issue for biopsy is the limited reliability of a negative or benign result. A recent systematic review of over 3000 renal lesion biopsies identified a negative predictive value of only 72% (Patel et al. 2016). One contributing factor is potential diagnostic uncertainty in the differentiation of benign renal oncocytoma (RO) from malignant chromophobe renal cell carcinoma (chRCC) (Yusenko 2010b) and, as an added difficulty, eosinophilic ccRCC. Consequently, there is a group of small renal lesions where increased confidence in characterization may defer or obviate the need for surgical intervention. RO and small chRCC are two such lesions.

ChRCC, although having a more favorable prognosis than other RCC subtypes, is a malignant tumor with the potential for metastatic spread and death. In comparison, there appears to be only one confirmed case report of metastases from RO (Oxley et al. 2007). Thus, due to its benign nature, RO can usually be monitored and treated expectantly. Similarly, small renal masses found to be chRCC may, in some situations, be suitable for active surveillance rather than immediate resection or ablation. RO and chRCC are often considered to be extremities of the same morphological spectrum (Delongchamps et al. 2009). Proper differentiation largely relies on haematoxylin and eosin (H&E) histochemistry of sections, and an experienced histopathologist to discern the characteristic histomorphologic features that separate the two entities. Immunohistochemistry (IHC) is used in selected instances with various biomarker antibodies. Electron microscopy was commonly performed in the past, but is done only in rare cases now, as these techniques are not widely available and costs of sample preparation and analyses are high. There is also the coexistence of RO with chRCC seen in sporadic cases of hybrid tumours, renal oncocytosis and Birt-Hogg-Dube (BHD) syndrome. Differentiation of RO and chRCC especially as small renal masses, from other more sinister forms of RCC like clear cell RCC (ccRCC), is also important for the appropriate management of these patients.

Currently, malignant chRCC and benign RO are two renal tumours that often are difficult to differentiate clinically, both at pre-operative diagnostic and post-operative histopathological stages. Current imaging modalities and pre-operative renal mass biopsy techniques cannot accurately differentiate chRCC and RO. Therefore, due to this diagnostic dilemma, these renal lesions undergo nephrectomy, with subsequent pathological reports revealing that approximately 20% of these small renal lesions (≤4 cm) were benign (Schachter et al. 2007).

With reliable characterisation of benign renal tumours, the rate of unnecessary renal surgery can be reduced with significant economic and health outcome benefits. Nephron preservation is an important strategy in reducing the incidence of end stage kidney disease and its associated cardiovascular mortality. Accurate diagnosis of pathological specimens also dictates long term surveillance requirements for malignant chRCC as compared to benign RO cases, where an expectant approach is sufficient. Improved accuracy and confidence in characterisation of benign and low malignant potential lesions will decrease unnecessary intervention and help curb expanding healthcare costs by avoiding overtreatment of benign and indolent lesions.

Better understanding of the molecular profiles of kidney cancers will help solve some of the diagnostic dilemma mentioned above. Therefore identification of such molecular biomarkers which can aid in the differentiation of chRCC and RO is crucial and forms the basis of this research project. “Biomarker” refers to a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (Biomarkers Definitions Working Group 2001). In this study, established and novel IHC biomarkers were analysed. This research result will hopefully be translated to provide useful intervention into day to day practice of clinical urology.

1.2 LITERATURE REVIEW

1.2.1 History

RO was first described by Zippel in 1942 as a neoplasm entirely composed of large eosinophilic cells called oncocytes (Zippel 1942). Later, in 1976, Klein and Valensi (Klein and Valensi 1976) identified another 13 cases as a distinct clinical pathological entity with a typical benign histological appearance and clinical course. RO was originally thought to derive from renal proximal tubules but most pathologists now suggest a distal tubular origin, (Storkel et al. 1988) most likely arising from intercalated cells of collecting ducts. The first description of chRCC, as distinct from ccRCC, was made by Thoenes *et al* in 1985 (Thoenes et al. 1985) and a year later, they added the chRCC subtype to the classification of renal tumours (Thoenes et al. 1986). The cell characteristic had been described prior to the 1985 publication but only in experimentally-induced adenomas in animals. The chromophobe cells had slightly opaque or finely reticular cytoplasm that resisted staining with H&E. They were able to be distinguished from ccRCC by a strongly-positive reaction within their cytoplasm to Hale's colloidal iron, and a weaker positive reaction with Alcian Blue, a distinction that has since been found to be unreliable. The authors, however, made a step forward for classification of RCC by suggesting that the descriptive term "light cell" RCC should be discarded and replaced by either "clear cell" or "chromophobe cell" as appropriate. They pointed out that chromophobe cell tumours were likely to have a different derivation from ccRCC and other RCC, and that they may also have a different prognosis, a fact that has since been established. Since the description of chRCC came a decade later than RO, there were many instances in that era where renal tumours, which were likely to be chRCC, were described as RO. This may have contributed to the confusion surrounding the original recognition of the benign nature of RO.

1.2.2 Epidemiology

Renal tumours are highly heterogeneous with at least 16 known subtypes, of which four subtypes predominate (Amin and Anthony 1999; Chawla et al. 2006). Recent recommendations from the classification working group of the International Society of Urological Pathology (ISUP) consensus conference on renal neoplasia stated that 5 entities should be recognized as new distinct epithelial tumours within the classification system: tubulocystic RCC, acquired cystic disease-associated RCC, clear cell (tubulo) papillary RCC, the MiT family translocation RCC (in particular t(6;11) RCC), and hereditary leiomyomatosis RCC syndrome-associated RCC. In addition, there are 3 rare carcinomas that were considered as emerging or provisional new entities: thyroid-like follicular RCC; succinate dehydrogenase B deficiency-associated RCC; and ALK translocation RCC (Srigley et al. 2013), as shown in Table 1.1 and 1.2.

Table 1.1: Proposed New Renal Epithelial Tumours and Emerging/Provisional Tumour Entities

<u>New epithelial tumours</u>

Tubulocystic renal cell carcinoma

Acquired cystic disease associated renal cell carcinoma

Clear cell (tubulo) papillary renal cell carcinoma

MiT family translocation renal cell carcinoma (including t(6;11) renal cell carcinoma)

Hereditary leiomyomatosis renal cell carcinoma syndrome associated renal cell carcinoma

Emerging/provisional entities

Thyroid-like follicular renal cell carcinoma

Succinic dehydrogenase B deficiency associated renal cell carcinoma

ALK-translocation renal cell carcinoma

Table 1.2: ISUP Vancouver Modification of WHO (2004) Histologic Classification of Kidney Tumours

Renal cell tumours

Papillary adenoma

Oncocytoma

Clear cell renal cell carcinoma

Multilocular cystic clear cell renal cell neoplasm of low malignant potential

Papillary renal cell carcinoma

Chromophobe renal cell carcinoma

Hybrid oncocytic chromophobe tumour

Carcinoma of the collecting ducts of Bellini

Renal medullary carcinoma

MiT family translocation renal cell carcinoma

Xp11 translocation renal cell carcinoma

t(6;11) renal cell carcinoma

Carcinoma associated with neuroblastoma

Mucinous tubular and spindle cell carcinoma

Tubulocystic renal cell carcinoma

Acquired cystic disease associated renal cell carcinoma

Clear cell (tubulo) papillary renal cell carcinoma

Hereditary leiomyomatosis renal cell carcinoma syndrome-associated renal cell carcinoma

Renal cell carcinoma, unclassified

Metanephric tumours

Metanephric adenoma

Metanephric adenofibroma

Metanephric stromal tumour

Nephroblastic tumours

Nephrogenic rests

Nephroblastoma

Cystic partially differentiated nephroblastoma

Mesenchymal tumours

Clear cell sarcoma

Rhabdoid tumour

Congenital mesoblastic nephroma

Ossifying renal tumour of infants

Leiomyosarcoma (including renal vein)

Angiosarcoma

Rhabdomyosarcoma

Malignant fibrous histiocytoma

Hemangiopericytoma

Osteosarcoma

Synovial sarcoma

Angiomyolipoma

Epithelioid angiomyolipoma

Leiomyoma

Hemangioma

Lymphangioma

Juxtaglomerular cell tumour

Renomedullary interstitial cell tumour

Schwannoma

Solitary fibrous tumour

Mixed mesenchymal and epithelial tumours

Cystic nephroma/mixed epithelial stromal tumour

Neuroendocrine tumours

Carcinoid (low-grade neuroendocrine tumour)

Neuroendocrine carcinoma (high-grade neuroendocrine tumour)

Primitive neuroectodermal tumour

Neuroblastoma

Phaeochromocytoma

Hematopoietic and lymphoid tumours

Lymphoma

Leukaemia

Plasmacytoma

Germ cell tumors

Teratoma

Choriocarcinoma

Metastatic tumours

Other tumours

ccRCC, arising from the proximal tubular epithelial cells, is the most common subtype constituting 70-80% of RCC, followed by papillary RCC (pRCC) (10-15%), chRCC (5%) and collecting duct RCC (<1%) (Kawaguchi et al. 2011; Kurup et al. 2012). RO accounts for approximately 3-7% of all adult renal neoplasms. The peak age of incidence for detection of RO tends to be in the 7^{th} decade of life. For chRCC, the peak incidence occurs in the 6^{th} decade. For cases of RO, men seem to be affected twice as often as females; for chRCC, the disease tends to affect men and women equally (Cindolo et al. 2005).RO and chRCC develop as either sporadic or familial forms, and both can be associated with distinct genetic mutations. The majority of RO and chRCC occur as sporadic cases (Lopez-Beltran et al. 2006; Vera-Badillo et al. 2012). There is also the occasional occurrence of familial renal cancers of oncocytoma with BHD syndrome. Familial oncocytoma is due to partial or complete loss of multiple chromosomes. BHD syndrome is an autosomal dominant inherited syndrome with the BHD gene locus located in the short arm of chromosome 17 (Khoo et al. 2001; Nickerson et al. 2002). This syndrome is characterised by fibrofolliculomas, lung cysts that can lead to spontaneous pneumothoraxes, and various subtypes of renal tumours including hybrid tumours, RO, chRCC and ccRCC.

In rare instances, patients can present with renal oncocytosis. Renal oncocytosis was first described in 1982 (Warfel and Eble 1982): multiple and bilateral oncocytic nodules and a spectrum of oncocytic changes are found diffusely throughout the renal parenchyma. A large

series investigating renal oncocytosis revealed that hybrid development of RO and chRCC was most common (Adamy et al. 2011). Hybrid oncocytic chromophobe tumours are tumours which display histological features of both chRCC and RO. They can occur in three clinicopathological scenarios: sporadic; in association with BHD syndrome; and in association with renal oncocytosis. All scenarios demonstrate indolent clinical behaviour (Hes et al. 2013).

1.2.3 Clinical presentation

Generally, patients with RO tend to be asymptomatic and present incidentally following cross-sectional imaging for an unrelated complaint. Similarly, the majority of patients with chRCC present incidentally with asymptomatic renal masses (Volpe et al. 2012). Less commonly chRCC may present with local symptoms of haematuria, flank mass and loin pain, and constitutional symptoms of weight loss and loss of appetite (Vera-Badillo et al. 2012). ChRCC can also present with paraneoplastic syndrome and metastases with predilection to the liver (Klatte et al. 2008). In the largest published series to date, chRCC present with metastases at a rate of 1.3% (Volpe et al. 2012). Generally, patients with chRCC tend to present in less advanced stages, less frequently with metastases and are usually of better performance status (Klatte et al. 2008) compared with other subtypes of RCC. It should be noted, however, that the local and constitutional symptoms for chRCC are similar to those seen for other RCC.Malignant chRCC have the propensity to metastasise whereas RO will almost always follow a benign clinical course with no significant risk of metastases. Previous published isolated case reports of metastatic RO on initial presentation or following resection of the RO (Amin and Anthony 1999; PerezOrdonez et al. 1997) have been noted, but these case reports have not been substantiated with proper histopathological confirmation of the

metastatic deposits, except for one liver metastasis (Oxley et al. 2007). In that case report by Oxley et al (2007), the patient presented with a large symptomatic left RO and subsequently developed liver metastases which were confirmed on histopathology from the liver biopsies. Therefore, accurate diagnosis of the benign nature of RO is crucial as no further surveillance or treatment will be required.

Renal tumours can be detected by radiological imaging using ultrasonography, computed tomography (CT), magnetic resonance imaging (MRI) and positron emission tomography (PET). Usually following the suspicion of a renal mass, either clinically or via ultrasound, a multiphase CT scan will be performed to delineate its nature (Szolar et al. 1997). Multiphase CT scans can clearly delineate the renal tumour, its local extension to surrounding tissues and detect any metastases to regional lymph nodes or other organs. Cases of small renal masses (lesions < 4cm) detected incidentally are increasing in incidence largely owing to the widespread utilisation of ultrasound and CT scans. Generally, there is no accurate differentiation between benign and malignant renal lesions using CT scans (except for angiomyolipoma), but retrospectively about 20% of these small renal masses will be found to be benign lesions (Remzi et al. 2006). Percutaneous biopsy of these small renal masses provides an enticing strategy to identify lesions of no or low malignant potential however widespread uptake of biopsy into clinical practice has been limited, at least in part due to the limited reliability of a negative or benign biopsy result. Predicting whether a small renal mass is malignant, based on its growth velocity, has been reported, but there is no good correlation of malignancy with growth rate (Kurup et al. 2012). A recent meta-analysis of small renal masses which included benign and malignant lesions, showed a mean growth rate of 0.28cm annually (range 0.09 to 0.86) for small renal masses followed with imaging (Chawla et al. 2006). RO increase in size with variable velocity, with one case series reporting an observed

growth rate of 0.20cm annually (Kawaguchi et al. 2011). The largest pool of 33 biopsy-proven benign RO demonstrated a growth rate similar to reported growth rates for RCC, thus highlighting again that observation of growth cannot distinguish between the benign or malignant nature of such lesions (Kurup et al. 2012). The locality and size of tumours may also be variable. Uncommonly, there have been case reports of large RO (25 x 15 x 12cm) (Akbulut et al. 2010), but the average size is normally around 4.9±2.7cm (Romis et al. 2004). Published reports worldwide show that RO can be multifocal in 6-11% (Dechet et al. 1999; Trpkov et al. 2010) and bilaterality was reported in about 3-5% (Davis CJ 1991; Dechet et al. 1999). In comparison, the median size of chRCC is about 6.0cm (Vera-Badillo et al. 2012), which is larger compared to other subtypes of RCC (Cheville et al. 2003). Multifocality of chRCC is usually around 10-12% (Yusenko 2010a).

1.2.4 Diagnostic dilemma

Following diagnosis of suspected renal tumours on ultrasound or other radiological modalities, a multiphase CT scan of the abdomen pelvis together with CT thorax or chest Xray or bone scan are usually performed to clinically stage these patients. For renal tumours, the current 2010 Tumour Node Metastasis (TNM) staging classification (Edge and Compton 2010) is as follows:

Table 1.3: 2010 Tumour Node Metastasis (TNM) staging classification
Primary tumours (T)

TX Primary tumour cannot be assessed
T0 No evidence of primary tumour
T1 Tumour ≤7 cm in greatest dimension, limited to the kidney
T1a Tumour ≤4 cm in greatest dimension, limited to the kidney
T1b Tumour >4 cm but ≤7 cm in greatest dimension, limited to the kidney
T2 Tumour >7 cm in greatest dimension, limited to the kidney
T2a Tumour >7 cm but ≤10 cm in greatest dimension, limited to the kidney
T2b Tumour >10 cm, limited to the kidney
T3 Tumour extends into major veins or perinephric tissues but not into the ipsilateral

adrenal gland and not beyond the Gerota fascia

T3a Tumour grossly extends into the renal vein or its segmental (muscle-containing) branches, or tumour invades perirenal and/or renal sinus fat but not beyond the Gerota fascia

T3b Tumour grossly extends into the vena cava below the diaphragm

T3c Tumour grossly extends into the vena cava above the diaphragm or invades the wall of the vena cava

T4 Tumour invades beyond the Gerota fascia (including contiguous extension into the ipsilateral adrenal gland)

Regional lymph node (N)

NX Regional lymph nodes cannot be assessed

N0 No regional lymph node metastasis

N1 Metastasis in regional lymph node(s)

Distant metastasis (M)

M0 No distant metastasis

M1 Distant metastasis

The increasing use of CT scans for small renal masses has led to a diagnostic dilemma of accurately characterising the nature of these renal lesions and their subsequent management. Typically on CT scans, RCC are solid heterogeneous masses with contrast enhancement showing areas of patchy uptake of contrast. Locally advanced tumours may directly invade the adrenal gland, renal vein, inferior vena cava and regional lymph nodes. ChRCC usually demonstrate homogenous enhancement, whereas ccRCC, papillary and collecting duct RCC tended to show heterogeneous or predominantly peripheral enhancement. Even though calcification was seen more commonly in chRCC (38%) than in papillary (32%) or conventional (cc)RCC (11%) (Kim et al. 2002), the differences between some subtypes were relatively small, and this feature was not reliable and not useful as a diagnostic criterion. On MRI, chRCC typically have heterogeneous T2 signal intensity and enhancement.In contrast, on CT scanning, RO typically show a well-defined, smooth, relatively homogeneous solid mass with a central area of hypo-attenuation due to the presence of a central stellate scar, and rarely show any extension to the renal vein, inferior vena cava or the adrenals. MRI scan will

typically reveal low to moderate homogeneous intensity on T1-weighted images and relatively high signal intensity on T2-weighted images (Remark et al. 1988). Classically, if renal angiography on RO were performed, it would show a typical spoke-wheel pattern, highlighting the marked peripheral vascularity in contrast with the relatively hypovascular central part of the tumour. However, classical hypo-attenuation of the central stellate scar on CT scan is seen in less than one third of RO, and although characteristic of RO, it is not diagnostic (Chawla et al. 2006; Khoo et al. 2001). Moreover, there are no consistently reliable pathognomic CT scan features that can safely differentiate RO from RCC (Choudhary et al. 2009). Therefore, most RO are treated as suspicious of RCC based on imaging, and thereafter are subjected to surgical resection. Examples of multiphase CT scans of chRCC and RO from our prospective cohort of patients are shown in Figures1.1 A-D. As can be seen on these CT scans, chRCC and RO lesions cannot be accurately differentiated based on features on multiphase CT scan alone.

A recent study on the ability of MRI to discriminate RO from chRCC showed that these two entities exhibited similar findings, and no MRI features were reliable in distinguishing between the two (Rosenkrantz et al. 2010). The ability of any renal lesion to uptake 18-fluorodeoxyglucose (FDG) is the basis of 18-FDG positron emission tomography/computed tomography (PET/CT) scans. However, in detection of renal tumours, the role of FDG PET is limited as there are high false negative rates (Aide et al. 2003). Benign ROs are also often FDG-avid, and thus this cannot be used in separating them from malignant renal tumours (Ramdave et al. 2001). Recently, multiphasic multi-detector CT scans have helped to discriminate ccRCC from RO, papillary RCC and chRCC by utilising the different enhancements at various phases of the scans (Young et al. 2013). This will aid somewhat to the distinction of ccRCC from RO, but not the discrimination of RO from chRCC. Arterial

phase enhancements >500% and washout values >50% in Hounsfield units obtained in multiphasic CT scans can be seen exclusively in RO and can aid in distinguishing RO from other subtypes of RCC (Bird et al. 2011).

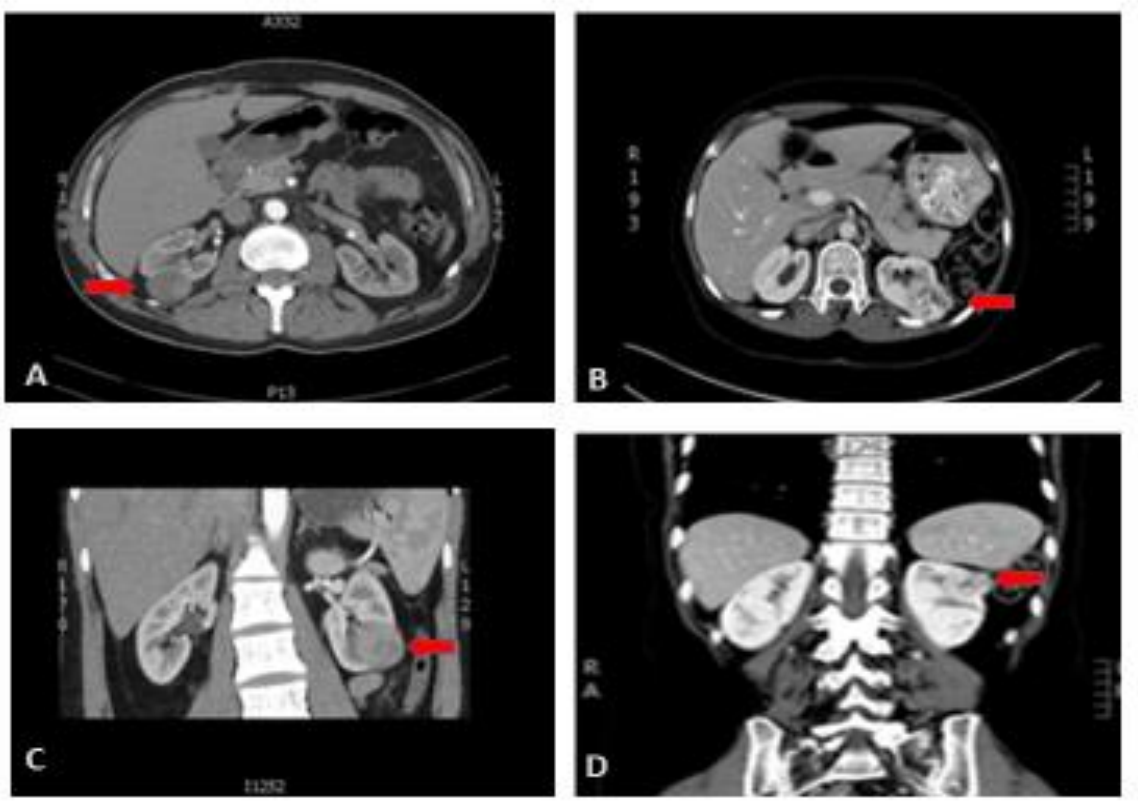

Figure 1.1: CT scan of renal tumourA. Axial CT scan (corticomedullary phase) of right chRCC; **B**. Axial CT scan (nephrographic phase) of left RO; **C**. Coronal CT scan (corticomedullary phase) of left chRCC; **D**. Coronal CT scan (nephrographic phase) of left RO (renal tumours shown with red arrows)

1.2.5 Pathology

Despite the non-invasive discriminatory features of multiphasic CT scans, renal mass biopsy provides the best opportunity for preoperative diagnosis. However, there are numerous potential shortcomings for this procedure, leading to the inevitability of surgical excision.

One of the main drawbacks of renal mass biopsy is the relative difficulty faced by pathologists to accurately and conclusively diagnose renal tumour subtype from the limited tissue biopsy samples, as usually an entire range of cytoarchitectural features is necessary for examination to arrive at a diagnosis (Barocas et al. 2006). However, as a general rule, if the lesion looks like chRCC on needle biopsy, it can be confidently reported as such. In comparison, a lesion that looks like an RO may be incompletely sampled, with other areas merging into the eosinophilic variant of chRCC. This may be a hybrid tumour or simply oncocytoma-like areas in a chRCC. Therefore most pathologists would not diagnose an RO outright on a needle biopsy, and make a comment as to the possibility of having chRCC elsewhere in the tumour. In addition to the difficulties in differentiating RO from chRCC clinically, the pathological features following surgical resection of these tumours often overlap and pose a diagnostic challenge to pathologists.

ChRCC are well-circumscribed encapsulated tumours which have a light-brown to tan cut surface. These are typically solid but cystic areas can be found. Central scarring may be seen. Histologically there are two types. The classic type has large polygonal cells with finely granular cytoplasm. These have prominent plant-like thick cell membranes. The eosinophilic variant is composed of polygonal cells with abundant eosinophilic cytoplasm. Nuclei are irregular, crinkled and angulated, often with perinuclear clearing. Binucleation is common. A solid sheet-like pattern with poor cellular cohesion is commonly found. RO are also well-circumscribed, but unencapsulated, tumours which are typically mahogany brown but sometimes tan-coloured. A central stellate scar is present in about one third of cases. Rarely, cystic change or haemorrhage can be found. Histologically there are large round polygonal cells with abundant eosinophilic cytoplasm and round nuclei. Nucleoli are inconspicuous.

Cells form nests, tubules, acini and microcysts. Focal degenerative nuclear atypia may be seen. Figure 1.2 demonstrates histopathology of chRCC and RO.

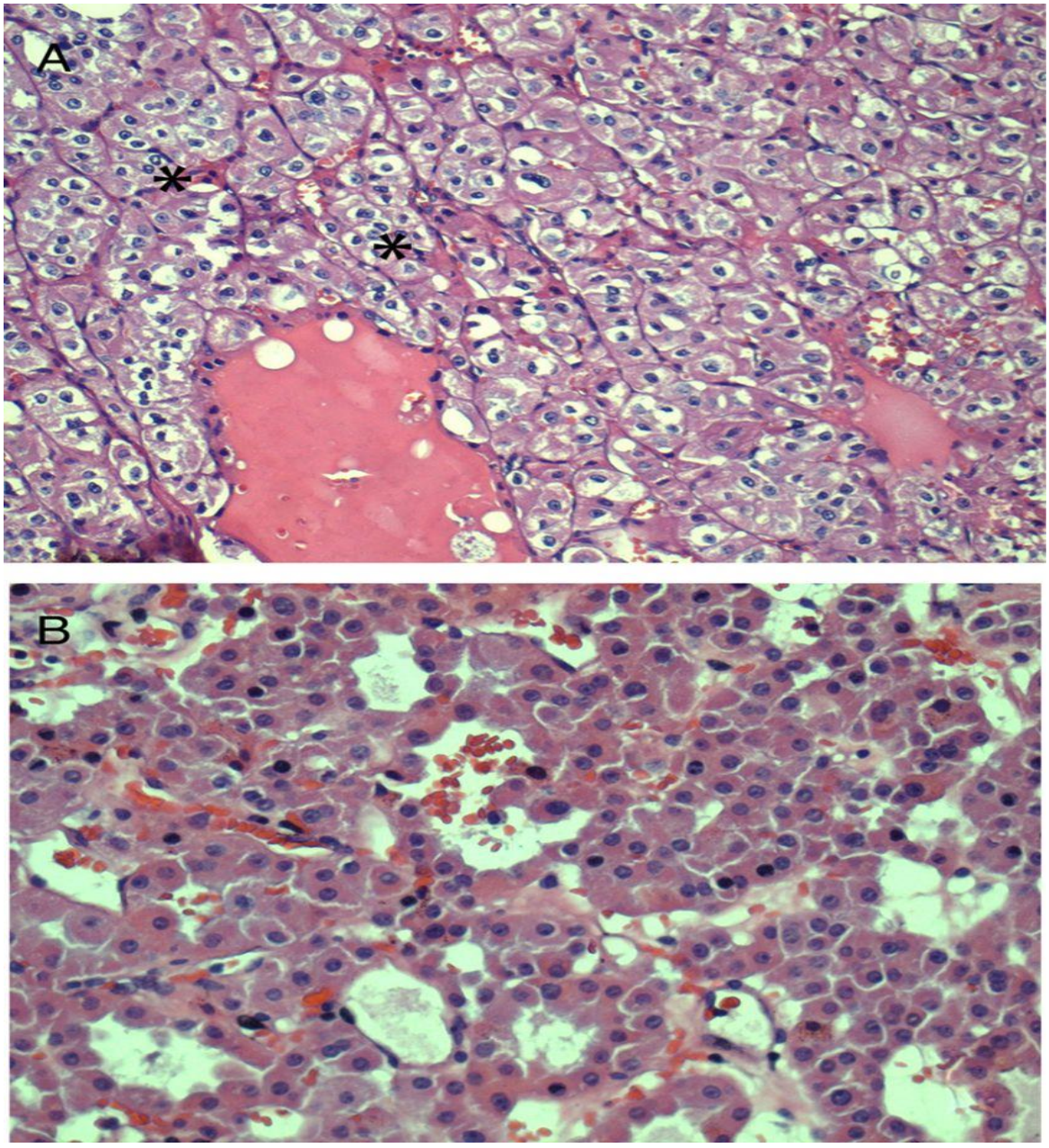

Figure 1.2: Histopathology of chromophobe renal cell carcinoma and renal oncocytoma

A. H&E-stained section of an example of eosinophilic variant of chromophobe renal cell carcinoma, showing typical large, pale, polygonal cells with prominent cell membranes. Nuclei tend to be irregular and wrinkled, and cells are sometimes binucleated (asterisks).

Perinuclear clearing can be prominent; **B**. H&E stained section of an example of renal oncocytoma, showing large oncocytes with densely granular eosinophilic cytoplasm. Cells are round to polygonal and nuclei are round and monotonous. Nucleoli are small and inconspicuous.

Table 1.4 describes the macroscopic and microscopic features of RO and chRCC. Despite having some subtle distinguishing macroscopic, microscopic and ultrastructural differences, there is often need to use ancillary histochemical and IHC stains to differentiate these two entities. Recently, a new oncocytic variant of chRCC was described, that morphologically resembles RO but has the biological characteristics of chRCC (Kuroda et al. 2013). In the 5 cases reported by Kuroda et al (2013), histologically the tumour cells had characteristics of RO; however positive cytokeratin 7 and mitochondrial antigen IHC staining and a subsequent fluorescence in situ hybridization study favoured chRCC. This "oncocytic variant" of chRCC adds to the difficulties for pathologists to discern RO from chRCC.

Table 1.4: Comparison of macroscopic, microscopic and ultrastructural features for oncocytoma and chromophobe renal cell carcinoma

Features	Oncocytoma	Chromophobe RCC
Macroscopic	Well-circumscribed, tan or mahogany brown, sometimes with a central stellate scar (Trpkov et al. 2010)	Usually circumscribed, homogenous, light brown, beige, yellow or tan colour (Latham et al. 1999).
Microscopic Cytoplasm Nuclei	Cells arranged in a nested or organoid pattern, but tubular, trabecular or solid structure can also be seen (Gudbjartsson et al. 2005). Granular eosinophilic cytoplasm Round, uniform nuclei (Tickoo and Amin 1998)	Variants: classic, eosinophilic and mixed. Cells arranged in sheets, with distinct or accentuated cell borders (Abrahams et al. 2004). Granular eosinophilic (eosinophilic variant) or pale, reticular and almost transparent appearance (classic) (Crotty et al. 1995) Presence of peri-nuclear halos, wrinkled nuclei (Crotty et al. 1995).
Ultrastructural	Abundant mitochondria with lamellar or focally-stacked cristae. Absent or sparse vesicles (Tickoo et al. 1998).	Scant mitochondria with tubule-vesicular cristae. Abundant microvesicles between mitochondria. (Latham et al. 1999).

To date, none of the histochemical, IHC or cytogenetic features has been proven to be reliable and specific (Mazal et al. 2005). However IHC biomarkers may be a cost-effective and valuable form of information for monitoring disease for both prognosis and planning treatment regimens. Tables 1.5-1.8 list some of the histochemical and IHC biomarkers that have been published. Hale's colloidal iron staining is still used. Currently, the most useful IHC markers for the differentiation of renal tumours are vimentin, cytokeratin (CK)7, CD10, and marker for RCC (RCCma). According to the literature, vimentin has been shown to be positive in ccRCC and negative in chRCC and RO, and CK7 is positive in chRCC and negative in RO and ccRCC. RCCma and CD10 are positive in ccRCC and negative in both chRCC and RO. Hale's colloidal iron staining with diffuse reticular pattern and peri-nuclear halo is present in chRCC but non-existent in RO and ccRCC (Geramizadeh et al. 2008). Colloidal iron and widespread CK7 positivity have been suggested to be useful in distinguishing chRCC from RO. In RO, colloidal iron staining is usually negative and CK7 shows only focal positivity. However, there is overlap in the staining patterns, preventing these stains to be of much practical value. Negative staining for vimentin and widespread staining for CK7 versus negative staining for CK7 and positive staining for vimentin can be useful in distinguishing chRCC from ccRCC.

However, as seen in Table 1.5 these IHC biomarkers still have their pitfalls in distinguishing between chRCC and RO. For example, the problems with Hale's colloidal iron in certain instances is its failure to stain adequately, or the staining pattern (diffuse cytoplasmic versus luminal) could not be adequately assessed (Latham et al. 1999). However, vimentin may be useful in discriminating chRCC from other RCC, and a panel of vimentin with glutathione S-transferase alpha (GST-α) and epithelial cell adhesion molecule (EpCAM) may achieve

100% sensitivity and specificity for the differential diagnosis of chRCC, RO and ccRCC (Liu et al. 2007).

RO and chRCC share not only histologic and cytologic features, but also share IHC markers for S100A1 and CD117 (KIT) (Bing et al. 2013). Several other studies with IHC markers, including kidney-specific cadherin, CK7, EMA, CD10, RCC, c-KIT, and *RON* proto-oncogene have been used to distinguish chRCC from RO, but the results of these studies are inconsistent and unsatisfactory (Lee et al. 2011).

Table 1.5: Histochemical and immunohistochemical biomarkers to differentiate chRCC and RO.

Method	No. of patients	Success as biomarker
Hale's colloidal iron stain	28 cases (11 chRCC, 12 RO, 6 ccRCC)	Colloidal iron was diffusely and strongly positive in 9/11 of chRCC, focally and weakly positive in 5/12 of RO, and negative in all granular cell variants of ccRCC (0/6). (Wang and Mills 2005)
Modified Mowry's colloidal iron stain better characterised chRCC	62 cases (14 chRCC, 19 RO, 11 ccRCC, 7 eosinophilic variants of pRCC)	Positive colloidal iron stain was not limited to chRCC, however a diffuse and strong, reticular staining pattern was observed only in chRCC (100%). Staining patterns less consistent in all other renal neoplasms. Most RO (84%) had focal, weak, fine dust-like positivity. 100% ccRCC had focal, coarse, droplet-like positivity. (Tickoo et al. 1998)
	76 cases (30 ccRCC, 16 pRCC, 21 chRCC, 8 RO, 1 cdRCC)	Fine reticular cytoplasmic pattern with peri-nuclear halo (87.5% chRCC; 16% ccRCC). 12.5% RO had focal, coarse, cytoplasmic staining without peri-nuclear halo. (Geramizadeh et al. 2008)
CD10 Outcome of CD10 to distinguish between chRCC and ROs is variable.	76 cases (30 ccRCC, 16 pRCC, 21 chRCC, 8 RO, 1 cdRCC)	CD10 positive, 79% ccRCC, 6.3% chRCC and 0% RO. CD10 reactivity favours ccRCC, and the absence of CD10 in RO shows CD10 could differentiate between chRCCs and RO in a panel of biomarkers. (Geramizadeh et al. 2008)
	83 cases (22 chRCC, 17 RO, and 45 ccRCC)	CD10 positive, ccRCC (91%), chRCC (45%) and RO (29%). (Liu et al. 2007)
	28 cases (11	CD10 positive, 100% ccRCC, 72% chRCC and 58%

	chRCC, 12 RO, 6 ccRCC)	RO. Not useful as a biomarker. (Wang and Mills 2005)
RCC marker (RCCma) RCCma is a relatively new IHC marker that has variable results.	76 cases (30 ccRCC, 16 pRCC, 21 chRCC, 8 RO, 1 cdRCC)	RCCma, positive in 62.5% ccRCC, 12.5% RO, but negative in chRCC. Holds potential as part of a panel to differentiate between chRCC and RO. (Geramizadeh et al. 2008)
	Renal cell neoplasm TMA (30 RO, 18 chRCC, 64 ccRCC, 50 pRCCs, 31 RO)	RCCma, positive in most RCC with granular/eosinophilic features. ccRCC (71%), pRCC (76%), negative in RO. (Huang et al. 2009)
	328 samples (256 ccRCC, 27 pRCC, 28 chRCC, 5 cdRCC, 5 unclassified RCC, 7 RO)	RCCma was negative in chRCC but was positive in 3/7 RO. (Kuroda et al. 2004)
	29 cases (11 chRCC, 12 RO, 6 ccRCC)	RCCma was observed in more than 80% of ccRCCs but was negative in all chRCCs and RO. (Wang and Mills 2005)
Vimentin	76 cases (30 ccRCC, 16 pRCC, 21 chRCC, 8 RO, 1 cdRCC)	Vimentin positive, 95% ccRCC, 6.3% chRCC, 12.5% RO. Negative staining for Vimentin, chRCC or RO. (Geramizadeh et al. 2008)
	83 cases (22 chRCC, 17 RO, 45 ccRCC)	Vimentin positive exclusively in ccRCCs. (Liu et al. 2007)
	Renal cell neoplasm TMAs (30 RO, 18 chRCC, 64 ccRCC, 50 pRCC, 31 RO)	Positive in most RCC with granular/eosinophilic features (ccRCC 78%, pRCC 85%). Negative in RO and chRCC. (Huang et al. 2009)

Cluster of differentiation (CD10), Collecting duct renal cell carcinoma (cdRCC), Renal cell carcinoma marker (RCCma), Papillary renal cell carcinoma (pRCC), Tissue microarray (TMA)

Table 1.6 describes emerging biomarkers used to differentiate chRCC from RO, directly or indirectly. BCA2, a RING H2 finger protein RING E3 ligase, holds potential as a tool to distinguish RO from its mimickers, like chRCC (Ehsani et al. 2013). In addition, RO has significantly higher expression of the cancer-testis antigens (CTAs), such as MAGE-A3/4 and NY-ESO-1 (Demirovic et al. 2010). Further investigation is needed to evaluate the potential diagnostic implications for these markers.

Table 1.6: Emerging biomarkers used to differentiate chRCC from RO

Method	No. of patients	Significance of success as biomarker
BCA2	158 patients (104 ccRCC, 8 chRCC, 2 pRCC, 38 RO, 6 oncocytic neoplasms	All RO and oncocytic neoplasms, which favour RO, were positive for BCA2 while all RCC were negative, including chRCC. (Ehsani et al. 2013)
C-kit (encodes the membrane-bound tyrosine kinase KIT)	mRNA levels, 17 chRCC, 20 RO from cDNA microarrays IHC analysis, 226 renal tumors in TMAs (40 chRCC, 41 RO, 40 ccRCC, 29 renal angio-myolipoma, 21 pRCC).	Significant increment of c-kit mRNA and overexpression of KIT protein by IHC in chRCC and RO hence low potential for differentiating between the two types. However there was potential for differentiating chRCC/RO from the other renal cell tumors (ccRCC and pRCC). (Huo et al. 2005)
EMA	86 retrospective nephrectomy specimens (15 ccRCCs, 15 pRCCs, 15 chRCCs, 10 ROs, 6 cdc)	EMA was positive in chRCC (75-100%), ccRCC (50-77%) and oncocytomas (51-86%), showing no major promise as a marker. (Comparison made with 3 tubulocystic carcinoma, 3 renal medullary carcinoma, 3 mucinous tubular and spindle cell carcinoma, 4 metanephric adenoma, 12 invasive high-grade urothelial carcinoma) (Skinnider et al. 2005)
	76 cases (30 ccRCC, 16 pRCC, 21 chRCC, 8 RO, 1 cdc)	EMA was positive in 100% of ChRCCs, 100% of ROs and 75% of ccRCC. So, we concluded that EMA is not a good marker for the differentiation of renal tumours. (Geramizadeh et al. 2008)
Carbonic anhydrase IX (CA IX)	TMAs, 20 cases of each ccRCC, chRCC, pRCC and RO	CA IX was highly sensitive for ccRCCs (90% positivity) and was negative in all other renal epithelial tumours except for 1 chRCC. (Bing et al. 2013)
Galectin-3	TMAs, 20 cases of each ccRCC, chRCC, pRCC and RO	Galectin-3 found mostly in renal tumours with oncocytic features, including RO (100%) and chRCCs (89%). May hold small promise to distinguish these from other RCC. (Bing et al. 2013)
Glutatione S-transferase alpha (GST-α)	22 chRCC, 17 RO, 45 ccRCC	GST-α exclusively observed in ccRCCs. (Liu et al. 2007)
KIT (CD117)	256 ccRCC, 29 chRCC, 25 pRCC, 6cdc, 6	83% chRCCs and 71% RO had membranous immunoreactivity for KIT, while none of the other RCC or the angiomyolipomas expressed.

	unclassified RCC, 7 RO, 20 UC, 7 NB, 2 AM	Cannot be used to differentiate chRCC and RO. (Pan et al. 2004a)
	11 chRCCs, 12 RO, 6 ccRCC	KIT was a very sensitive marker for both chRCC and RO, but not useful to differentiate between the two. KIT with RCCma may be useful when trying to differentiate ccRCCs from chRCCs or ROs. (Wang and Mills 2005)
	22 chRCC, RO & ccRCC	CD117, strongly expressed in chRCC (82%) and RO (100%), whereas none of the ccRCCs were immunoreactive. (Liu et al. 2007)
CD15	10 ccRCC, pRCC, chRCC and RO	CD15 was able to distinguish between chRCCs and RO. 7/10 RO (70%) stained positive for CD15 and none of the chRCC stained for CD15. (Ray et al. 2011)
MAGE-A3/4 cancer testis antigen/CTA	35 patients (17 RO, 18 chRCC)	88% RO stained positively for MAGE-A3/4; 39% chRCC stained positively. (Demirovic et al. 2010)
RON proto-oncogene, encoding a receptor tyrosine kinase,	TMAs (55 RO, 52 chRCCs).15 & 5 conventional sections of RO & chRCC were also analysed	69 of 70 RO and 55 of 57 chRCC had strong, diffuse cytoplasmic stain. (Patton et al. 2004)
	11 chRCC, 12 RO, 6 ccRCCs	11/11 chRCCs, 12/12 RO, but only 3/6 of ccRCC. (Wang and Mills 2005)
NY-ESO-1 CTA	35 patients (17 RO, 18 chRCC)	15/17 RO stained positive, and 6/18 chRCC were positive. (Demirovic et al. 2010)
Interphase fluorescence in situ hybridization (FISH)	11 chRCC, 12 RO, compared with conventional metaphase cytogenetics by karyotyping.	RO often show normal DNA content by interphase and metaphase analyses. The loss of 2 or more of chromosomes 1, 2, 6, 10, and 17 favours the diagnosis of chRCC over RO. FISH analysis is shown to be a useful tool that helps identify differences between these 2 tumour types. (Brunelli et al. 2010)
Endogenous avidin-binding activity (EABA)	Renal TMAs (30 RO, 18 chRCC, 64 ccRCC, 50 pRCC, 31 benign renal tissues)	97% RO, 26% ccRCC, 35% pRCC with granular/eosinophilic (GE) features and 6% of chRCCs positive for EABA. RCC without GE features were negative. EABA is an excellent marker for RO, and so useful in differentiating RO from chRCC. (Huang et al. 2009)
PAX8 and MUC-1	TMAs of 36 chRCC, 20 RO	Expression of PAX8 more frequent in RO than

		in chRCC (55% vs 25%). MUC1 expressed more diffusely and frequently in chRCC than RO (94% vs. 55%). (Bing et al. 2013)

Breast cancer-associated gene 2 (BCA2), epithelial membrane antigen (EMA), proto-oncogene that encodes for a transmembrane tyrosine kinase receptor KIT (Ckit), carbonic anhydrase IX (CAIX), glutatione S-transferase alpha (GST), transmembrane tyrosine kinase receptor (KIT), cluster of differentiation (CD15), melanoma-associated antigen A3/4 (MAGE-A3/4), Recepteur d'Origine Nantais (RON), NY-ESO-1 type of cancer-testis antigen (NY-ESO-1 CTA), Endogenous avidin-binding activity (EABA), paired box gene 8 (PAX 8), mucin-1 (MUC-1),Fluorescence in situ hybridization (FISH).

The cadherins comprise of a family of transmembrane glycoproteins that function as calcium-dependent homotypic adhesion molecules and are expressed by the majority of epithelium. Currently, over 20 different tissue-specific cadherins have been identified (Langner et al. 2004). The promise of cadherin proteins in distinguishing chRCC from RO is shown in Table 1.7. CKs are a family of intermediate filaments that are characteristic markers of epithelial differentiation. Currently, 20 distinct CKs have been identified. They can be useful in the differential diagnosis of neoplasms of epithelial origin, and consequently several CKs have been investigated in renal neoplasms (Skinnider et al. 2005). The CKs that have been trialed to discriminate chRCC from other RCC and also RO are listed in Table 1.8, but none holds major promise, including CK7. Caveolin-1 (Cav-1) is a scaffolding protein encoded by the *Cav-1* gene. This has demonstrated better promise in differentiating chRCC from RO than CK7 (Liu et al. 2007).

Table 1.7: Biomarkers from the cadherin family (also known as calcium-dependent adhesion)

Method	No. of patients	Significance of success as biomarker
Kidney-specific cadherin (Ksp-cad)	102 ccRCC, 46 pRCC, 30 chRCC, 3 cdRCC, 31 RO	Ksp-cad was expressed almost exclusively in chRCCs (97.7% of cases). Ksp-cad offers a quick, dependable approach for differentiating between RO and chRCCs. (Mazal et al. 2005)
	42 ccRCC, 30 pRCC, 13 chRCC, 20 RO using whole sections	In contrast to Mazal et al., 2004, here both chRCC (13/13) and RO (19/20) were positive for Ksp-cad. Ksp-cad not a useful marker for differentiating. (Shen et al. 2005)
	15 chRCC, 15 RO for mRNA analysis & IHC on TMAs containing 36 chRCC, 41 RO	Ksp-cad differentiate RO from chRCC. Ksp-cad was present in chRCCs and ROs at mRNA (89% chRCC and 64% RO) and IHC (31/36 chRCCs and 31/41 RO). (Adley et al. 2006b)
N-Cadherin	21 Japanese cases chRCC, ccRCC, RO.	chRCC and RO were positive for E-cadherin but not for N-cadherin. All ccRCCs were negative for E-cadherin, and 58% were positive for N-cadherin. Useful to distinguish chRCC from ccRCC but not between chRCC and RO. (Taki et al. 1999)
E-Cadherin		
Ep-CAM (epithelial cell adhesion molecule)	22 chRCC, 17 RO,45 ccRCC	Expressed in all chRCC in more than 90% of cells. EpCAM-positive RO (5/17; 29%) had single cell or small cell cluster positivity. The homogeneous EpCAM expression assists to diagnosis chRCC from RO. (Liu et al. 2007)
	10 each of ccRCC, pRCC, chRCCs, RO	EpCAM distinguished between RO and chRCC. RO were negative for EpCAM but positive in 8/10 (80%) of chRCC. (Ray et al. 2011)

Kidney specific (Ksp), Neural-cadherin (N-cadherin), Epithelial-cadherin (E-cadherin), Epithelial cell adhesion molecule (EpCAM), Tissue microarray (TMA).

Table 1.8: Biomarkers from the cytokeratin family

Method	No. of patients	Significance of success as biomarker
CK7 (Basic or neutral cytokeratin)	6 chRCC, 11 RO	All chRCC, strong cytoplasmic staining with peripheral cell accentuation. 8/11 RO, negative, 3 weakly staining. (Leroy et al. 2000)
	21 chRCC, 26 RO	chRCCs (100%) and almost all RO (96%) were positive for CK7. (Garcia and Li 2006)
	11 chRCC, 21 RO from 4 hospitals	73% chRCC, 25% RO positive for CK7; 33% RO focally positive for CK7. No consistency in differentiating the 2 neoplasms. (Wu et al. 2002)
		Positive in 100% chRCC, 8% ccRCC and negative in RO. (Geramizadeh et al. 2008)
	22 chRCC, 17 RO, 45 ccRCC	Positive in 80% chRCC, 0% RO. (Liu et al. 2007)
	TMAs (20 each ccRCC, chRCC, pRCC, RO)	Positive in pRCC (90%), chRCC (89%), and RO (90%). (Bing et al. 2013)
	TMAs (36 chRCC, 20 RO)	Expressed significantly more often in chRCC than RO, both diffusely (53% vs. 10%) and focally (42% vs. 15%). (Bing et al. 2013)
	TMAs (30 RO, 18 chRCC, 64 ccRCC, 50 pRCC)	81% pRCC, 63% chRCC, essentially negative in ccRCC and RO. (Huang et al. 2009)
	10 each ccRCC, pRCC, chRCC, RO	Distinguished RO and chRCC. RO were not stained 80% chRCCs were positive. (Ray et al. 2011)
CK8 (Basic or neutral cytokeratins)	76 cases (30 ccRCC, 16 pRCC, 21 chRCC, 8 RO, 1 cdRCC)	Positive in 70% ccRCC, 93% chRCC and 87.5% RO. (Geramizadeh et al. 2008)
CK18 (Acidic cytokeratin)	76 cases (30 ccRCC, 16 pRCC, 21 chRCC, 8 RO, 1 cdRCC)	Positive in 87% ccRCC, 100% chRCC and 87.5% RO. (Geramizadeh et al. 2008)
CK19 (Acidic cytokeratin)	76 cases (30 ccRCC, 16 pRCC, 21 chRCC, 8 RO, 1 cdc)	Positive in 41% ccRCC, 37.5% chRCC and 62.5% RO. Not a useful marker for differentiation among these subtypes. (Geramizadeh et al. 2008)
CK20 (Acidic cytokeratin)	15 RO only from archives	12/15 RO were positive for CK20. (Stopyra et al. 2001)
	11 chRCC, 21 RO from 4 hospitals	chRCC and RO were uniformly negative for CK20. (Wu et al. 2002)
	76 cases (30 ccRCC, 16 pRCC, 21	Positive in only 8% ccRCCs, 12.5% chRCCs, negative in RO. Not a useful marker for

	chRCC, 8 RO, 1 cdRCC)	differentiation among these subtypes. (Geramizadeh et al. 2008)

Cytokeratin (CK), Tissue microarray (TMA), Collecting duct carcinoma (cdc)

Other recently investigated IHC biomarkers which could aid in the differentiation of these two entities include: amylase α1A (Jain et al. 2013), FXYD2 (Gaut et al. 2013) and transforming growth factor β1 (TGF-β1) (Demirovic et al. 2014). These IHC biomarkers will also be discussed in the meta-analysis section in Chapter 3.

The ISUP recently convened a consensus conference on renal cancer, preceded by an online survey, to address issues relating to the diagnosis and reporting of renal neoplasia (Tan et al. 2013). In their report, the role of biomarkers in the diagnosis and assessment of prognosis of renal tumors is addressed. In particular the study consensus group focused upon the use of IHC markers and the approach to specific differential diagnostic scenarios (Tan et al. 2013). Tan *et al* noted that although no individual antibody or panel of antibodies reached consensus for classifying renal tumors, or for confirming renal metastatic disease, it was noted from the online survey that 87% of respondents used IHC to subtype renal tumors sometimes or occasionally. The selection of these IHC antibodies depends on the familiarity of pathologists as well as ready availability of the antibodies. In their report, Tan *et al* listed the commonly used IHC differential staining patterns for differentiating chRCCs and ROs as: CK7, MOC31, EpCam, Cav-1, EABA, CD82, S100A1, parvalbumin, Ksp-cadherin and CD117 (Tan et al. 2013).

One of the interesting biomarkers in RCC is nuclear factor – kappa B (NF-κB). NF-κB is a collective term for transcription factors of the reticuloendotheliosis (Rel) family of DNA-binding proteins that recognize a common sequence motif (5′GGG(A/G)NN(T/C)(T/C)CC-3′,

where N is any base), called the κB site (Makarov 2000). NF-κB was first described as a B-cell factor that binds to a site in the enhancer region on the gene encoding the immunoglobulin κ light chain (Sen and Baltimore 1986). All five NF-κB members (p65, p50 p52, RelB, c-Rel) contain a Rel homology domain (RHD) of 300 amino acids in the amino terminal, which is essential for dimerization, DNA-binding, and transcription. The RHD contains a nuclear localization sequence towards the carboxyl end. These proteins fall into two categories based on the mode of synthesis, proteolytic cleavage and transcription activities: those that do not, and those that do, require proteolytic cleavage. p65, RelB and c-Rel proteins do not require proteolytic cleavage and are synthesized in their mature form. The second group consists of NF-κB1 and NF-κB2, which are synthesized in the immature form as p105 and p100 respectively and their activation requires proteolytic cleavage. These proteins have ankyrin repeats (AR) at their carboxyl terminals making them inactive. Ubiquitin-dependent proteolytic cleavage removes the carboxyl terminal domain, resulting in the production of the mature p50 from NFκB1 and p52 from NF-κB2 (Hayden and Ghosh 2004; Karin and Ben-Neriah 2000).

NF-κB transcription factors have been implicated in various cancers, including RCC. In tumours, NF-κB affects target genes involved in immunity, cellular proliferation, pro- or anti-apoptotic functions and carcinogenesis. In addition, NF-κB is unique in RCC as it regulates all important aspects of RCC biology that pose a challenge to conventional therapy: resistance to apoptosis; angiogenesis; and multi-drug resistance (Morais et al. 2011). Therefore we investigated the expressions of NF-κB subunits in renal tumour subtypes and their normal counterparts, and will be further discussed in Chapter 4.

Similarly, IHC with established (CK7, caveolin-1, S100A1) and novel biomarkers (kidney injury molecule-1, leptin and leptin receptor) were also investigated to further elucidate the differences in expressions between RCC tumour subtypes (ccRCC, chRCC and RO). The reasons for these selection will be further discussed in Chapter 5.

1.2.6 Conclusion

The current clinical paradigm remains treatment of all localised renal lesions suspicious for renal cell carcinoma on the assumption they are malignant and the standard treatment for these lesions remains surgical resection with either complete or partial nephrectomy when feasible. The increasing detection of small renal masses with a significant chance of benign aetiology provides a diagnostic and management challenge. RO and to a lesser extent small chRCC are two lesions that could be managed conservatively, in many situations, avoiding the morbidity inherent to resection of renal lesions. However, a very high level of diagnostic certainty is required if surgical intervention is to be avoided. Current imaging and biopsy techniques do not always provide this certainty as evidenced by the number of benign small renal lesions reported in contemporary surgical series. If confident diagnosis of renal lesions with low or no malignant potential can be achieved then active surveillance will usually be appropriate, with intervention reserved for tumours demonstrating excessive growth or symptoms. The ability to diagnose RO and chRCC with a high level of confidence may lead to improved utility of preoperative diagnostic techniques and reduced intervention rates for indolent renal lesions. Importantly, identification of reliable and reproducible IHC biomarkers which can aid in the differentiation between chRCC and RO, will pave the way for more accurate pathological diagnoses, which will determine the further management strategies for patients.

Therefore, there is need for further research into the identification of molecular profiles of renal tumours to address not only diagnostic issues mentioned above; but also further understanding of tried and novel biomarkers can be translated into diagnostic and therapeutic targets, thus making a difference in patients with renal tumours.

1.3 HYPOTHESIS

This PhD research is centred upon the hypothesis that there are distinct differences in the molecular signatures between renal cancers that can be exploited. The differences in the unique molecular signatures of various renal tumours can be utilised to distinguish between malignant chRCC and benign RO phenotypes.

1.4 AIMS

The aims of this research include:

1) Identification of panel of IHC biomarkers which can effectively differentiate chRCC from RO through a comprehensive literature search and meta-analysis approach;

2) Assessment of the different molecular profiles of renal cancers via immunohistochemistry and morphometry techniques using selected biomarkers on renal tumour and normal tissue samples;

3) Analyses of IHC biomarkers that are useful in differentiating chRCC from RO via IHC and Aperio ImageScope morphometry techniques; and

4) Creation of comprehensive Renal Tumour Biobank (clinical data, urine, sera, renal tumour and normal tissue) from patients with renal tumours undergoing nephrectomy.

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2

MATERIALS AND METHODS

In this chapter, general materials and methods are presented, including the use of prospective RCC patient samples, IHC of archived RCC tissue blocks, morphometry and statistical analyses. The materials and methods involved in original research chapters 4 and 5 generally follow the details mentioned in this chapter. However, specific materials and methods are described in the meta-analysis in chapter 3. Prospective RCC patients' samples which included renal tumour, normal renal tissue, sera and urine were collected, processed and stored in the CKDR at the TRI, Brisbane Australia, leading to the creation of the Renal Tumour Biobank, which is located in the CKDR. This work comprised Aim 4 of the thesis and will be a legacy of this PhD research. Although the Biobank itself will not be described, its formation involved a significant amount of time and resources throughout the PhD research project.

With ethics approvals and patient consent, archived human RCC tissue blocks were obtained from the University Malaya Medical Centre (UMMC) in Kuala Lumpur Malaysia and Aquesta Pathology, Toowong Australia. IHC of the biomarkers on the tissue slides was performed with assistance from Mr. Clay Winterford in Queensland Institute of Medical Research (QIMR), Dr. David Small (CKDR and Bonventre Lab, Boston USA) and Ms Crystal Chang in Histology Core Facility, TRI.

2.1 Prospective RCC patient samples – the Kidney Tumour Biobank

This component of research involved recruitment of patients, collection, processing and storage of samples. The success of this part of the research was achieved after months of planning, application with approval of ethics and invaluable team coordination and effort of various departments. Ethics approval for collection of patient samples was obtained from Metro South Human Research Ethics Committee (HREC/05/QPAH/95) for Princess Alexandra Hospital and from Greenslopes Research and Ethics Committee (protocol 13/23) for Greenslopes Private Hospital. Relevant ethics approval for investigational research work into these samples and other relevant work involved in this PhD was obtained from Metro South Human Research Ethics Committee (HREC/12/QPAH/125) and also from the University of Queensland Institutional Human Research Ethics Committee (approval number 2013001265). These ethics approvals are included in Appendix 2. The collection of samples started around mid June 2013 and the process is still ongoing. We are proud to report that at time of writing this thesis, there have been approximately 200 samples obtained from RCC patients and stored in the Renal Tumour Biobank in CKDR, TRI with comprehensive clinical data of the patients stored in a secure database.

2.1.1 Clinical data

Prospective patients with renal tumours undergoing nephrectomy that presented to Princess Alexandra Hospital and Greenslopes Private Hospital were recruited following informed consent and discussion about the research project. As per ethics protocols, patients were given the Patient Information and Consent Form (PICF) to be signed. Along with these, clinical data (Queensland Renal Tumours Clinical Record) were recorded by the attending clinician. These forms (PICF and Queensland Renal Tumours Clinical Record) are included in Appendix 3. The comprehensive clinical data included patient characteristics, biochemical

parameters, tumour characteristics, pathology report and also follow up data on tumour progression. The clinical data are then stored in a de-identified coded form in soft and hard copies locked away in the office at CKDR. Corresponding H&E-stained histology slides of these patients are also scanned with Aperio ScanScope digital imaging and stored in de-identified coded forms as soft copy in the CKDR computer files in the TRI.

2.1.2 Collection, processing and storage of serum samples

Pre-operatively, approximately 6-10 mls of venous or arterial blood were collected from the patient in EDTA blood collection tubes. This was transported back to the CKDR laboratory in ice. The samples were then centrifuged for 15 minutes at 2000 rpm at 25°C. Resultant plasma was then aliquoted into 3 Eppendorf tubes with each tube containing 1ml of plasma. The buffy coat was also stored in another Eppendorf tube. The plasma, buffy coat and remnant whole blood cells were then coded accordingly to the corresponding patient and stored in the -80°C Biobank freezer.

2.1.3 Collection, processing and storage of urine samples

Pre-operatively, approximately 15-20 ml of fresh urine were collected from the patient. This was transported in ice immediately to the laboratory and then centrifuged at 1200 rpm for 10 min at 25°C. The resultant supernatant was then divided into 1ml aliquots and placed in Eppendorf tubes with the corresponding unique patient's de-identified code. These were stored in the -80°C Biobank freezer in CKDR.

2.1.4 Collection, processing and storage of kidney tissue samples

Following nephrectomy, the kidney was transported fresh in a cooler box filled with ice to the Pathology Department at the Princess Alexandra Hospital (if nephrectomy from Greenslopes Hospital, then transported to Aquesta Pathology in Toowong). The pathologist then located the tumour and 2 pieces of tumour tissue with 2 pieces of normal renal cortical tissue were retrieved for tumour biobanking (approximately 5x5 mm size). These were transported back in a cooler filled with ice to the CKDR laboratory.

Back in the laboratory, fresh normal kidney tissue was divided into 4 pieces. One piece was fixed in 4% buffered formalin and stored in a 4°C fridge. Within 24 h, this piece of tissue was removed from formalin and placed into phosphate buffered saline (PBS) and stored in the 4°C fridge. This was later paraffin-embedded into tissue blocks using routine histology procedures (Histology Core Facility, TRI) and kept in locked storage at the CKDR. The other normal fresh piece of kidney tissue was divided into 3 smaller tissue cubes and stored into individual Eppendorf tubes (with unique patient's code) at -80°C freezer as part of the Renal Tumour Biobank.

On occasions where there were metastatic and tumour thrombus tissues available, these samples were also retrieved in similar fashion to above from the pathologist and transported back in container filled with ice to the TRI. There, the samples were divided into smaller pieces and stored similarly (fresh at -80°C and fixed in formalin) as described above for the renal tissue.

All tissue samples stored were recorded in soft copy and hard copy in CKDR. These Renal Tumour Biobank samples, together with the clinical database of the patients, provide a

comprehensive collection of RCC patients. The clinical database is continually updated and provides an ongoing database of progression in these patients. It is envisioned that this clinical database will be an invaluable resource for future studies. Likewise, the Renal Tumour Biobank will be a rich resource (sera, urine, tumour and normal tissue) for future research into renal tumours. I believe that this Renal Tumour Biobank is the first dedicated exclusive renal tumour tissue biobank in Australia. There are many other established tissue banks that store renal tumour tissue (for example, Wesley Research Institute Biobank, Victorian Cancer Biobank, Australasian Biospecimen Network), but they do not store renal tissue exclusively, unlike ours.

Currently at time of writing we have approximately 200 patient samples stored in our Biobank and along with that, the corresponding clinical data of these patients. The snapshot summary of patient clinical characteristics is listed in Table 2.1.

Table 2.1: Clinical data from prospective patients

Patients	N = 202
Male : Female	127 : 75
Mean age at presentation (years)	57.0 ± 13.5
BMI	28.9 ± 6.0
Hypertension (%)	126 (62.4)
Mean preoperative eGFR (ml/min/1.73m^2)	72.7 ± 26.4
Median tumour size (cm)	4.5 (1.2 – 22)
Clinical T stage (%)	T1 = 158 (78.2) T2 = 16 (7.9) T3 = 17 (8.5) T4 = 6 (2.9) M1 = 5 (2.5)
Pathology (%)	Clear cell RCC = 131 (64.8) Papillary RCC = 22 (10.9) Chromophobe RCC = 20 (9.9) Clear cell tubulopapillary RCC = 4 (2) Multilocular cystic RCC = 5 (2.5) Oncocytoma = 9 (4.5) Others (benign and malignant) =11 (5.4)

Body mass index (BMI); Tumour (T), Metastates (M), Estimated glomerular filtration rate (eGFR); Renal cell carcinoma (RCC)

2.2 Immunohistochemistry of archived human renal tumour samples

Archived human renal tumour tissue paraffin blocks were obtained from University Malaya Medical Centre (UMMC), Kuala Lumpur Malaysia (for IHC of nuclear factor–kappa B/NF-κB) and Aquesta Pathology Toowong Australia (for IHC of cytokeratin7, caveolin-1, leptin (Ob), leptin receptor (ObR), S100A1, kidney injury molecule-1/KIM-1). The archived tissue blocks were collected retrospectively from a period of 2003-2013 for UMMC and from 2009-2014 for Aquesta Pathology. The ethics approvals for scientific use of archived pathology blocked samples were obtained from University Malaya Ethics Committee (Ref: 848.17) and Aquesta Pathology Ethics Committee (protocol 14/02). These are included in Appendix 4. The clinical data collected retrospectively from these two sets of archival pathology renal tumour tissue blocks are listed in Table 2.2 (clinical data of UMMC) and Table 2.3 (Aquesta Pathology). The clinical staging system used for UMMC and Aquesta Pathology was the 2010 Tumour Node Metastasis (TNM) staging classification as previously described in chapter 1 (Edge and Compton 2010). Fuhrman grading of ccRCC was based on the nuclear features: Grade 1. Small nuclear diameter, round nuclear shape and absent nucleoli; Grade 2. Larger nuclear diameter, irregular nuclear outline and visible nucleoli at x400; Grade 3. Even larger nuclear diameter, obvious irregular nuclear outline and prominent nucleoli at x100; Grade 4. Bizarre large often multilobed nuclei with or without spindle cells (Fuhrman et al. 1982).

Table 2.2 Clinical data for University of Malaya Medical Centre tissue blocks

Period	2003 - 2013			
Patients	96			
M : F	67.7% : 32.3%			
Median age	62 (39-83)			
Median size	6cm (1.5-17)			
Clear cell RCC (stage)	43 (T1)	22 (T2)	9 (T3)	2 (T4)
Clear cell RCC (grade)	10 (G1)	37 (G2)	22 (G3)	7 (G4)
Papillary RCC (stage)	5 (T1)	3 (T2)	2 (T3)	1 (T4)
Papillary RCC (grade)	0 (G1)	7 (G2)	4 (G3)	0 (G4)
Chromophobe RCC (stage)	3 (T1)	0 (T2)	2 (T3)	0 (T4)
Multilocular cystic RCC (stage)	1 (T1)	0 (T2)	0 (T3)	0 (T4)
Clear cell tubulopapillary RCC (stage)	1 (T1)	1 (T2)	1 (T3)	0 (T4)
Stage T1	53 (55.2%)			
T2	26 (27.1%)			
T3	14 (14.6%)			
T4	3 (3.1%)			
Metastases (M1)	22 (22.9%)			

Tumour (T); Metastases (M)

Table 2.3 Clinical data for Aquesta Pathology tissue blocks

Patients	N = 75			
Period	2009 – 2014			
Gender	49 Male : 26 Female			
Median age (years)	64 (18-88)			
Median size (cm)	3.8 (1.2-18)			
Nephrectomy	Partial = 25 (33.3%) Radical = 50 (66.7%)			
Subtype	30 ccRCC	30 chRCC	15 RO	
T stage	ccRCC	chRCC	RO	
T1 = 47 (62.7%)	20(66.7%)	15(50%)	12(80%)	
T2 7 (9.3%)	0	5(16.7%)	2(13.3%)	
T3 = 20 (26.7%)	9(30%)	10(33.3%)	1(6.7%)	
T4= 1 (1.3%)	1(3.3%)	0	0	
M1 stage	2 (2.67%)			
Fuhrman (ccRCC)	Grade1	Grade2	Grade 3	Grade 4
	0	63.3%	20%	16.7%
Poor prognostic histological features	21 (28%)			

Tumour (T); Metastases (M)

2.2.1 Haematoxylin and eosin staining of renal tumour sections

All histology sections were cut from the formalin-fixed paraffin-embedded renal tumour tissue blocks at 3-4µm thickness using the Leica microtome and placed onto Menzel-Glaser Superfrost® Plus slides (Thermo Scientific, USA). This work was performed in the Histology Core Facility, TRI, with aid from Ms Crystal Chang. Histology sections for each specimen were also stained with H&E for general morphology and pathological analysis. This work was carried out at the Histology Facility in QIMR by Mr. Clay Winterford. Sections were dewaxed in xylene and rehydrated in descending grades of alcohol. Sections were briefly washed in distilled water before staining in Mayer's haematoxylin for 5-10 minutes. Sections were then washed in water for 2 minutes before the nuclear stain was blued in Scott's solution (potassium bicarbonate 2g/ml, magnesium sulphate 20g/ml in distilled water) and washed in water for 2 minutes. Sections were then washed in 70% alcohol before alcoholic eosin was added as a counter stain for 1-3 minutes. Absolute alcohol was used to dehydrate the sections before being cleared in xylene and mounted using Depex (Sigma-Aldrich, USA) for the coverslips.

2.2.2 IHC for Nuclear factor kappa B (NF-κB), leptin receptor (ObR), S100A1, kidney injury molecule -1 (KIM-1)

The IHC for NF-κB, ObR, S100A1 was manually batch-stained at QIMR with help of Mr. Clay Winterford. IHC of KIM-1 was manually batch-stained by Dr. David Small in the Bonventre Lab, Harvard USA. Batch staining allows comparison of kidney tumour samples using semi-quantitative IHC. Antibody optimisation and positive control tissue samples (tissue microarray of human liver, kidney and gut) were used to verify the staining activity of the biomarker in human tissue. Negative controls without primary antibody were prepared for

each batch stain. An example of positive and negative controls for S100A1 IHC is shown in Figure 2.1. The principles of IHC are similar and generally follow the steps listed. The buffer solutions, dilutions of primary and secondary antibodies and detection kits will differ and these are listed in Table 2.4. Mentioned briefly here are the general steps for IHC for NF-κB, ObR and S100A1.

Sections were dewaxed in xylene and rehydrated through descending graded alcohols to water using standard protocol. Then the sections were transferred to Tris buffered saline (TBS) pH 7.6. Endogenous peroxidase activity was blocked by incubating the sections in 2.0% hydrogen peroxide (H_2O_2) in TBS for 10 minutes. Sections were then washed in three changes of water, and transferred into buffer and subjected to 15 minutes heat antigen retrieval at 105°C using a Biocare Medical decloaking chamber. On completion of the cooling cycle the slides were allowed to cool for a further 20 minutes on the bench before transferring back to TBS. Then they were washed in 3 changes of TBS. Nonspecific antibody or peroxidase binding was inhibited by incubating the sections in Biocare Medical Background Sniper for 15 minutes. In a humidified chamber excess Sniper was decanted from the sections and the primary antibody was applied for 60-90 minutes at room temperature. Sections were washed in three changes of TBS. Detection kit of specific secondary antibodies of MACH 1 Universal HRP-Polymer Detection (Biocare Medical, USA) was applied for 30 minutes. Sections were then washed in three changes of TBS. Signals were developed in Betazoid diaminobenzidine hydrochloride (DAB) (MACH1 kit) for 5 minutes, with DAB as the chromogen. Sections were then washed in water three times to remove excess chromogen, then lightly counterstained in haematoxylin, washed in water, dehydrated through ascending graded alcohols, cleared in xylene, and mounted using DePex.

For KIM-1 IHC, paraffin sections were deparaffinised and rehydrated by routine methods as described above. Endogenous peroxidase activity was ablated by incubation in 2% H_2O_2 in methanol for 20 minutes. Then sections were washed with water. Antigen retrieval was carried out in a pressure cooker, in buffer pH 8. Sections were allowed to cool for 30 minutes, then they were washed in PBS 10 minutes (PBS x3 changes). Blocking was carried out in a humidifier chamber in 3% bovine serum albumin (BSA) in PBS for 60 minutes at room temperature. Sections were incubated with primary antibody diluted in 3% BSA:PBS, overnight at 4^oC in a humidifier chamber, then washed in PBS (x3 times) and incubated with Biotin anti-mouse secondary antibody diluted in PBS for 30 minutes at room temperature. Slides were then washed in PBS and incubated with Avidin-Biotin-Complex (Vectorstain Elite ABC kit) diluted in PBS for 60 minutes at room temperature. Slides were then washed in PBS (x3 changes), and then DAB substrate (in 2.5 mL dH_2O, add 1 drop buffer, 2 drops substrate, 1 drop H_2O_2) was added to slides generously and incubated for 5 minutes. Slides were thoroughly washed with dH_2O to remove all DAB, and then counter stained with hematoxylin, blued as described previously, dehydrated in alcohols and cleared in xylene before DePex to mount coverslips.

2.2.3 IHC for CK7, leptin (Ob), caveolin-1 (Cav-1)

The IHC for CK7 and Ob, Cav-1 was performed with an automatic Ventana Discovery ULTRA Stainer (Ventana Medical Systems Inc, Roche) using their set protocols. This was done with help of Mr David Small and Ventana representative, Ms Janet Thompson. The slides were placed into the Ventana automated stainer. Primary and secondary antibodies were added to the autostainer at specific stages. Following staining, the slides were then dehydrated and cleared in xylene before coverslips were mounted automatically. The

protocols of IHC by the Ventana automated stainer have been included in Appendix 5. The details of the primary and secondary antibodies are given in Table 2.4.

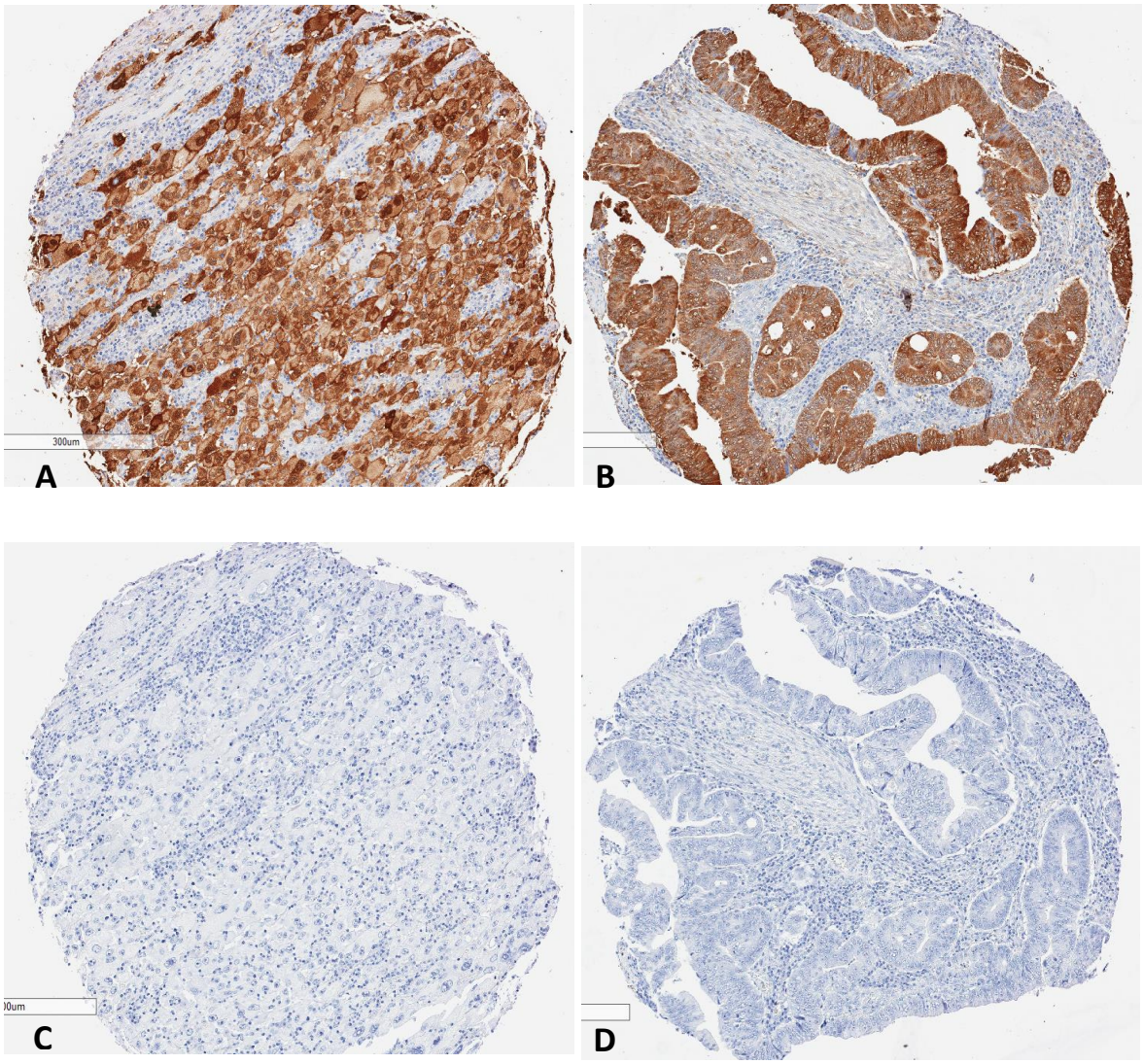

Figure 2.1: TMA S100A1 positive and negative controls

A. TMA of S100A1 positive control of melanoma; **B**. TMA of S100A1 positive control of colon; **C**. TMA of S100A1 negative control of melanoma; **D**. TMA of S100A1 negative control of colon cancer. (x10 Aperio magnification)

Table 2.4: Primary and secondary antibodies for IHC

IHC	Retrieval buffer	Primary antibody with dilution	Secondary antibody
NF-κB	For p50, p52, RelB and c-Rel: EDTA/Tris buffer (1mM/0.01M, pH9.0) 15 minutes at 105°C For p65: citrate buffer (0.1M, pH6) at 125°C for 5 minutes	rabbit anti-human antibodies from Santa Cruz : p50 (sc-7178, dilution 1:100), p52 (sc-298, 1:100), p65 (sc-372, 1:150), RelB(sc-226, 1:100) and c-Rel (sc-71, 1:400)	MACH 1 Universal Rabbit HRP-Polymer Detection
ObR	Dako pH 6 Epitope Rerieval buffer 30 mins at 95°C	Santa Cruz purified goat anti- ObR (1:50 dilution) (sc-1834)	Goat HRP secondary Ab 40 mins room temperature
S100A1	Dako pH 6 Epitope Rerieval buffer 15 mins at 105°C	Sigma purified rabbit anti-S100A1 (1:125 dilution) (HPA006462)	MACH 2 Rabbit HRP secondary Ab for 30 minutes (105°C)
KIM-1	0.1M citrate buffer pH8 R-UNIVERSAL Epitope Recovery Buffer	anti-KIM-1 mouse monoclonal antibody AKG7 (neat)	Vector biotinylated anti-mouse IgG
Caveolin-1	0.01M citric acid buffer pH6	Santa Cruz rabbit anti-Cav-1 (1:250 dilution) (sc-894)	MACH2 HRP anti-rabbit polymer
CK7	CC 1 buffer pH6	Santa Cruz mouse anti-CK7 (1:75 dilution) (sc-23876)	Anti-mouse HQ 16 mins
Ob	CC1 buffer pH6	Santa Cruz rabbit anti-Ob (1:60dilution) (sc-842)	Anti-rabbit HQ 16 mins

2.3 MORPHOMETRY ANALYSIS

Stained slides were scanned with an Aperio ScanScope XT slide scanning system (Aperio Technologies, USA) at 20x magnification. Digital images of the sections were captured using Aperio ImageScope software (Leica Biosystems, Germany) (Staniszewski 2009). A quantitative scoring of expression intensity and localisation of the various IHC biomarkers

was analysed with respect to overall expression, nuclear expression and membrane expression, following advice from Aperio staff and as demonstrated in peer-reviewed publications from our laboratory (Rajandram et al. 2012; Rajandram et al. 2014; Gobe et al. 2016).

2.3.1 Overall positive pixel expression analysis

Three random fields of the same size were selected for each RCC and paired normal kidney section, using DAB positivity as the positive chromogen. Analysis was carried out using the Positive Pixel Count v9 algorithm (for total staining intensity) from the Aperio ImageScope software. Staining (% positive pixels) was scored according to the intensity and percentage of cells stained. The intensity output for Positive Pixel Count v9 algorithm was given as number of negative, weak positive, positive or strong positive pixels. The output was analysed in Excel. Overall positive pixels (%) were calculated by adding the values for "positive %" and "strong positive %" pixels staining.

The average of the 3 overall positive pixels % from the 3 respective scanned fields of renal tumour sections was obtained. Similarly the average overall % positive pixels for 3 random fields in the normal kidney sections, paired to a particular tumour, were obtained. Subsequently, the intensities of tumour and normal kidney values were normalised against respective normal kidney regions and the data were expressed as the percentage of overall normal values.

These results of normal kidney overall % change and tumour overall % change were then tabulated and analysed with Graphpad Prism 6 (GraphPad Software, Inc). Graphs were generated to show the % expression change for tumour versus normal kidney.

2.3.2 Nuclear expression analysis

Similarly, three random fields of the same size were selected for each RCC and paired normal kidney section. Analysis was carried out using the algorithm IHC Nuclear v1.0 from the Aperio ImageScope software. The output for IHC Nuclear v1 algorithm was given as a percentage of pixels with 0, 1+, 2+ or 3+ staining intensity. These results were analysed using Excel. Nuclear positive pixels (%) were calculated by adding the values for 2+ % and 3+ % staining. The average of the 3 nuclear positive pixels from 3 normal sections and average of 3 nuclear positive pixels from 3 tumour regions was then calculated. These were then made into average nuclear percentage. The nuclear intensities of tumour and normal kidney were normalised against respective normal regions and the data were expressed as the percentage of overall normal values.

The results of normal nuclear % change and tumour nuclear % change were then tabulated and analysed with Graphpad Prism 6 (GraphPad Software, Inc) programme. Graphs were generated to show the nuclear expression % change for tumour versus normal kidney.

2.3.3 Membrane expression analysis

Three random fields of the same size were selected for each RCC and paired normal kidney section. Analysis was carried out using algorithm IHC Membrane v1.0 from the Aperio

ImageScope software. The output for IHC Membrane v1 algorithm was given as percentage of pixels with 0, 1+, 2+ or 3+ staining intensity. These results were analysed in Excel. Membrane positive pixels (%) were calculated by adding the values for 2+ % and 3+ % staining. The average of the 3 membrane positive pixels from 3 normal sections and average of 3 membrane positive pixels from 3 tumour regions was then calculated. These were then made into average membrane percentage. The membrane intensities were normalised against respective normal regions and the data were expressed as the percentage of overall normal values.

These results of normal kidney membrane % change and tumour membrane % were then tabulated and analysed with Graphpad Prism 6 (GraphPad Software, Inc). Graphs were generated to show the membrane expression % change for tumour versus normal kidney.

2.3.4 Survival analysis for NF-κB subunits

For survival analysis, the median positive pixel score was used to determine cut-off scores for 'high' or 'low' staining for each biomarker subunit. These results were correlated against the patient cancer specific survival in months from treatment. Cancer specific survival is defined as net survival (in months) from death caused by the cancer rather than any other causes. It measures mortality directly due to cancer (Dickman and Adami 2006).

2.4 STATISTICAL ANALYSIS

Statistical analysis was performed using Graphpad Prism 6 (GraphPad Software, Inc). Data analysis comparing intensities of expression between 2 groups was carried out using Student's t-test. A one way analysis of variance (ANOVA) was used for comparison among more than two groups, to determine the difference in positive pixels (%) or staining intensity between several groups. Statistical significance was determined at $p<0.05$. For survival analysis for NF-κB subunits, the survival curves were obtained using Kaplan-Meier. Survival differences between groups were evaluated using the log rank test. The Cox proportional hazards regression was used to analyse subunits of biomarkers that showed significance in the log rank test. For multivariate analysis, confounding variables included tumour subtype, grade and clinical TNM stage because all these factors affect survival.

2.5 META-ANALYSIS

The methodology for the meta-analysis employed in Chapter 3 will be discussed in detail in that chapter. Dr. Anne Bernard, biostatistician from the Queensland Facility for Advanced Bioinformatics (QFAB), TRI, Brisbane Australia advised on the methods involved in R studio statistical analysis required for this part of the research.

CHAPTER 3

META-ANALYSIS OF IMMUNOHISTOCHEMICAL BIOMARKERS THAT DIFFERENTIATE CHROMOPHOBE RENAL CELL CARCINOMA FROM RENAL ONCOCYTOMA

CHAPTER 3

META-ANALYSIS OF IMMUNOHISTOCHEMICAL BIOMARKERS THAT DIFFERENTIATE CHROMOPHOBE RENAL CELL CARCINOMA FROM RENAL ONCOCYTOMA

Following the discussion in Chapter 1 in which numerous novel and existing IHC biomarkers had been reported as useful to differentiate chRCC from RO, the literature was examined for a detailed assessment of IHC biomarkers that are reliable and effective. Therefore, a meta-analysis was conducted to answer this question. This meta-analysis has been published (Ng et al. 2016) (Appendix 6).

3.1 INTRODUCTION

As discussed in Chapter 1, both RO and chRCC arise from intercalated cells of the collecting ducts. Due to considerable morphological and histological overlap between the two entities, they are often considered to be extremes of the same morphological spectrum (Delongchamps et al. 2009). Accurate differentiation between benign RO and malignant chRCC will obviously lead to better patient management and follow-up strategies in the clinical setting. Following excision of RO, patients will not require any further surveillance imaging and are managed expectantly. On the other hand, despite having a more favourable prognosis compared with other counterparts of RCC, patients with chRCC will still require future surveillance imaging protocols to assess local recurrence or metastases. Proper differentiation largely relies on H&E histochemistry of sections, and an experienced histopathologist to

discern the characteristic histomorphological features between the two entities. However, pathologists generally have difficulties discriminating RO, especially from the eosinophilic variant of chRCC. Despite having some subtle distinguishing macroscopic, microscopic and ultrastructural differences, there is often a need to use ancillary histochemical and IHC stains to differentiate these two entities. To date, however, none of the histochemical, IHC or cytogenetic features has been proven to be reliable and specific (Mazal et al. 2005). Other techniques, for example, electron microscopy, fluorescence *in situ* hybridisation, proteomics and cytogenetics have been used to delineate the two entities, but they are costly, not easily available and require more technical expertise. Therefore, IHC has been the mainstay of laboratory techniques due to its accessibility, ease of use and cheaper costs. Numerous biomarkers have been employed for IHC to differentiate RO from chRCC. However, consistent accurate diagnosis differentiating RO from chRCC is likely to remain elusive until modern molecular biomarkers are identified and applied routinely to ensure reproducibility (Ng et al. 2014).

Therefore, the aim of this study was to analyse and summarise selected results from published literature regarding the discriminatory role of IHC biomarkers in differentiation of chRCC from RO. Following this, we identify and propose IHC biomarkers that are useful in this respect so as to assist in the important distinction between chRCC and RO. This distinction will affect management pathways of the two clinically distinct entities and also perhaps be useful in future implications for preoperative diagnostic modalities.

3.2 METHODS

3.2.1 Literature search

For the assessment of research that involved IHC biomarkers that differentiated chRCC from RO, a literature search via the PubMed medical literature database was performed up to 19 January 2015, with the help from an expert librarian from Princess Alexandra Hospital, Queensland. The main criteria for the literature search centred on the differential ability of biomarkers in discriminating chRCC and RO. The search strategy was based on the combination of terms used: 'chromophobe renal cell carcinoma and renal oncocytoma'; and 'differentiation or diagnoses'; and 'biomarkers or proteins or antibodies'. Following the search and with help from the librarian, the full texts were obtained of selected articles, with both soft and hard copies available for further analysis. These publications were initially scrutinised through inspection of their contents for their relevance to the aim of this review, which was the differential ability of IHC biomarker(s) to identify chRCC and RO. Publications that did not conform to the main aim of this review were discarded.

3.2.2 Quality appraisal of publications

There were strict inclusion and exclusion criteria set out to assess the validity of the publications obtained from the literature search. For inclusion eligibility, publications were English articles from 1991 which had to contain: clear objectives in prospective or retrospective cohort design in the assessment of IHC biomarkers in differentiating renal cancers; description of IHC for biomarkers/proteins/antibodies used; human renal tumour tissue of histology slides/tissue microarray/tissue core biopsies; techniques and analyses of the IHC on subtypes of renal cancers but must include chRCC and RO; clear documentation

of IHC results of biomarkers with negative results (no difference between chRCC and RO) also recorded and statistical analyses of results, which included p values or sensitivity and specificity data. Publications that were excluded : 3 non-English articles (2 Chinese, 1 German); 6 non-IHC methods; 14 single case reports or limited case series (<10 cases); 3 analyses of other subtypes of RCC without inclusion of chRCC and RO; 5 reply or letter to editor; 11 studies involving familial RCC syndromes; and abstracts or conference proceedings. Quality assessment of these publications was made and some were discarded from further analyses, if found lacking in the criteria mentioned above. Publication bias was actively avoided, with all publications pertaining to the use of IHC biomarkers in renal tumours included, and studies with negative or inconclusive results also analysed.

3.2.3 Extraction of data

All the eligible publications were then fully reviewed. Data from the included publications were then extracted into an Excel spreadsheet. Study characteristics that were gathered included: title; first author's name; journal site; publication year; biomarker(s) studied; IHC design; sample size (total and individual chRCC and RO); measurement of IHC analyses (staining intensity or differential staining expression); results of IHC biomarker for chRCC and RO and methods and significance of statistical analyses (p value, sensitivity and specificity). Data were then further analysed and publications were ranked 1–4, according to the quality of the IHC results, which had the best differential ability in discriminating chRCC from RO based on their objective of the study, IHC results and statistical strength of their results.

Rank 1: publications in which the objective was to assess the role of IHC biomarker in differentiating exclusively chRCC and RO, with good significant discriminatory final results.

Rank 2: publications in which the objective was to assess IHC biomarker(s) in differentiating subtypes of renal cancers, which included chRCC and RO with final significant discriminatory results.

Rank 3: publications in which the objective was to assess IHC biomarker in only chRCC and RO, but final results were unclear or did not show any discriminatory value.

Rank 4: publications in which the objective was to assess IHC biomarker in subtypes of renal cancers which included chRCC and RO, but final results were inconclusive.

Once these publications were ranked, only the rank 1 and 2 publications were further analysed. The results (both qualitative and descriptive expression) of the biomarkers from these publications were further evaluated for odds ratios (ORs) and 95% confidence intervals (CIs). Some biomarkers (termed repeated biomarkers) had been investigated in numerous publications and were further analysed in a subset analysis with pooled ORs. Finally, a panel of biomarkers was selected based on the strength of their statistical results and reproducibility of such results in various studies.

3.2.4 Statistical analyses

Statistical analyses were performed with the help of Dr Anne Bernard, biostatistician from QFAB Bioinformatics, Institute for Molecular Bioscience, University of Queensland. Following the ranking and selection of the most relevant biomarkers, statistical analyses of ORs for chRCC compared with RO and 95% CIs were calculated using R statistical software (http://www.r-project.org). When the biomarkers were investigated in at least two publications, the pooled ORs with 95% CI and I^2 test for heterogeneity were computed using

the R function ‘metabin’ available in the ‘meta’ R package. The statistical test of heterogeneity among studies was performed using the Q test and result represented by I^2 percentage (derived from the Q test). The I^2 is a measure of the degree of inconsistency in the study results and represents the percentage of total variation across studies that is due to heterogeneity rather than chance (Higgins et al. 2003). A value of 0% indicates no observed inconsistency, and larger values show increasing heterogeneity. We considered heterogeneity to be present if p value was <0.1. Forest plots of repeated biomarkers were also prepared. For studies with a zero cell count, a treatment arm continuity correction is used instead (Diamond et al. 2007; Sweeting et al. 2004). Studies with zero or infinite OR are not presented on the plot, as their variance cannot be calculated sensibly. Nevertheless, their significant results based on different staining patterns are discussed to provide the readers with the understanding behind the value of the biological results, despite the calculated zero or infinite OR. Throughout the work in this review, published guidelines outlined by PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) (Juni and Egger 2009), were adhered to.

3.3 RESULTS

3.3.1 Relevant studies and flow chart

Following the literature search, 109 manuscripts were available for review. From this, 42 manuscripts were excluded based on inclusion and exclusion criteria mentioned above. Sixty-seven relevant publications from 1991 to 2014 were then analysed. The full texts of all 67 manuscripts that were deemed most appropriate and relevant in achieving the aim of this meta-analysis were then reviewed. After data extraction of the 67 publications, further

assessment and ranking of these publications were made as described above. Consequently, 40 publications which presented significantly discriminatory IHC results of biomarkers were ranked accordingly. Only rank 1 and 2 publications were further analysed as these studies revealed biomarkers that could differentiate chRCC and RO appropriately as shown in Tables 3.1 and 3.2. There were 20 publications ranked 1, 20 ranked 2, 4 ranked 3 and 23 ranked 4. The 40 rank 1 and 2 publications yielded 49 biomarkers: 31 biomarkers that were studied once and 18 biomarkers that had been investigated in at least 2 or more studies. OR and 95% CI of these 49 biomarkers were calculated. Biomarkers that did not have statistically significant differentiating results were noted and filtered, leaving behind 25 biomarkers studied once (single biomarkers) and 12 repeated biomarkers. Finally, the 6 most-effective single biomarkers were chosen from the 25, and 4 most-effective repeated biomarkers were chosen from the 12 repeated biomarkers. This is depicted in the flow chart in Figure 3.1 below. This final selection was based on strength of the study where the biomarkers exhibited the best differentiating ability for chRCC and RO in regards to larger sample size ($n>34$; as the median sample size from the studies was calculated to be 34), significant p value <0.05, high OR and pooled OR with 95% CI, high sensitivity and specificity and distinctive staining patterns.

Table 3.1: Rank 1 publications

Author	Journal	Year/Vol/Pages	Biomarkers
(Cochand-Priollet et al. 1997)	Arch Pathol Lab Med	1997,121,1081-1086	Peanut agglutinin antigen, UEA-1, cytokeratin KL1, epithelial membrane antigen, vimentin, S100 protein, lysozyme
(Leroy et al. 2000)	European Urology	2000,37,484-487	CK7
(Kuroda et al. 2004)	Histology and Histopathology	2004,19,23-28	CK 7,8, 10, 10/13, 14, 18 19,20 AE1/AE3
(Mazal et al. 2005)	Human Pathology	2005,36,22-28	Ksp cadherin, CK7, EMA
(Mete et al. 2005)	Virchows Arch	2005,447,38-946	anti-mitochondrial, caveolin 1, CD63, CK14
(Garcia and Li 2006)	Am J Clin Pathol	2006,125,392-398	caveolin 1, CK7
(Adley et al. 2006a)	Anal Quant Cytol Histol	2006,28,228-236	CK7, parvalbumin
(G. Li et al. 2007)	Histopathology	2007,50,642-647	S100A1
(Sukov et al. 2009)	Human Pathology	2009,40,1296-1303	cyclin D1
(Osunkoya et al. 2009)	Human Pathology	2009,40,206-210	Claudin 7, claudin 8
(Kim et al. 2009)	Histopathology	2009,54,633-635	cytokeratin 7, S100A1 and claudin 8
(Demirovic et al. 2010)	Pathology-Research and Practice	2010,26,695-699	MAGE-A3/4, NY-ESO-1
(Kuroda et al. 2011)	Med Mol Morphol	2011,44,111-115	S100A1
(Carvalho et al. 2011)	Histopathology	2011,58,169-179	CK7, vimentin,S100A1 and C-kit
(Ohe et al. 2012)	Med Mol Morphol	2012,45,98-104	KAI1, epithelial specific antigen, and epithelial related antigen, claudin 7, claudin 8
(Zheng et al. 2013)	Exp and Mol Path	2013 , 94, 29-32	LMP2
(Gaut et al. 2013)	Modern Pathology	2013, 26, 716-724	FXYD2, Ksp-cadherin
(Ehsani et al. 2013)	Tumor Biology	2013, 34,787-791	BCA2
(Jain et al. 2013)	AM J Surg pathol	2013,37,1824-1830	amlyase alpha1A

(Demirovic et al. 2014)	Eur J Histochemistry	2014 58:2265	TGF β1

Ulex europaeus agglutinin-1 (UEA-1), cytokeratin (CK), anti-cytokeratin AE1/AE3 clone antibodies (AE1/3), kidney specific (Ksp), epithelial membrane antigen (EMA), cluster of differentiation (CD), S100 calcium binding protein A1 (S100A1), melanoma-associated antigen A3/4 (MAGE-A3/4), NY-ESO-1 type cancer-testis antigen (NY-ESO-1), proto-oncogene that encodes for a transmembrane tyrosine kinase receptor KIT (C-kit), immunoproteasome LMP2 (LMP2), distal tubule regulator of the trimeric Na+/K+ -transporting ATPase (FXYD2), breast cancer-associated gene 2 (BCA2), transforming growth factor beta1 (TGF β1).

Table 3.2: Rank 2 publications

Author	Journal	Year/Vol/pages	Biomarkers
(Bonsib et al. 1991)	Modern Pathology	1991,4,16-23	CK7, CK18
(Tickoo et al. 1997)	Am J Surg Pathol	1997,21,922-930	antimitochondrial antibody (113-1)
(Liu and Fanning 2001)	Cancer (Cancer Cytopathology)	2001,93,390-397	cytokeratin cocktail (AE1/3, CAM5.2,MNF116), vimentin, Hale colloidal iron
(Rampino et al. 2003)	Am J Surg Pathol	2003,27,779-785	Ron, Ki-67, p53,Bcl-2
(Pan et al. 2004b)	Histopathology	2004,45,452-459	Pan-CK, HMCK,LMCK, CK7, EMA, MOC31, BerEP4, RCCma, CD10, E cadherin, CD15, vimentin
(Skinnider et al. 2005)	Am J Surg Pathol	2005,29,747-754	CK (1,5,5/6, 7,8,10,13,14,17,18,19,20), vimentin
(Liu et al. 2007)	Arch Pathol Lab Med	2007,131,1290-1297	vimentin, glutathione S-transferase α, CD10, CD117, CK7, epithelial cell adhesion molecule
(Shomori et al. 2007)	Modern Pathology	2007,20,199-207	ARPP (ankyrin-repeated protein with a proline rich region), EMA, CD10
(Choi et al. 2007)	J Korean Med Sci	2007,22,305-310	Claudin 7, parvalbumin
(Geramizadeh et al. 2008)	Indian J Pathol Microbiol	2008,51,167-171	Hale colloidal iron, CK7, CK8, CK18, CK19, CK20, vimentin, EMA, CD10,RCC marker
(Rao et al. 2010)	Tumori	2010,96,304-309	Wnt-5a
(Sari et al. 2012)	APMIS	2011,120,187-194	nucleophosmin/B23 (NPM)
(Al-Ahmadie et al. 2011)	Am J Surg Pathol	2011,35,949-961	CAIX, CD117,AMACR, CK7, CD10
(von Brandenstein et al. 2012)	Am J of Pathology	2012,180,1787-1797	protein kinase C α
(Yasir et al. 2012)	Appl Immunohistochem Mol Morphol	2012,20,454-461	CD10, CK7, c-KIT, E-cadherin, N cadherin, Ksp-cadherin, Recepteur d'origine nantais (RON)
(Cui et al. 2012)	European J of Histochemistry	2012,56,245-249	parafibromin
(Hu et al. 2012)	J Clin Pathol	2012,65,254-256	PAX8
(Han et al. 2013)	Annals of Diagnostic	2013, 172-175	HP-1α/β

	Pathology		
(Bing et al. 2013)	Annals of Diagnostic pathology	2013, 58-62	carbonic anhydrase IX, α-methylacyl coenzyme a racemase, cytokeratin 7, and galectin-3
(Patricio et al. 2013)	J of Cellular and Molecular Medicine	2013,8,1048-1058	PAX2

Cytokeratin (CK), anti-cytokeratin AE1/AE3 clone antibodies (AE1/3), Recepteur d'origine nantais (RON), high molecular weight cytokeratin (HMCK), low molecular weight cytokeratin (LMCK), epithelial membrane antigen (EMA), monoclonal antibody that recognises epithelial glycoprotein 2 (MOC31), renal cell carcinoma marker (RCCma),epithelial cadherin (E-cadherin), cluster of differentiation (CD), Ankyrin-repeated protein with a proline-rich region (ARPP), nucleophosmin (NPM), carbonic anhydrase IX (CAIX), alpha-methylacyl-Coenzyme A racemase (AMACR),neural cadherin (N-cadherin), paired box gene (PAX), heterochromatin-associated protein - 1 alpha/beta (HP-1α/β).

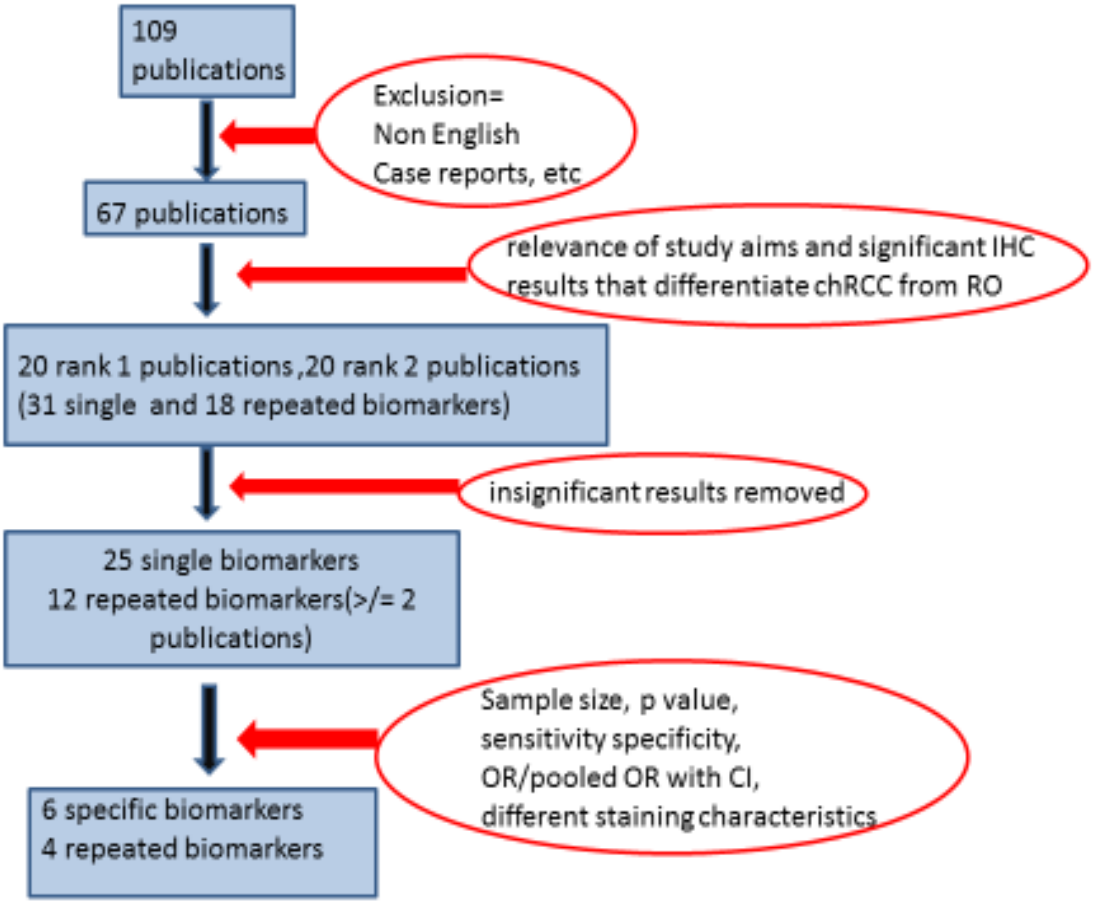

Figure 3.1 Flow chart

Flow chart reveals the algorithm by which publications were selected, excluded and analysed.

Final results showed there were 6 specific and 4 repeated biomarkers.

3.3.2 Quality of the studies

The characteristics of the studies extracted from rank 1 and 2 publications are summarised in Table 3.3. Not surprisingly, there was an increase in the number of studies published in the last two decades in regards to biomarkers differentiating renal tumour subtypes. Twenty-nine of the 40 publications (72.5%) were reported in the last decade. Forty per cent of the publications involved studies investigating 1 IHC biomarker, with 11 studies conducted on 5 or more biomarkers (range of 1–12 biomarkers investigated per study). Eleven out of 40 studies (27.5%) investigated only chRCC and RO tissue samples, whereas most studies assessed biomarkers in a variety of subtypes of renal tumours including chRCC and RO. Across the 40 studies analysed, there were adequately large sample sizes (range 10–321) used in assessing the IHC biomarkers, with 40% of studies having more than 90 samples.

All studies described the IHC staining protocols for the tissue slides or tissue microarrays. The majority of the studies (28/40, 70%) detailed the specific measurements for staining intensities, while 12 studies described only number of positively stained slides from the total number of samples. The number and percentage of positively-stained slides in chRCC and RO were compared in all 40 studies. Twenty-one studies included the statistical p value for the difference in staining between the two entities. Four studies also included sensitivity and specificity of the IHC biomarker and eight studies described differences in staining patterns between chRCC and RO. There were 31 biomarkers (single biomarkers) that were studied only once. Some biomarkers have been repeatedly investigated by various researchers (repeated biomarkers), with CK7 being investigated in 11 previous studies.

Table 3.3: Characteristics of 40 studies ranked 1 and 2

Year	No. of pubs	Biomarkers	No. of pubs	Renal tumours	No. of pubs
1991-1995	1 (2.5%)	1	16 (40%)	only chRCC and RO	11(27.5%)
1996-2000	3 (7.5%)	2	7 (17.5%)	other RCC subtypes	29(72.5%)
2001-2005	7 (17.5%)	3	2 (5%)		
2006-2010	12 (30%)	4	4 (10%)		
2011-2014	17 (42.5%)	>/= 5	11 (27.5%)		

Sample size	no. of pubs	Results	no. of pubs	no. of rpt studies	biomarkers
1-10	1 (2.5%)	% positive slide	40	repeated 11	CK7
11-30	6 (15%)	p value	21	repeated 7	vimentin
31-50	5 (12.5%)	sens/spec	4	repeated 6	CD10
51-70	4 (10%)	stain pattern	8	repeated 5	EMA
71-90	8 (20%)			repeated 4	S100A1, c-KIT
>90	16 (40%)			repeated 3	claudin 7, claudin 8, Ksp-cadherin
				repeated 2	RCC marker, caveolin-1, parvalbumin, antimitochondrial antibody, AMACR, E cadherin, Ron, CAIX
				single study	31

Alpha-methylacyl-CoA racemase (AMACR), carbonic anhydrase IX (CAIX), cytokeratin 7 (CK7), cluster of differentiation 10 (CD 10), epithelial membrane antigen (EMA), Kidney specific (Ksp), Renal cell carcinoma (RCC), Recepteur d'Origine Nantais (Ron), sensitivity (sens), specificity (spec), pubs = publications.

3.3.3 Meta-analysis

The individual and pooled statistical results (p value and OR) of 25 single biomarkers and 12 repeated biomarkers are shown in Tables 3.4 and 3.5. The OR is calculated as the odds of chRCC staining with the particular biomarker as compared with RO. OR <1 means that RO will stain more than chRCC counterparts. Some OR will be equal to zero or infinity because the mathematical calculation and their variance cannot be calculated to reach a finite ratio number. However, the relevant studies with OR=0 will be discussed in depth and their significant results described.

As seen in Table 3.4, the results of the 25 single biomarkers are significant. The differentiation ability of these biomarkers is reflected by their statistical results of p value <0.05 obtained from the studies and the calculated significant OR with 95% CI. The single IHC biomarkers are Amylase α1A, Wnt 5a, FXYD2, Ankyrin-repeated protein with a proline-rich region (ARPP) which displayed significant calculated OR and CI; while cluster of differentiation 63 (CD63), and TGFβ1 were chosen for their differential staining patterns rather than calculated ORs which were zero (**bold** in Table 3.4).

3.3.4 Amylase α1A

In the study by Jain *et al* (Jain et al. 2013), amylase α1A, a salivary type amylase enzyme, was investigated. A large sample size (chRCC = 54, RO = 75) was obtained from whole slides and tissue microarray (TMA) cores. While 87% of chRCC cases were negative for the immunostain, all RO were positive for amylase α1A ($p<0.001$). The sensitivity and specificity for distinguishing RO from chRCC was 100% and 90.74%, respectively. The

computed OR (for chRCC compared to RO) was 0.001 (95% CI 0.0001-0.019). Conversely, the odds ratio of RO being stained with amylase α1A when compared to chRCC was 1000.

3.3.5 Wnt-5a

Wnt-5a, a member of the Wnt family of proteins, acts as a tumour suppressor or a tumour stimulator depending on tumour types in various non-renal tumours (Kremenevskaja et al. 2005; Kurayoshi et al. 2006). Wnt-5a was investigated in a study of 18 chRCC and 20 RO (Rao et al. 2010). Wnt-5a was expressed only in 16% of chRCC compared to expression in all oncocytomas ($p<0.01$). The calculated OR for chRCC compared to RO for Wnt-5a staining was 0.0076 (95% CI 0.0004 - 0.15). Therefore, the OR for Wnt-5a immunostaining for RO compared to chRCC was 131.

3.3.6 FXYD2

FXYD2, a distal tubule regulator of the trimeric Na^+/K^+ -transporting ATPase (Gaut et al. 2013), was expressed in 96% (26/27) of chRCC whereas this biomarker expression was absent in 83% (25/30) of ROs. In this series, the sensitivity for differentiating chRCC from RO was 97% and specificity was 83%. The OR calculated was 130 (95% CI 14.2 – 1192.3).

3.3.7 Ankyrin-repeated protein with a proline-rich region (ARPP)

ARPP, highly expressed in skeletal and cardiac muscles, was originally discovered in oesophageal carcinoma cells (Moriyama et al. 2001) and was investigated by Shomori *et al* as a biomarker to distinguish RO and chRCC (Shomori et al. 2007). From the analysis, all chRCC failed to express ARPP while 12/14 (85.7%), while RO had high expression of

ARPP. The computed OR for chRCC compared to RO in ARPP IHC reactivity was 0.0054 (95% CI 0.002 – 0.12). In other words, RO will express ARPP more than chRCC by an OR of 200.

3.3.8 Cluster of differentiation 63 (CD63)

CD63 is a lysosomal membrane glycoprotein translocated to the plasma membrane after platelet activation (Rous et al. 2002). Mete *et al* investigated the IHC expression of CD63 in renal tumours of epithelial origin with eosinophilic cytoplasm, especially RO from eosinophilic chRCC (Mete et al. 2005). In that study, 94% of RO had apical and/or polar CD63 immunostaining compared to 96% of chRCC with diffuse staining ($p< 0.0005$). The differential staining patterns of CD63 in RO versus chRCC had 95% sensitivity and 100% specificity recorded by the authors. OR calculated in this case was zero because the chRCC and RO had similar numbers of slides with positive immunostaining. Nevertheless, the distinction here is the biological nature of staining pattern difference rather than intensity or positivity of staining.

3.3.9 Transforming growth factor beta 1 (TGFβ1)

TGFβ1 is a multi-potent cytokine involved in regulating a number of cellular processes. Demirovic *et al* studied the expression of TGFβ1 in 18 RO and 16 chRCC (Demirovic et al. 2014). In that study, the intensity of TGFβ1 expression in chRCC was weaker compared to RO ($p<0.05$), with chRCC predominantly staining in a membranous pattern while RO had predominantly cytoplasmic staining ($p<0.05$). There was no conclusive OR as it equalled zero as the chRCC and RO slides had similar number of positive stained slides. Again the

discriminatory ability of TGFβ1 is the differential staining pattern between the two tumour subtypes, displaying its biological distinctive staining nature.

Table 3.4: Results of 25 single IHC biomarkers

Biomarker n(chRCC)/n(RO)	**p value/ sens/spec% (obtained from studies)**	**Calculated OR (chRCC >RO)**	**Calculated 95%CI**
amylase alpha1A 54 / 75	**p<0.001; sens 100% spec 90.74%**	**0.001 (RO>chRCC 1000)**	**0.0001-0.019**
Wnt-5a 18 / 20	**p<0.01**	**0.0076 (RO>chRCC 131)**	**0.0004-0.15**
FXYD2 27 / 30	**sens 97%; spec 83%,**	**130**	**14.2 - 1192.3**
ARPP 21 / 14	**0**	**0.0054 (RO>chRCC 200)**	**0.0002-0.12**
TGF β1 16 / 18	**p<0.05**	**0 Different stain pattern**	**-**
CD63 27 / 35	**P=0.0005**	**0 Different stain pattern**	**-**
Bcl2 5 / 18	-	0.0007	0-0.39
MAGE-A3/4 18 / 17	p 0.0013	0.0848485	0.01-0.49
Parafibromin 22 / 19	p=0.00198	0.03	0.0016-057
Ki 18 / 18	p<0.005	0	-
PAX2 30 / 30	p<0.01; sens 67% spec 90%	0.0555556	0.01-0.23
KAI1 20 / 10	P<0.001	81	6.45-1017.14
p53 18 / 18	p<0.05	0	-
protein kinase C α 18 / 5	p<0.05	0.0025	0-0.14
NY-ESO-1 18 / 17	p0.0008	0.0666667	0.01-0.39
ESA 20 / 10	p<0.001	11	9.57-3055.52
BCA2 8 / 38	-	0.0008	0-0.41
HP-1α/β 20 / 20	-	0.1428571	0.04-0.57

ERA 20 / 10	p<0.001	11	9.57-3055.52
Lysozyme 21 / 103	-	3.72	1.18-11.74
EpCAM 22 / 17	-	102.27	5.21-2006.44
nucleophosminB23 18 / 9	p<0.001	0	-
MOC 31 28 / 7	-	64.09	3.16-1299.57
CD15 28 / 7	-	0.09	0.01-0.61
PAX8 66 / 16	-	0.2717949	0.03-2.25

Ankyrin-repeated protein with a proline-rich region (ARPP), Transforming growth factor beta 1 (TGF β1), cluster of differentiation (CD), melanoma-associated antigen A3/4 (MAGE A3/4), paired box gene (PAX), tumour metastasis suppressor gene (KAI1), epithelial specific antigen (ESA), breast cancer-associated gene 2 (BCA2), heterochromatin-associated protein - 1 alpha/beta (HP-1α/β), epithelial-related antigen (ERA), epithelial cell adhesion molecule (EpCAM), monoclonal antibody that recognises epithelial glycoprotein2 (MOC 31); number (n).

3.3.10 Cytokeratin 7 (CK7)

CK7 is a low molecular weight keratin expressed in various epithelia and related neoplasms. Eleven relevant studies that investigated CK7 were analysed with the individual and pooled OR results shown graphically in the Forest plot in Figure 3.2 (Adley et al. 2006a; Al-Ahmadie et al. 2011; Carvalho et al. 2011; Geramizadeh et al. 2008; Kim et al. 2009; Kuroda et al. 2004; Leroy et al. 2000; Liu et al. 2007; Mazal et al. 2005; Skinnider et al. 2005; Yasir et al. 2012). In combining those studies, there were 448 samples (chRCC 242, RO 206) with a calculated pooled OR (chRCC compared to RO) of 44.22 (95% CI 22.52 – 86.64).The derived I^2 percentage from Q test of heterogeneity I^2=15%, suggested that there was no significant level of heterogeneity amongst the studies.

3.3.11 S100A1

There were 4 studies that evaluated the IHC of S100A1 in renal tumours. S100 proteins are involved in different biological activities such as transduction of intracellular calcium signalling, cytoskeleton-mediated interactions, cell cycle progression and cell differentiation (Li et al. 2007). Individual and pooled OR results are shown in a Forest plot in Figure 3.3. (Carvalho et al. 2011; Kim et al. 2009; Kuroda et al. 2011; Li et al. 2007) The pooled OR (chRCC compared to RO) was 0.01 (95% CI 0-0.03), with I^2 = 0%. Therefore, the OR of RO compared to chRCC for S100A1 staining is 100 and I^2 study heterogeneity revealed similar consistency between the effects of the 4 studies.

3.3.12 Caveolin-1 (Cav-1)

Cav-1 is a membrane protein present in most cells. Two previous studies of Cav-1 are included in this analysis. In the study by Garcia *et al*, all 21 (100%) of chRCC were stained positively for Cav-1 compared to 88% (23/26) of RO which were negative (Garcia and Li 2006). The OR from that study was 265.66 (95% CI 7.79-9061.79). Another study produced positive immunostains in all 27 chRCC and 34 out of 35 of RO. The OR calculated therefore was 2.85 but the 95% CI (0.1-85.04) which included 1. However in that study, the authors showed that there was significantly-different staining patterns in chRCC (diffuse peripheral) and RO (diffuse) (p0.0005) (Mete et al. 2005). The pooled OR calculated was 32.95 (95% CI 3.67-296.1) with I^2 = 70%, suggesting heterogeneity between the two study results.

3.3.13 Claudin-7

Claudin-7 together with claudin-8 code for tight junction proteins that are expressed in distal nephrons. (Li et al. 2004) There were 3 studies that investigated claudin-7 in renal tumours. The individual and pooled OR results are shown in Figure 3.4 (Choi et al. 2007; Ohe et al. 2012; Osunkoya et al. 2009). The pooled OR was 24.7 (95% CI 6.28-97.1) with I^2 = 0%, displaying no significant heterogeneity between the 3 study results.

Similarly, repeated biomarkers that were investigated in various studies have their results documented with calculated pooled OR with 95% CI and Q test of heterogeneity with I^2 % described. The summary of repeated IHC biomarkers are cytokeratin 7 (CK7), S100A1, caveolin-1 and claudin-7 are shown in **bold** in Table 3.5.

Table 3.5: Twelve repeated IHC biomarkers

Repeated biomarkers	no. of pubs	pooled OR (chRCC>RO)	95% CI	I^2 (%)
cytokeratin 7	**11**	**44.22**	**22.52 - 86.64**	**15**
S100A1	**4**	**0.01 (RO>ch 100)**	**0 - 0.03**	**0**
caveolin-1	**2**	**32.95**	**3.67-296.10**	**70**
Claudin7	**3**	**24.7**	**6.28-97.1**	**0**
Ksp-cadherin	3	4.43	2 -9.79	90.7
claudin8	4	0.63	0.36-1.09	91.7
anti-mitochondrial	2	0.11	0.01-2.23	NA
EMA	5	1.87	0.89-3.96	81.7
Ron	2	0.04	0.01-0.14	81.4
Carbonic anhydrase IX	2	4.25	0.32-55.82	0
CD10	3	10.46	1.24-88.04	0
Hale colloidal iron	3	3.87	1.87-8	93.7

Cluster of differentiation 10 (CD10), epithelial membrane antigen (EMA), kidney specific (Ksp), epithelial membrane antigen (EMA), Recepteur d'Origine Nantais (Ron).

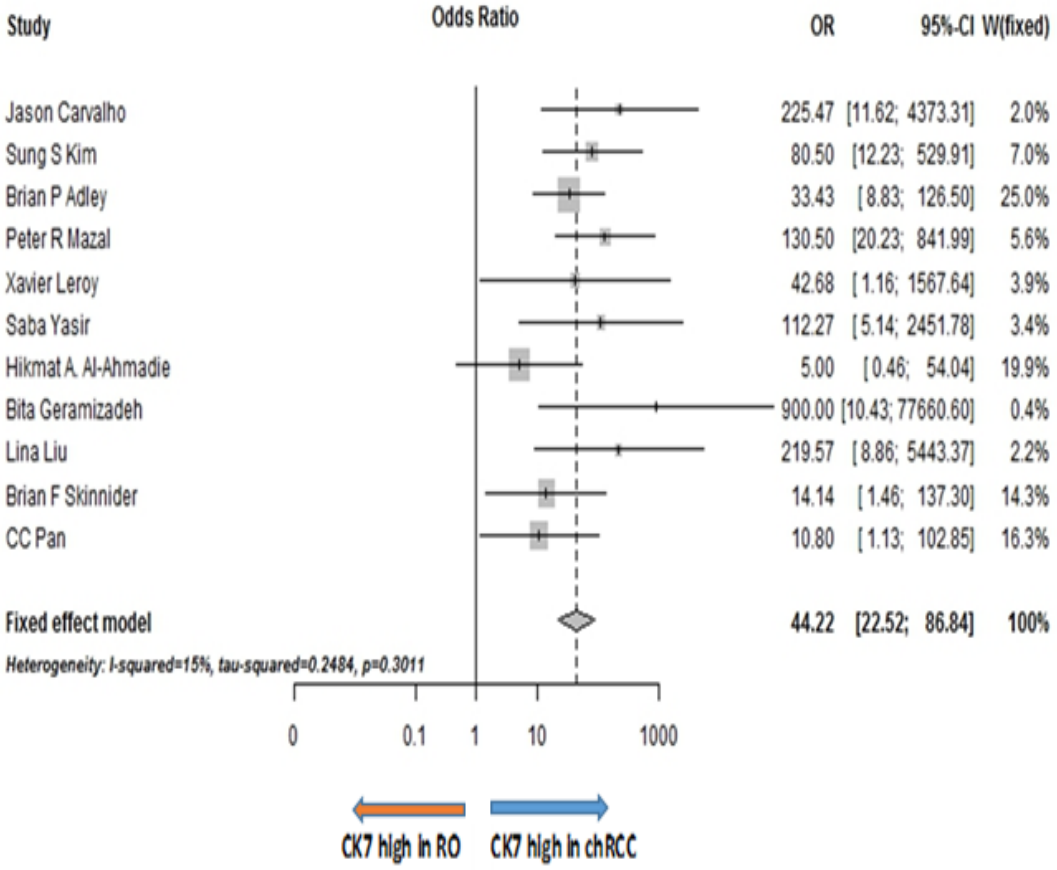

Figure 3.2: Forest plot for CK7

Forest plot of CK7 showing the respective OR and CI together with the pooled OR and pooled CI. Noted that all studies shown here revealed higher CK7 expression in chRCC.

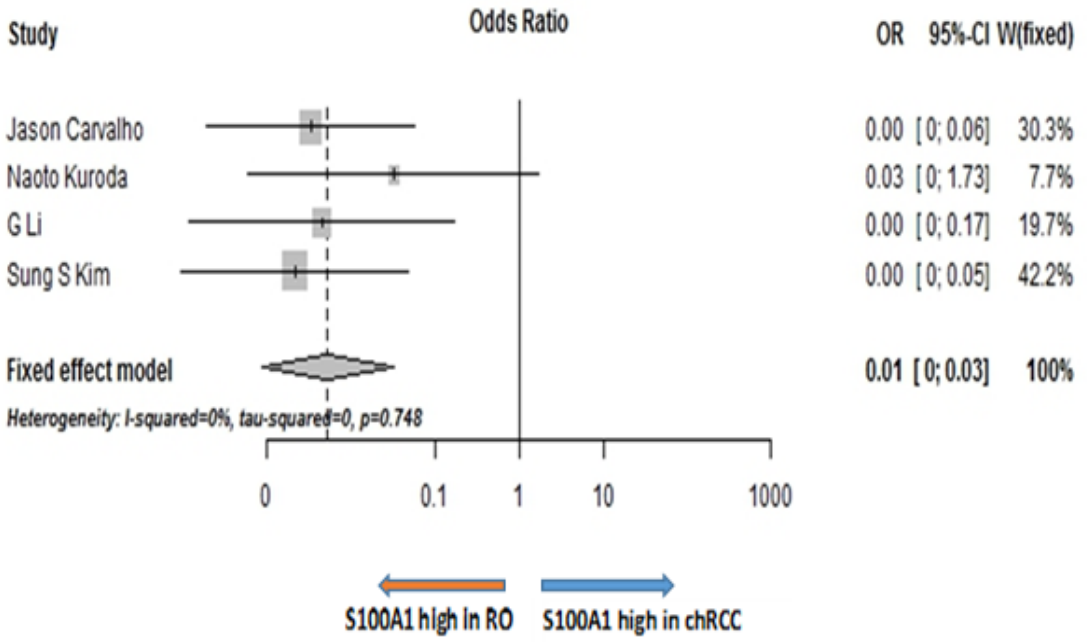

Figure 3.3: Forest plot for S100A1

Forest plot of S100A1 showing the respective OR and CI together with the pooled OR and pooled CI. Noted that all studies shown here revealed higher S100A1 expression in RO.

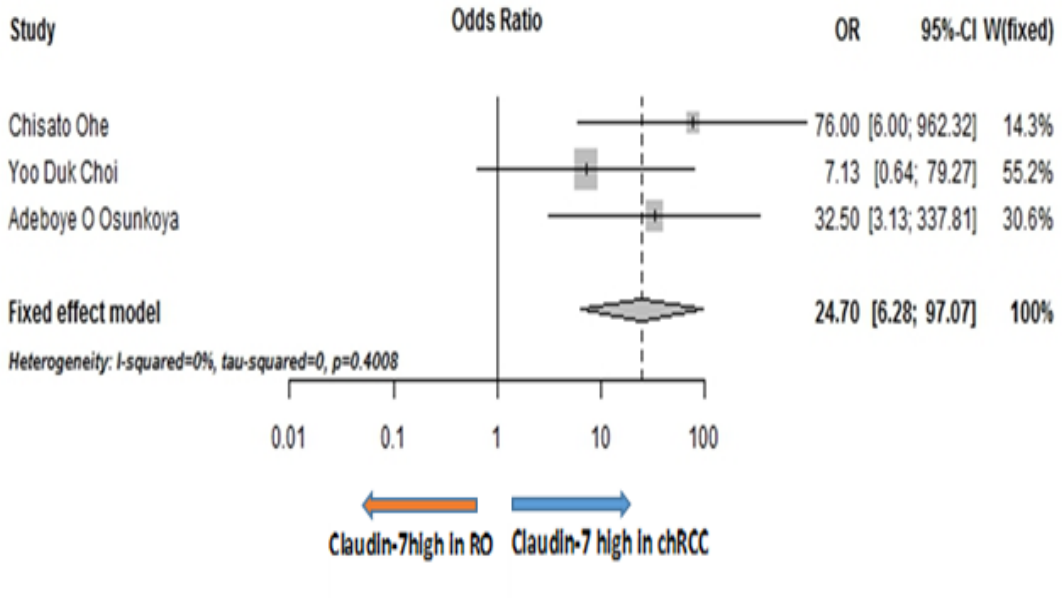

Figure 3.4: Forest plot for Claudin-7

Forest plot of claudin-7 showing the respective OR and CI together with the pooled OR and pooled CI. Noted that all studies shown here revealed higher claudin-7 expression in chRCC.

3.4 DISCUSSION

It is worthwhile mentioning from the outset that the gold standard for differentiating chRCC from RO remains careful discernment of histopathological features on H&E staining. In fact, all studies in this meta-analysis which investigated IHC biomarkers were performed on slides or microarrays of known tumour tissue subtype based on H&E. Nevertheless, IHC is still beneficial and relevant in cases where differentiation of chRCC from RO is too difficult on routine H&E.

From this analysis, there are already numerous IHC biomarkers that have been used routinely across laboratories, which can aid in the differentiation between chRCC and RO. Often some of the results of trialled and novel IHC biomarkers are not consistent or reproducible across laboratories. The meta-analysis indicates that it is unlikely that a single molecule can consistently differentiate the two entities. It is more likely a panel of biomarkers with differential expression would be required to assist in the differentiation. Consequently, we identified 10 biomarkers that are differentially expressed in these two entities: 6 specific biomarkers (amylase α1A, Wnt-5a, FXYD2, ARPP, CD63, TGFβ1); and 4 repeated biomarkers (CK7, S100A1, Cav-1, claudin-7), as outlined in Table 3.6.

Seventeen of the 40 studies (42.5%) were conducted in the last 5 years (Table 3.3). This may be because clinicians are increasingly faced with the diagnostic dilemma of differentiating the two entities and therefore more research has been generated to resolve this difficult distinction. There were also various biomarkers (CK7, vimentin, CD10, EMA, S100A1, c-KIT, claudin 7 and 8, Ksp-cadherin, RCC marker, Cav-1, parvalbumin, antimitochondrial antibody, AMACR, E-cadherin, Ron and carbonic anhydrase IX) that have been repeatedly

investigated in different studies. This is not surprising as some of these biomarkers have been implicated or involved in important cellular mechanisms pertaining to cell growth, apoptotic pathways and tumorigenesis. However, from our results of studies involving repeated biomarkers, only CK7, S100A1, Cav-1, claudin-7 have been shown to be consistently useful in this differentiation. These repeated biomarkers are crucial in this analysis as they provide us the ability to compare results between studies of the same biomarker and assess their consistency, reproducibility and heterogeneity.

CK7 was involved in 11 previous studies in this analysis, with all studies in the Forest plot showing similar trend of OR for CK7 staining when chRCC was compared to RO (Figure 3.2). This suggests that CK7 is a reliable biomarker that numerous researchers have relied upon to differentiate the two tumour entities. Cytokeratins are a large family of structural polypeptides that are the fundamental markers of epithelial differentiation. They consist of at least 20 distinct molecules, the expression of which depends on cell type and differentiation status, making them useful in differential diagnosis of many epithelial tumours (Chu and Weiss 2002). The CKs found in simple epithelia (CK7, CK8, CK18, and CK19) were widely expressed in normal kidney and renal neoplasms (Skinnider et al. 2005).

In this meta-analysis, the calculated pooled ratio was 44.22 (95% CI 22.52-86.64) which showed that chRCC are 44 times more likely to express CK7 when compared to RO. All studies revealed that chRCC were more likely to express CK7 than RO with individual ORs ranging from 5 to 900. The large combined sample size of 448 chRCC and RO only strengthened this analysis and proved the usefulness of CK7. In fact, the low level of heterogeneity of I^2 = 15%, suggests that all 11 studies consistently provided similar reliable

reproducible results in chRCC and RO. From Figure 3.2, all studies recorded an OR of >1 in 95% CI. Only one study had 95% CI that included 1 (0.46-54.04) in which the OR was 5 (Al-Ahmadie et al. 2011). However in that study, the small sample size of 5 RO, where 4 out of 5 RO showed negative expression of CK7, would have influenced the result. Therefore, despite that study, all the other 10 studies showed that chRCC had significantly higher expression of CK7 when compared to RO.

Another biomarker that was investigated in 4 different studies was S100A1. S100A1 is a member of the S100 family of calcium binding molecules, most of which are clustered on chromosome 1q21, and expressed in RCC (Teratani et al. 2002). Importantly, these proteins are involved in cell cycle progression and cell differentiation (Li et al. 2007) and therefore implicated in tumorigenesis, a basis for investigation of S100A1 in renal tumour subtypes. The calculated pooled OR (chRCC vs RO) was 0.01, with $I^2 = 0\%$ (Figure 3.8). In other words, RO will express S100A1 biomarker a hundred fold more than chRCC. As per the Forest plot in Figure 3.3, RO showed higher expression of S100A1 compared to chRCC. Even with a fairly large sample of 124 (chRCC 59, RO 65) from the combined 4 studies, there is hardly any heterogeneity detected amongst all studies. In one study, the OR 0.03 with 95% CI 0 – 1.73 which included 1 therefore rendering the OR insignificant (Kuroda et al. 2011). However that study analysed only 4 RO and 10 chRCC and therefore, the weightage of that study only contributed 7.7% for the pooled OR analysis. When all data were combined, 61/65 (93.8%) of RO expressed S100A1 immunoreactivity compared to only 4/59 (6.8%) of chRCC. Therefore, S100A1 is another reproducible IHC biomarker that can differentiate RO from chRCC.

Another repeated biomarker that was selected was claudin-7. Claudin-7 belongs to a family of tight junction proteins and is expressed in the cell membrane of distal tubules and collecting ducts. The tight junction structure is important for restricting lateral effusion of lipids and membrane proteins, thereby physically defining the border between the apical and basolateral components of the cell (Choi et al. 2007). The tissue specificity of claudins strongly suggests that they may have other functions, in addition to being structural components of tight junctions (Zheng et al. 2003). The results of the 3 studies that were analysed for claudin-7 are shown in Figure 3.9 (Choi et al. 2007; Ohe et al. 2012; Osunkoya et al. 2009). The pooled OR was 24.7 (chRCC compared to RO) with 95% CI (6.28-97.1) and I^2 = 0% reflecting no significant heterogeneity between the results of all 3 studies. The combined sample size was 89 (51 chRCC, 38 RO). The Forest plot (Figure 3.4) revealed all studies showing that chRCC have higher expression of claudin-7 compared to RO. Therefore chRCC is 24 times more likely to express claudin-7 than RO and thus can aid in the IHC differentiation of chRCC from RO.

Cav-1, a 24-kd membrane protein, is a major component of membrane caveolae. Functionally, Cav-1 serves important roles in macromolecular transcytosis, endocytosis of pathogens, lipid metabolism, and cellular signal transduction (Cohen et al. 2004). In two studies that investigated Cav-1, all the chRCC stained positively for Cav-1 (Garcia and Li 2006; Mete et al. 2005). The calculated pooled OR was 32.95 (95% CI 3.67-296.1). In contrast to the 100% immunostaining of chRCC, 88% of RO failed to express Cav-1 (Garcia and Li 2006). In the other study by Mete *et al*, 34/35 RO also expressed Cav-1 (compared to 27/27 of chRCC), but chRCC had diffuse and peripheral Cav-1 staining while 88% of RO had diffuse cytoplasmic staining (p=0.0005) (Mete et al. 2005). However, as a reflection of differing results between the two studies, I^2 was moderate at 70%. Therefore the pooled OR

ratio should be interpreted cautiously as there is significant heterogeneity. Nevertheless, both studies portrayed different strengths of results in quantitative and qualitative aspects (which accounted for the high I^2), and so Cav-1 is considered to be a useful marker for differentiating chRCC from RO.

Of the 6 specific biomarkers selected (amylase α1A, Wnt-5a, FXYD2, ARPP, CD63, TGF-β1), amylase α1A, FXYD2, Wnt-5a and TGFβ1 were investigated in the last 5 years. Perhaps one reason for this discovery of novel biomarkers stems from better understanding of the molecular basis of renal tumour profiling, coupled with newer technologies that are available. As can be seen in Table 3.4, RO significantly express amylase α1A, wnt-5a and ARPP more, when compared to chRCC, with ORs (RO compared to chRCC) of 1000, 131 and 200, respectively. In contrast, chRCC had higher expression of FXYD2 when compared to RO (OR of 130). The 95% CIs of these 4 studies were significant and valid as none included the value of 1.These significant results prove that these biomarkers are useful in distinguishing between chRCC and RO in quantitative IHC. In both CD63 and TGFβ1, there were significantly-different staining patterns between chRCC and RO. The sample sizes of chRCC and RO involved in these 6 studies of specific single biomarkers are relatively large with a range from 32 – 129. Obviously these large sizes add power to the studies and provide further strength to their results.

The significance and functions of these 6 single biomarkers would have led the investigators to study their expressions in renal tumour subtypes. In the case of amylase α1A, chRCC have deletions in the 1p21.1 region including the amylase α1A gene, with no such deletions in oncocytomas. The AMY1A gene encodes the salivary gland–type amylase isoenzyme that

hydrolyzes the 1,4-a-glucoside bonds in oligosaccharides and polysaccharides to produce maltose, which is cleaved to 2 molecules of glucose by the enzyme maltase (Jain et al. 2013). For the biomarker Wnt-5a, it belongs to a large family of cysteine-rich secreted molecules that play diverse biological roles in the regulation of several normal and pathological processes including cell growth, differentiation and tumorigenesis (Kurayoshi et al. 2006).

In regards to FXYD2, the protein is a single pass type III membrane protein and the gamma subunit of trimeric Na^+/K^+-transporting ATPase whose regulatory function is to modulate the kinetic properties and stabilize the renal tubular Na+/K+-transporting ATPase. Previous studies have shown that the FXYD2 protein is highly enriched in kidney tissue (Floyd et al. 2010; Geering 2006). Whereas in the investigation of ARPP, it is characterized by the presence of four ankyrin-like repeat motifs in its middle portion and proline (P), aspartic and glutamic acids (E), serine (S) and threonine (T) (PEST-like) sequences in the amino-terminal regions. ARPP is implicated in the regulation of protein turnover (Shomori et al. 2007). Lastly, TGF-β1 is a potent, pleiotropic cytokine involved in regulating a number of cellular processes including proliferation, differentiation, apoptosis, development, tissue repair, cell motility, extracellular matrix formation, inflammation, immunosuppression, and tumorigenesis (Demirovic et al. 2014).

Consequently from this study, 10 biomarkers were identified with their results of IHC staining summarised in Table 3.10. Typically chRCC shows positive expression for CK7, FXYD2, Cav-1, claudin-7 and diffuse CD63 and membranous TGFβ1 staining patterns. In contrast, RO will usually express positivity for amylase α1A, Wnt-5a, ARPP, S100A1 and apical/polar CD63 and cytoplasmic TGF-β1 staining patterns. Obviously, this positivity and

negativity staining expression holds true in most instances and there will be inevitably small samples that do not conform to these results. However, the specificity and accuracy of differentiation will be enhanced if two or more of these biomarkers were utilised in difficult situations of equivocal staining results. It is recommended that laboratories utilise a few of these biomarkers in future to distinguish difficult cases of chRCC from RO.

There are notable strengths and limitations in this meta-analysis. To our knowledge, our study is the first to report a meta-analysis and systematic review in this highly specialised yet clinically challenging field of the IHC differentiation between chRCC and RO. From the diverse unbiased survey of the current available literature on differentiation of chRCC and RO, with standardised approach in meta-analysis methods, we were able to summarise the robust data extracted, and select out the panel of most relevant IHC biomarkers. This will in turn provide clinicians with the most sensitive IHC biomarkers that can distinguish the two entities and guide further management.

There are several limitations in our study. The small number of studies, which investigated specific biomarkers, except for the 11 studies of CK7, may not produce robust available data for analysis. However, this is because of the paucity of successful usage of available IHC biomarkers in the differentiation. With evaluation of studies pertaining to only IHC method of biomarkers in the differentiation, some studies which report significant results from combination of IHC with another different technique (such as *in situ* RNA hybridisation) might not have been analysed appropriately. In the present analysis, an aspect of publication bias was unavoidable as we only included published studies and did not search unpublished studies or abstracts due to our methodology strategy. Another potential source of bias is that

there was no uniformity in the measurement of the IHC expression in the samples and inter-observer variability in interpretation of positivity across all studies.

In summary, our findings show that these 10 biomarkers have demonstrated their ability to differentiate chRCC and RO. It is hoped that larger scale studies will be performed on these 10 biomarkers in the future to further consolidate or affirm the reproducibility of similar results in differentiation of RO from chRCC. Hopefully, there will also be further novel biomarkers investigated in this respect.

3.5 CONCLUSION

The clinical diagnostic dilemma and difficult histopathological differentiation of RO from chRCC still persist. This systematic review and meta–analysis have revealed numerous IHC biomarkers that have been investigated and regularly used across laboratories to aid differentiating chRCC and RO. Despite this, there are no consistently-reproducible robust IHC biomarker(s) that can accurately differentiate the two tumour entities. However, this analysis has provided us with a panel of the most relevant IHC biomarkers that may help to discriminate the two entities. Following this meta-analysis, we set out to replicate and validate the differential ability of certain IHC biomarkers (CK7, Cav-1 and S100A1) on our Australian cohort of patients in our laboratory (discussed in chapter 5). Further large international collaborative studies are needed to further validate the clinical usefulness of these biomarkers. Currently, when faced with difficult histopathological distinction of chRCC and ROs, we suggest the application of IHC of some biomarkers from the panel presented in Table 3.6.

Table 3.6:- Final panel of discriminatory IHC biomarkers

Biomarker	ChRCC	RO
amylase α1A	-	+
Wnt-5a	-	+
FXYD2	+	-
ARPP	-	+
CD63	Diffuse	Apical/polar
TGFβ1	Membranous	Cytoplasmic
CK7	+	-
S100A1	-	+
Caveolin-1	+ (diffuse peripheral)	- (diffuse if +)
Claudin-7	+	-

+ = majority positive; - = majority negative

Ankyrin-repeated protein with a proline-rich region (ARPP), cluster of differentiation 63 (CD 63), transforming growth factor beta1 (TGFβ1).

CHAPTER 4

IMMUNOHISTOCHEMICAL ANALYSIS OF NUCLEAR FACTOR –KAPPA B

IN RENAL TUMOURS

CHAPTER 4

IMMUNOHISTOCHEMICAL ANALYSIS OF NUCLEAR FACTOR –KAPPA B IN RENAL TUMOURS

As mentioned in the first chapter, better understanding of the molecular profiling of renal tumours will ultimately translate into better diagnostic, therapeutic and prognostic management pathways. Discovery of newer and novel biomarkers will add to further delineation of molecular signatures of renal tumours. The objective of this chapter was to achieve the 2nd aim of the research: assessment of the different molecular profiles of renal cancers via IHC and morphometry techniques using selected biomarkers on renal tumour and normal tissue samples. Therefore in this chapter, nuclear factor kappa B (NF-κB) was investigated in RCC disease. Furthermore, the expressions of the five NF-κB subunits have not been investigated in such an extensive set of patients with RCC and this study provided the opportunity to carefully compare expressions and localisations in different subtypes of RCC (including chRCC). From our close collaboration with Surgery Department of University Malaya, we had the opportunity to assess the expression of NF-κB in human RCC tissue via IHC analyses. The IHC research work was performed here in CKDR, TRI with the help of Mr. Clay Winterford from QIMR. This is the first and largest study to report on the IHC expressions of NF-κB subunits (p65, p50, p52, cRel) in human RCC tissue.

4.1 INTRODUCTION

Among the biomarkers of interest implicated in RCC are the NF-κB subunits. The NF-κB family of proteins comprises p65 (RelA), NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelB and

c-Rel. The NF-κB subunits may form homo or heterodimers in the cell. They act as transcription factors regulating inflammation, angiogenesis, immunity, cell proliferation and apoptosis (Morais et al. 2011). NF-κB is present in the cytoplasm in an inactive form, bound to one of the IκB inhibitor molecules, such as IκB-α, IκB-β, IκB-γ, IκB-ε, p100, p102 or Bcl-3 (Morais et al. 2011). NF-κB is then released into the nucleus in its active form to activate its transcriptional target when IκB is degraded by IκB kinase (IKK).

The p65 subunit is the most studied NF-κB subunit in cancers, including RCC. Studies showed that p65 expression is upregulated and constitutively activated in RCC tissue (Djordjevic et al. 2008; Peri et al. 2013). NF-κB p65 and p50 have both been correlated with apoptotic and proliferation markers in RCC tissue (Kankaya et al. 2015). Activation of NF-κB induces anti-apoptotic factors such as inhibitors of apoptosis (IAPs) or anti-apoptotic members of the Bcl-2 family (Chen et al. 2000; Dolcet et al. 2005). NF-κB activity is enhanced in the absence of functional pVHL and furthermore, expression of NF-κB p65 is associated with vascular endothelial growth factor (VEGF) in ccRCC, the most common type of the RCC (Djordjevic et al. 2008; Meteoglu et al. 2008; Qi and Ohh 2003). Hence, the NF-κB family likely plays an important role in the carcinogenesis and progression of RCC. However, the prognostic implications of NF-κB in RCC are contradictory, based on previous research where NF-κB was associated with poorer prognosis (Oya et al. 2003; Peri et al. 2013; Djordjevic et al. 2008) while others did not correlate NF-κB with survival or prognosis (Kankaya et al. 2015; Meteoglu et al. 2008;; Sourbier et al. 2007). Currently to our knowledge, there is no published literature on NF-κB2 (p100/p52), RelB and c-Rel expression in human RCC and their prognostic value. Additionally, most studies did not assess the survival outcome of RCC patients in relation to NF-κB expression.

The aim of the current study was to report the expression of p65 (RelA), NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelB and c-Rel in RCC tissue in comparison with the corresponding normal kidney. The association of the NF-κB subunits with the tumour characteristics and survival outcome was also evaluated.

4.2 MATERIALS AND METHODS

Ethics approval for retrospective and prospective tissue collection was obtained from the University Malaya Ethics Committee (Ref: 848.17) (Appendix 4). The present study examined 96 cases of formalin fixed and blocked RCC tissue with paired normal kidney from patients who have undergone nephrectomy in University of Malaya Medical Centre (UMMC) from 2003-2013. The clinicopathological data and demographics of patients were collected from UMMC online database or patients' medical record folders. Survival information was acquired from patients' medical records or the National Registration Department, Malaysia. The discussion of the IHC methodology and description of the morphometry analyses with statistical analysis have been detailed in chapter 2.

4.3 RESULTS

Table 4.1 shows the clinical and pathological data from 96 renal tumour archived specimens. Two thirds of patients were males who underwent nephrectomy in the UMMC between 2003 till 2013 (male:female = 2:1). Median age was 62 (range 39-83 years) with median renal tumour size of 6 cm (1.5 – 17 cm). Approximately half the patients presented with clinical stage T1, with 22 patients (22.9%) having metastases at presentation. As expected, the majority of the tumour pathological subtype was ccRCC (n=76), followed by papillary RCC (n=11), chRCC (n=5), clear cell tubulopapillary RCC (n=3) and one multilocular cystic RCC.

Median follow up for this cohort of RCC patients was 54.5 months (0.2 – 135 months), with 28 deaths noted at time of analysis.

Table 4.1: Clinical and pathological data

Patients	96			
M : F	67.7% : 32.3%			
Median age	62 (39-83)			
Median size	6cm (1.5-17)			
Clear cell RCC (stage)	43 (T1)	22 (T2)	9 (T3)	2 (T4)
Clear cell RCC (grade)	10 (G1)	37 (G2)	22 (G3)	7 (G4)
Papillary RCC (stage)	5 (T1)	3 (T2)	2 (T3)	1 (T4)
Papillary RCC (grade)	0 (G1)	7 (G2)	4 (G3)	0 (G4)
Chromophobe RCC (stage)	3 (T1)	0 (T2)	2 (T3)	0 (T4)
Multilocular cystic RCC (stage)	1 (T1)	0 (T2)	0 (T3)	0 (T4)
Clear cell tubulopapillary RCC (stage)	1 (T1)	1 (T2)	1 (T3)	0 (T4)
Stage T1	53 (55.2%)			
T2	26 (27.1%)			
T3	14 (14.6%)			
T4	3 (3.1%)			
Metastases (M1)	22 (22.9%)			

Renal cell carcinoma (RCC); Tumour (T); Metastases (M)

The IHC staining characteristics and morphometric results of 4 NF-κB subunits (p50, p52, p65 and c-Rel) are shown in Figures 4.1 - 4.16. Based on the immunostaining patterns of the tumour and normal cells, overall, nuclear and membrane morphometric expression analyses were performed with Aperio ImageScope. Despite the positive control slides (human tonsil) showing staining for the fifth NF-κB subunit: RelB; normal kidney and RCC tissue had minimal RelB immunostaining. Therefore these could not be analysed for positive pixels using morphometry, and consequently results for RelB are not reported. Cancer survival analyses were performed for high and low overall and nuclear NF-κB subunits expressions and results portrayed as Kaplan Meier curves; except for membrane expressions as there were no significant differences between tumour expression results.

4.3.1 NF-κB p65 subunit analysis

IHC staining characteristics of p65 subunit in normal and renal tumour tissues are shown in Figure 4.1. The immunostaining pattern was mainly cytoplasmic and nuclear. Overall positive pixel, nuclear and membrane expression of p65 were significantly higher in RCC compared to normal renal tissue ($p < 0.0001$, $p = 0.0015$ and $p < 0.0001$ respectively) (Figure 4.2A, 4.3A and 4.4A).

For overall, nuclear and membrane immunostaining p65 expression of renal tumour subtypes, chRCC recorded the least intensity ($p=0.004$, $p=0.02$, $p=0.004$ respectively) (Figures 4.2D, 4.3D and 4.4D). There was no significant correlation of overall or nuclear or membrane expression of tumours in regards to clinical T staging, tumour grading or metastases.

With regards to cancer-specific survival analysis, high p65 nuclear expression (higher than median cut-off value) was significantly associated with worse survival outcome ($p = 0.03$) (Figure 4.3F). Using Cox regression analysis, nuclear p65 was a significant prognostic factor in univariate analysis (HR 2.39, 95% CI 1.08-5.29, $p = 0.03$). For multivariate analysis adjusted for subtype, stage and grade (Hazards ratio 2.77, 95% CI 1.12-6.86, $p = 0.02$); higher nuclear p65 was noted to be an independent prognostic factor. No significant association of overall and membrane expression of p65 was seen with cancer-specific survival.

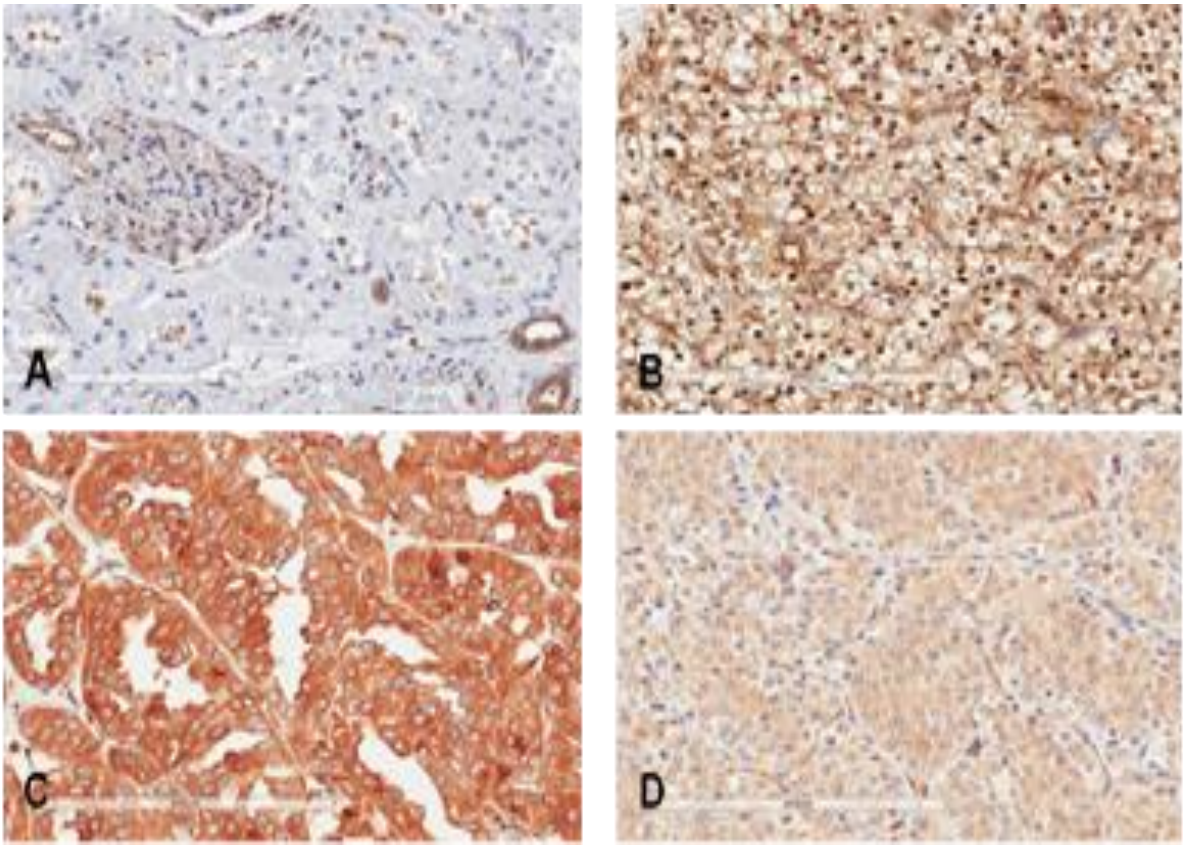

Figure 4.1: p65 immunostaining for renal tumours and normal kidney

A. Immunostaining of p65 in normal kidney; **B**. Immunostaining of p65 in ccRCC; **C**. Immunostaining of p65 in pRCC; **D**. Immunostaining of p65 in chRCC. Scale bar 200µm. (x20 Aperio magnification)

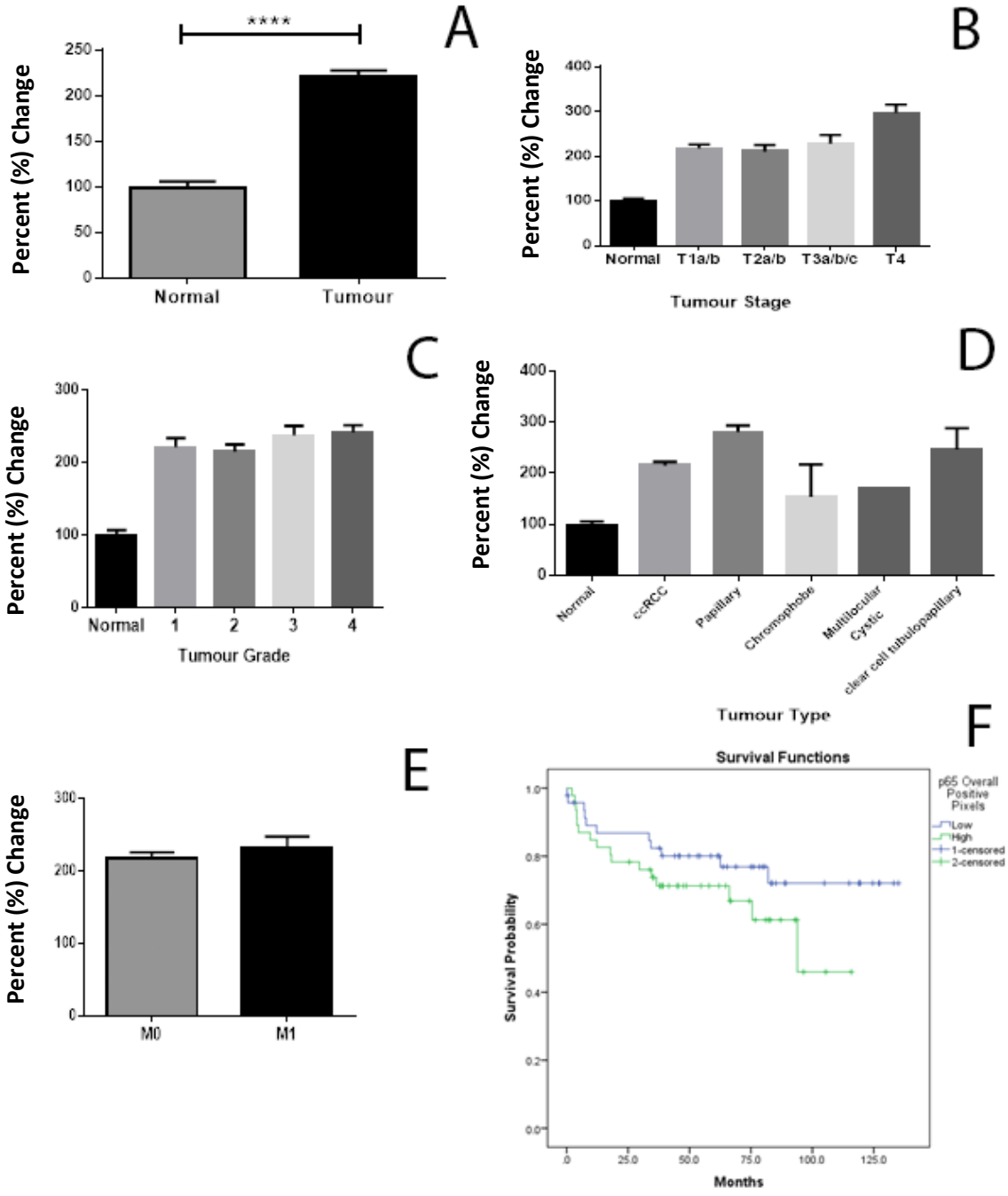

Figure 4.2: Overall positive pixel expression of p65 with survival analysis

A. p65 overall expression in tumour vs normal (****p<0.0001); **B**. p65 overall expression in various tumour stages; **C**. p65 overall expression in various tumour grades; **D**. p65 overall expression in tumour subtypes; **E**. p65 overall expression in M1 vs M0 stages; **F**. Kaplan Meier cancer specific survival analysis for p65 overall expression. (**--- low, --- high**)

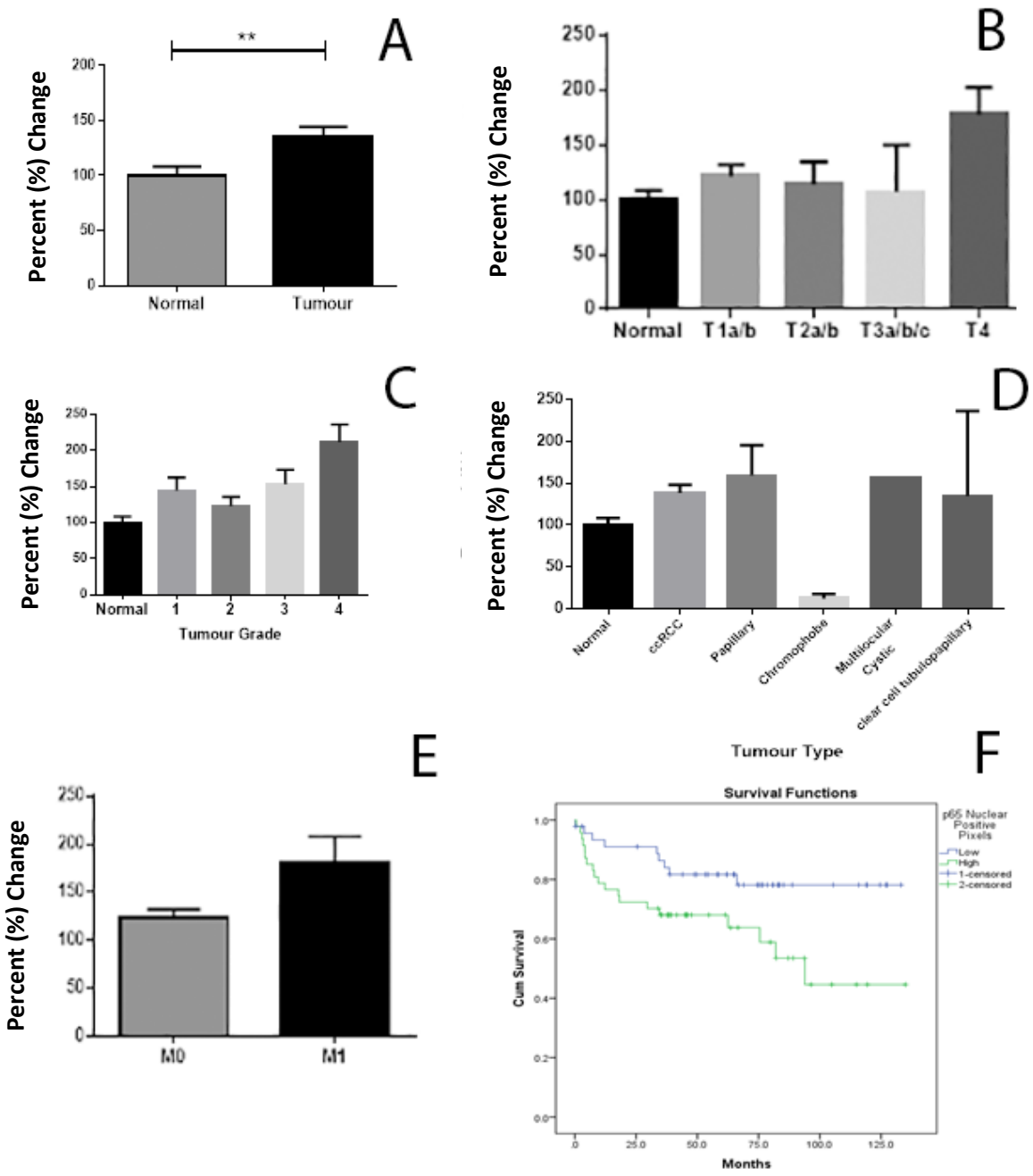

Figure 4.3: Nuclear expression of p65 with survival analysis

A. p65 nuclear expression in tumour vs normal (**p0.0015); **B**. p65 nuclear expression in various tumour stages; **C**. p65 nuclear expression in various tumour grades; **D**. p65 nuclear expression in tumour subtypes; **E**. p65 nuclear expression in M1 vs M0 stages; **F**. Kaplan Meier cancer specific survival analysis for p65 nuclear expression (p=0.03). (**--- low, --- high**)

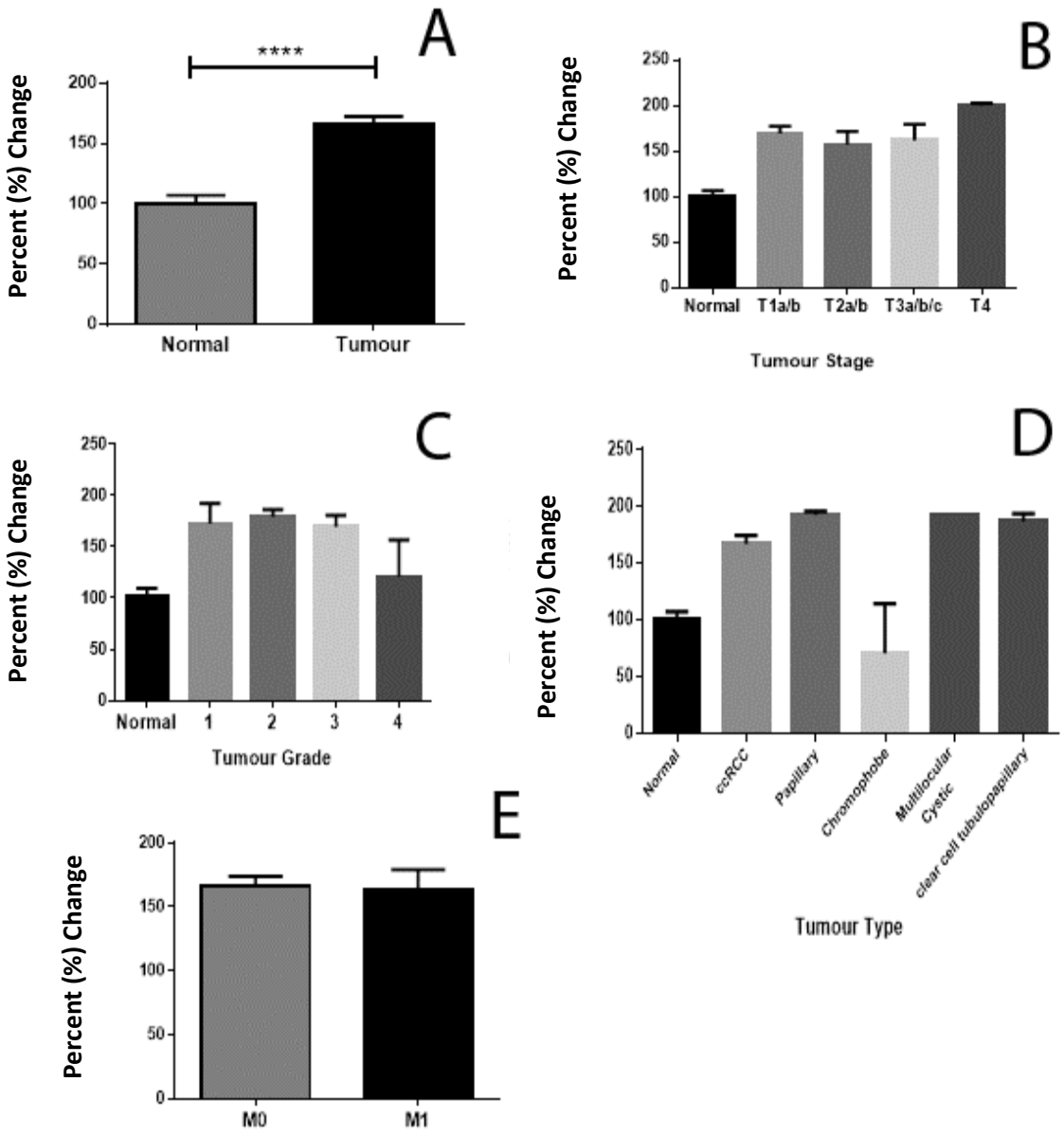

Figure 4.4: Membrane expression of p65

A. p65 membrane expression in tumour vs normal (***p<0.0001); **B**. p65 membrane expression in various tumour stages; **C**. p65 membrane expression in various tumour grades; **D**. p65 membrane expression in tumour subtypes; **E**. p65 membrane expression in M1 vs M0 stages.

4.3.2 NF-κB p50 subunit analysis

The staining characteristics of p50 was mainly cytoplasmic and nuclear as shown in Figure 4.5 (A-D). The overall, nuclear and membrane expression of p50 IHC were lower in all RCC subtypes compared to normal renal tissue ($p < 0.0001$, $p < 0.0001$ and $p = 0.0001$ respectively) as shown in Figure 4.6A, 4.7A and 4.8A. Among the tumour subtypes, clear cell tubulopapillary RCC (ccpRCC) had highest overall and nuclear expression of p50 compared to the rest of the subtypes; with multilocular cystic RCC (mcRCC) recording the lowest expression as shown in Figure 4.6D, 4.7D and 4.8D. There were no significant differences in expression between tumour subtypes for overall expression, nuclear or membrane analyses. There were no significant differences in p50 expression noted in clinical T stage, tumour grading and metastatic nature.

Higher p50 overall expression was significantly associated with worse survival outcome ($p = 0.005$) (Figure 4.6F). Using Cox regression analysis, overall p50 was a significant prognostic factor in univariate analysis (Hazards ratio 3.23, 95% CI 1.42-7.34, $p = 0.005$). For multivariate analysis adjusted for subtype, stage and grade (Hazards ratio 3.42, 95% CI 1.34-9.02, $p = 0.01$); overall p50 was noted to be independent prognostic factor. No significant association for nuclear or membrane expressions of p50 was seen with cancer-specific survival.

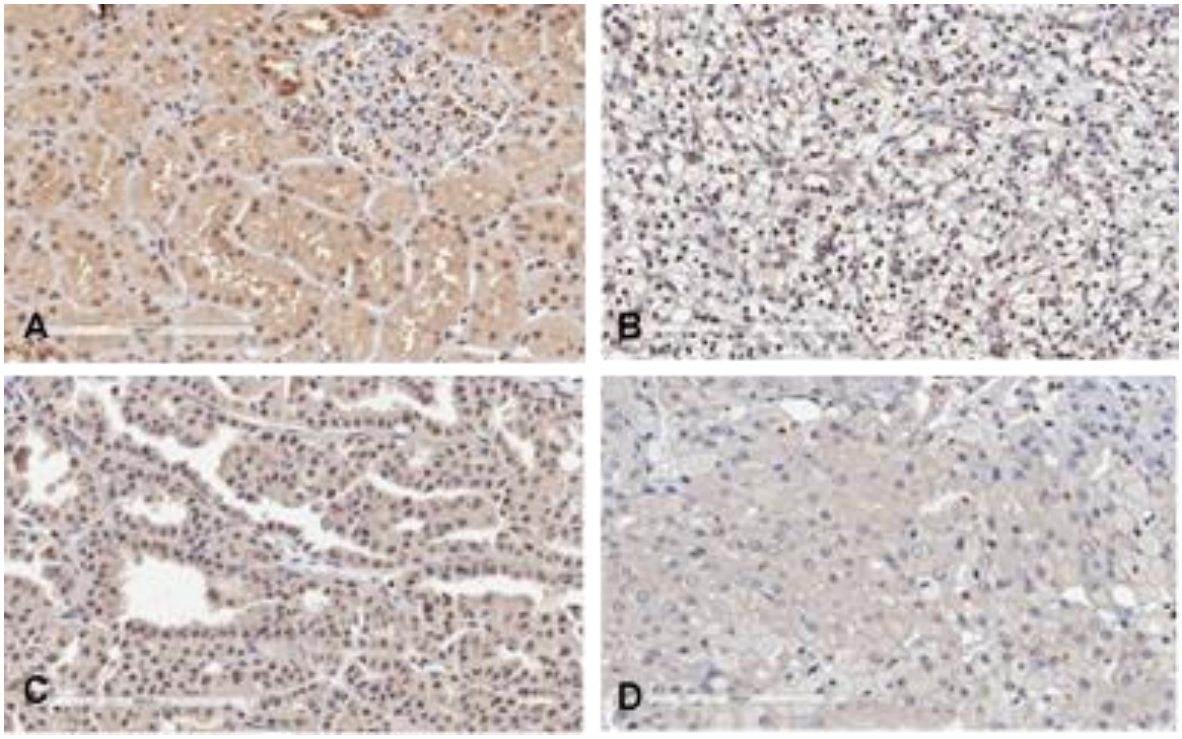

Figure 4.5: p50 immunostaining for renal tumours and normal kidney

A. Immunostaining of p50 in normal kidney; **B**. Immunostaining of p50 in ccRCC; **C**. Immunostaining of p50 in pRCC; **D**. Immunostaining of p50 in chRCC. Scale bar 200µm. (x20 Aperio magnification)

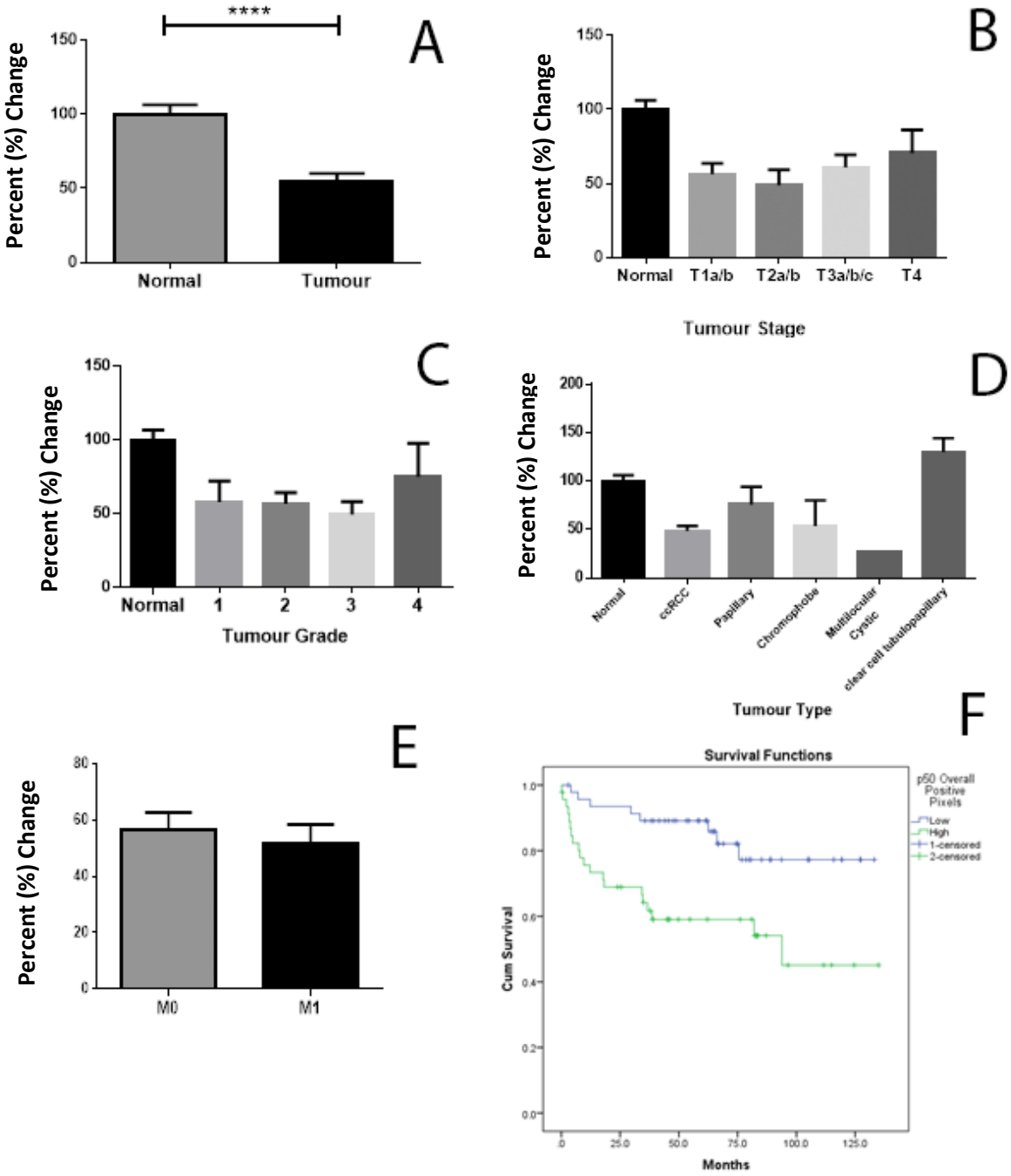

Figure 4.6: Overall positive pixel expression of p50 with survival analysis

A. p50 overall expression in tumour vs normal (****p<0.0001); **B**. p50 overall expression in various tumour stages; **C**. p50 overall expression in various tumour grades; **D**. p50 overall expression in tumour subtypes; **E**. p50 overall expression in M1 vs M0 stages; **F**. Kaplan Meier cancer specific survival analysis for p50 overall expression (p 0.005) (**--- low, --- high**)

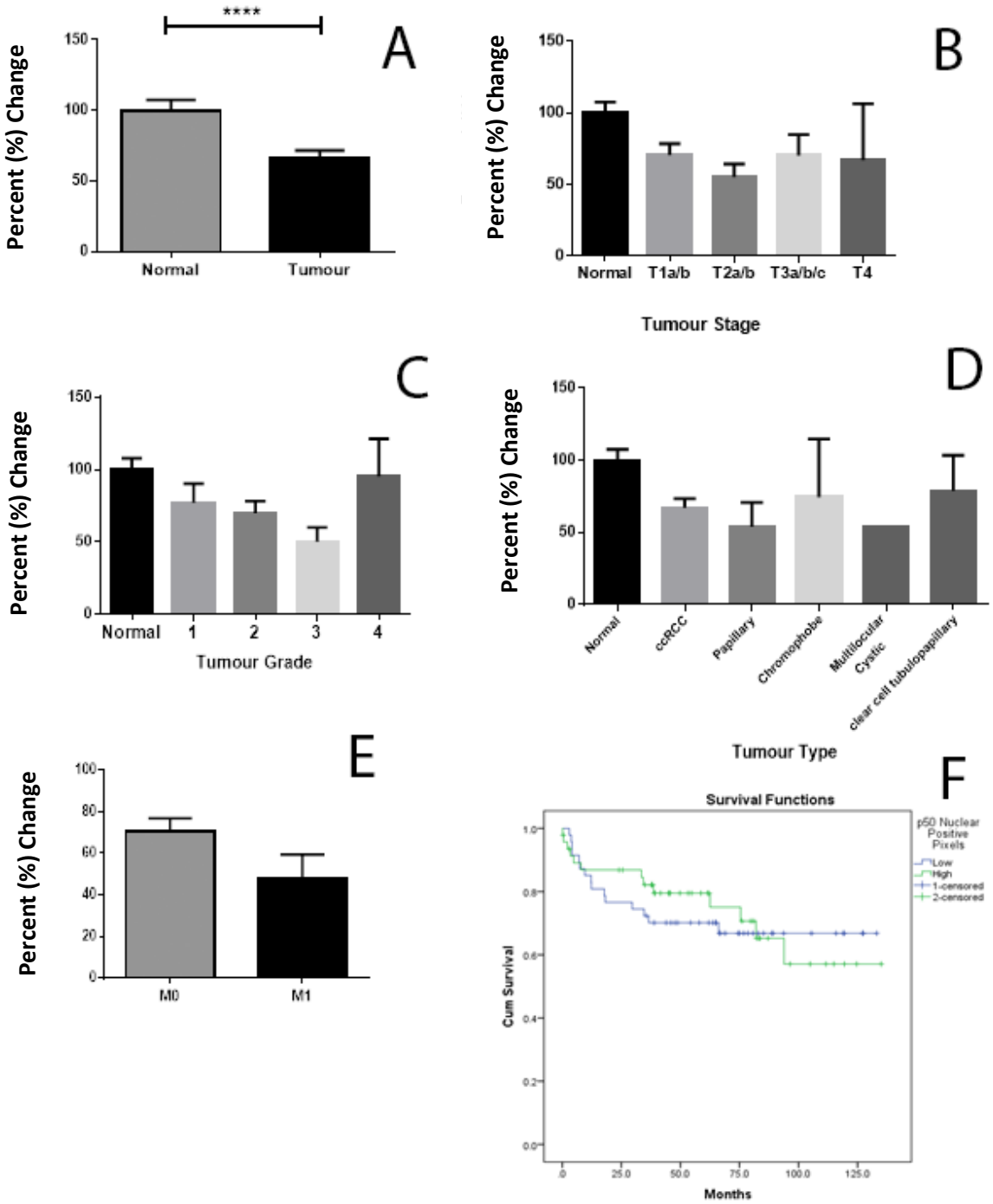

Figure 4.7: Nuclear expression of p50 with survival analysis

A. p50 nuclear expression in tumour vs normal (****p<0.0001); **B**. p50 nuclear expression in various tumour stages; **C**. p50 nuclear expression in various tumour grades; **D**. p50 nuclear expression in tumour subtypes; **E**. p50 nuclear expression in M1 vs M0 stages; **F**. Kaplan Meier cancer specific survival analysis for p50 nuclear expression (**--- low, --- high**)

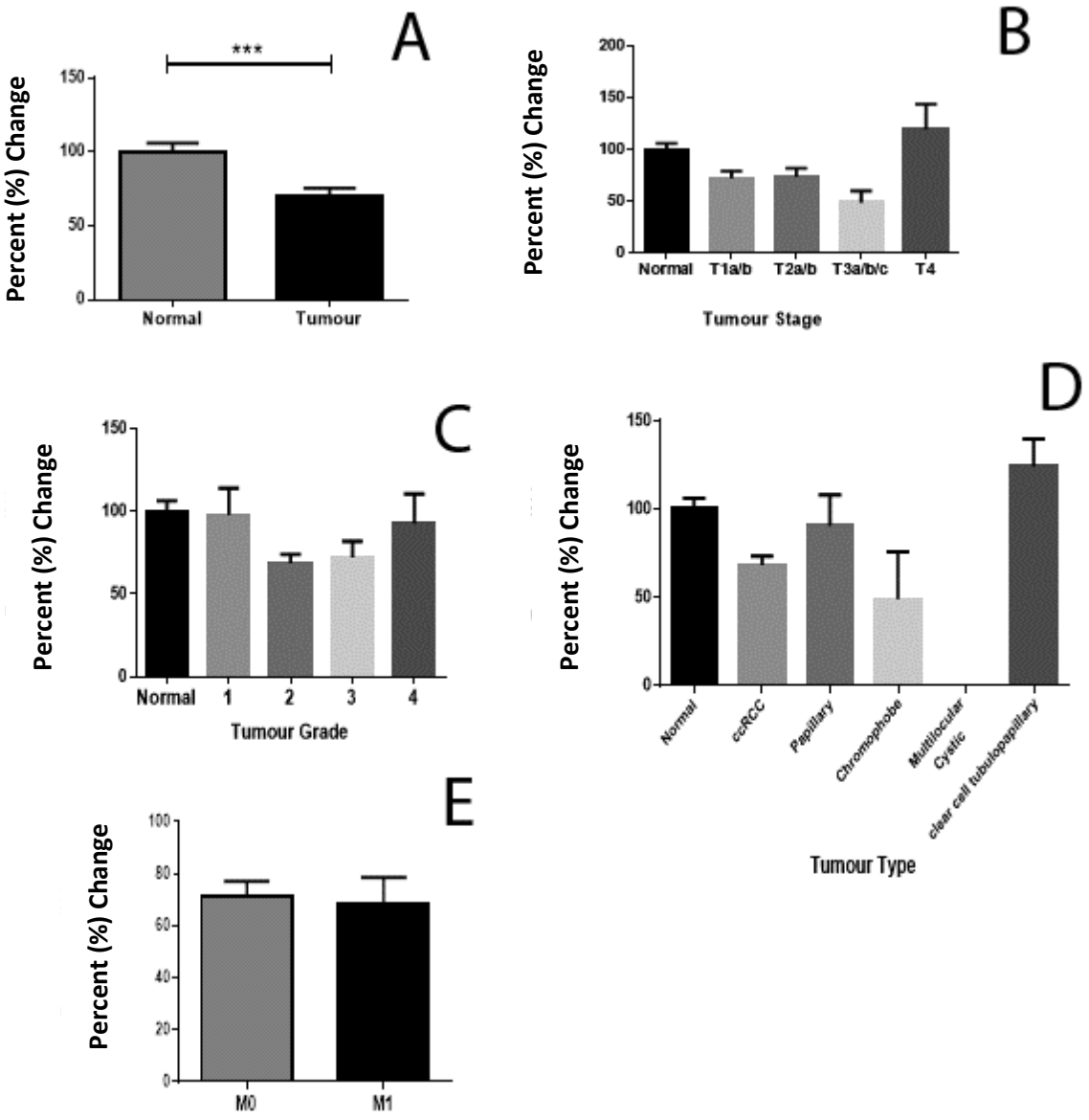

Figure 4.8: Membrane expression of p50

A. p50 membrane expression in tumour vs normal (***p = 0.0001); **B**. p50 membrane expression in various tumour stages; **C**. p50 membrane expression in various tumour grades; **D**. p50 membrane expression in tumour subtypes; **E**. p50 membrane expression in M1 vs M0 stages.

4.3.3 NF-κB p52 subunit analysis

The p52 subunit expression was mainly cytoplasmic and occasionally nuclear in both normal and tumour tissues as shown in Figure 4.9 (A-D). Similar to p50 expression, RCC tumour tissues had lower expression in overall, nuclear and membrane p52 intensity when compared to normal renal tissue ($p < 0.0001$, $p = 0.003$, $p < 0.0001$ respectively) (Figures 4.10A, 4.11A and 4.12A). No differences in p52 expression were noted in RCC subtypes, clinical T stages and evidence of metastasis.

Patients with high p52 overall expression (higher than median cut off value) were significantly associated with worse survival prognosis ($p = 0.02$) (Figure 4.10F). Using Cox regression analysis, overall p52 expression was a significant prognostic factor in univariate analysis (Hazards ratio 2.74, 95% CI 1.17-6.4, $p = 0.02$). In addition, high nuclear p52 expression (higher than median cut off value) was significantly associated with worse survival prognosis ($p = 0.03$) (Figure 4.11F). However on multivariate analysis adjusted for subtype, stage and grade; both overall and nuclear p52 expression were not independent prognostic factors.

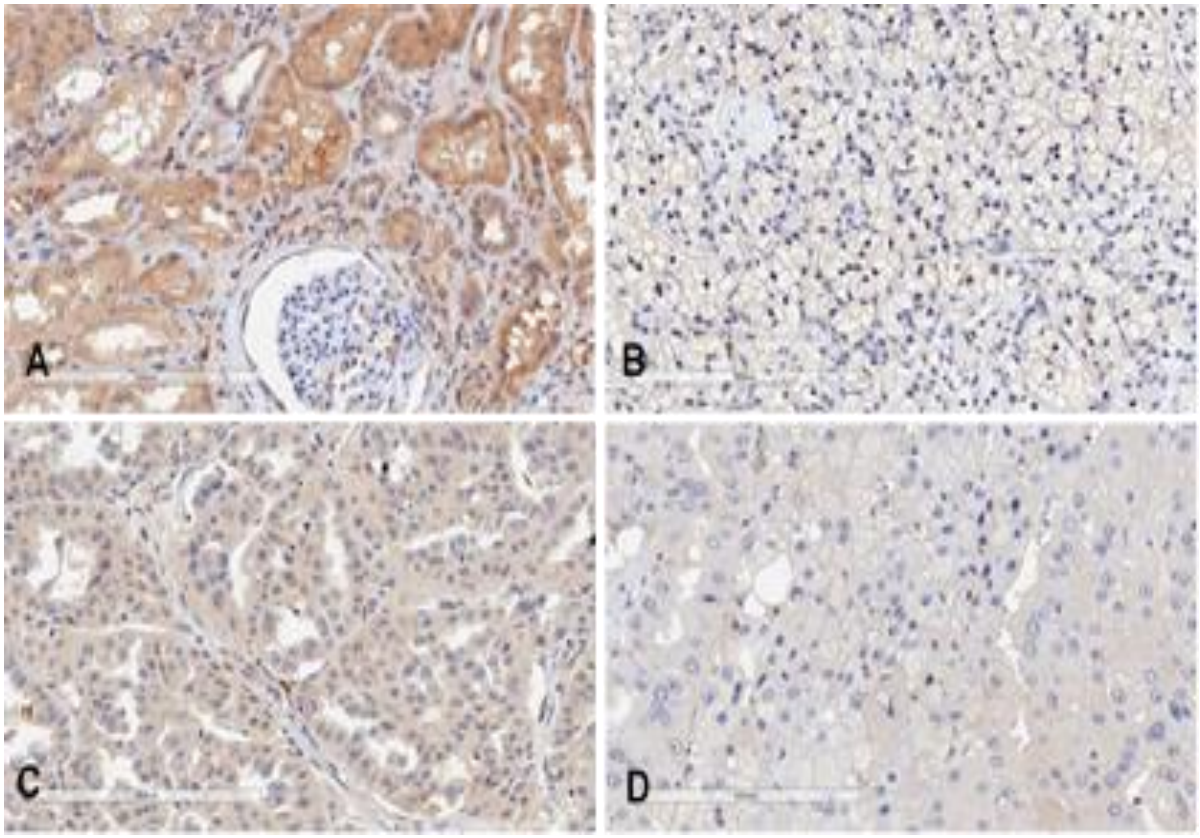

Figure 4.9: p52 immunostaining for renal tumours and normal kidney

A. Immunostaining of p52 in normal kidney; **B**. Immunostaining of p52 in ccRCC; **C**. Immunostaining of p52 in pRCC; **D**. Immunostaining of p52 in chRCC. Scale bar 200µm. (x20 Aperio magnification)

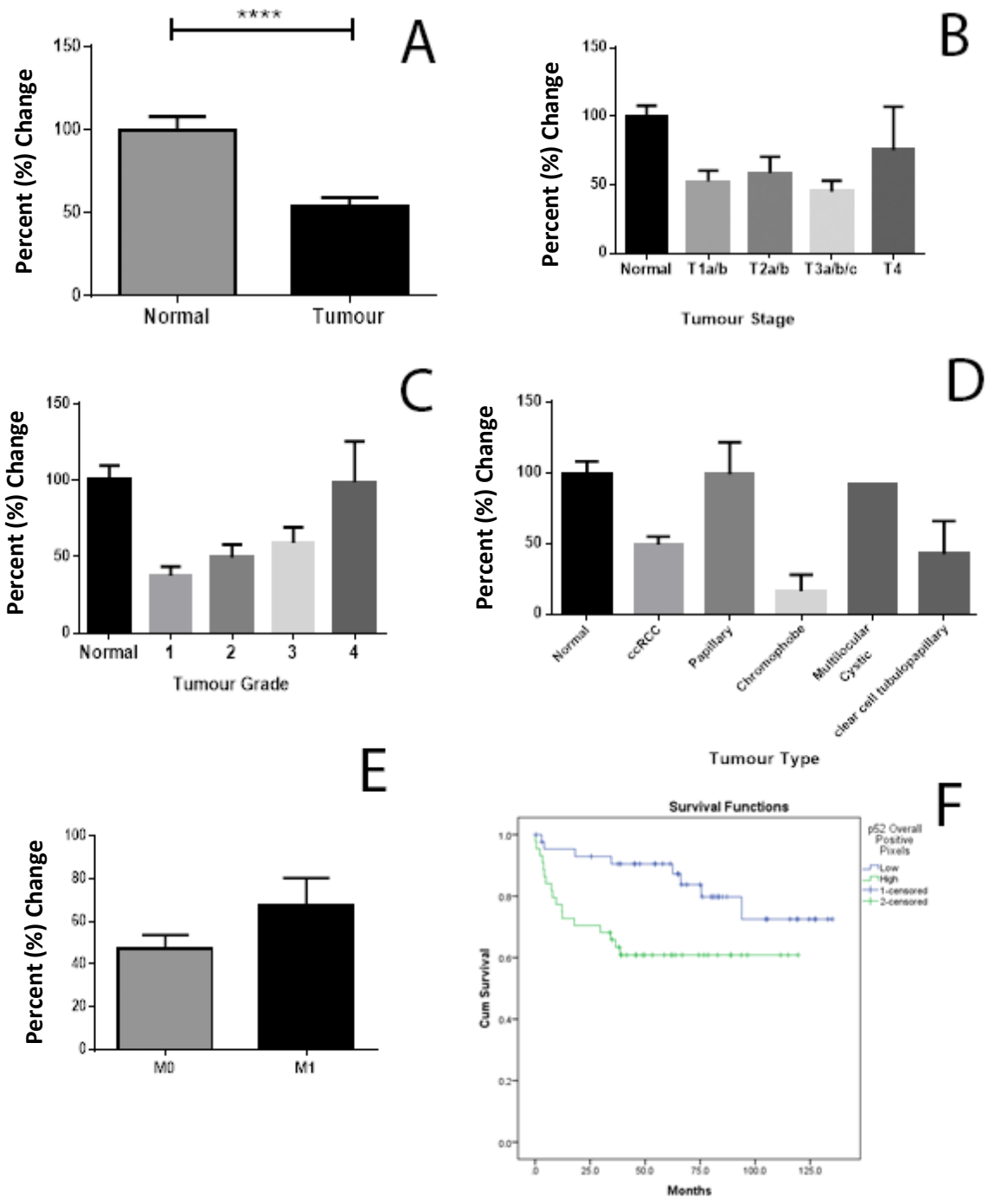

Figure 4.10: Overall positive pixel expression of p52 with survival analysis

A. p52 overall expression in tumour vs normal (****p<0.0001); **B**. p52 overall expression in various tumour stages; **C**. p52 overall expression in various tumour grades; **D**. p52 overall expression in tumour subtypes; **E**. p52 overall expression in M1 vs M0 stages; **F**. Kaplan Meier cancer specific survival analysis for p52 overall expression (p0.02) (**--- low, --- high**)

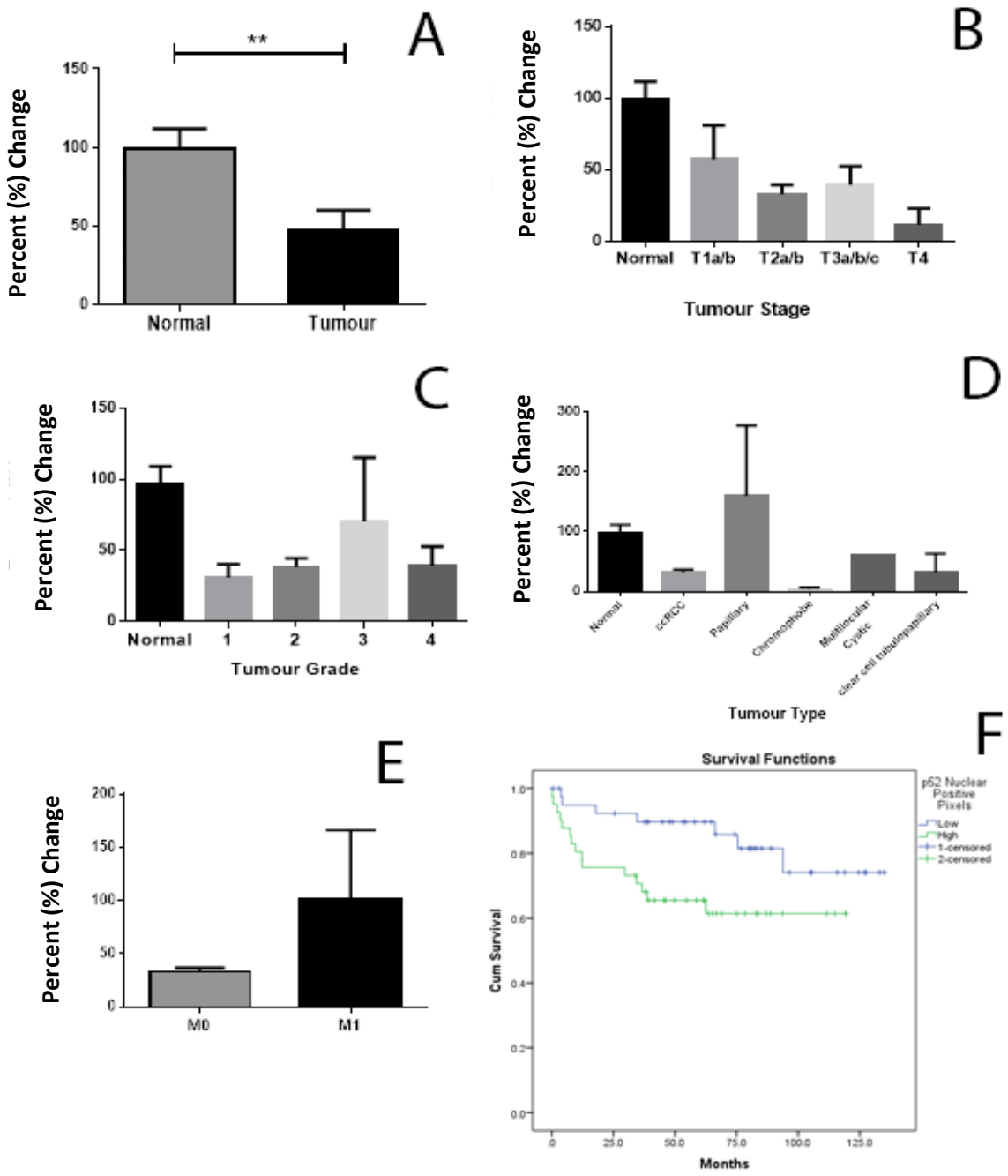

Figure 4.11: Nuclear expression of p52 with survival analysis

A. p52 nuclear expression in tumour vs normal (**p=0.003); **B**. p52 nuclear expression in various tumour stages; **C**. p52 nuclear expression in various tumour grades; **D**. p52 nuclear expression in tumour subtypes; **E**. p52 nuclear expression in M1 vs M0 stages; **F**. Kaplan Meier cancer specific survival analysis for p52 nuclear expression (p0.03) (**--- low, --- high**)

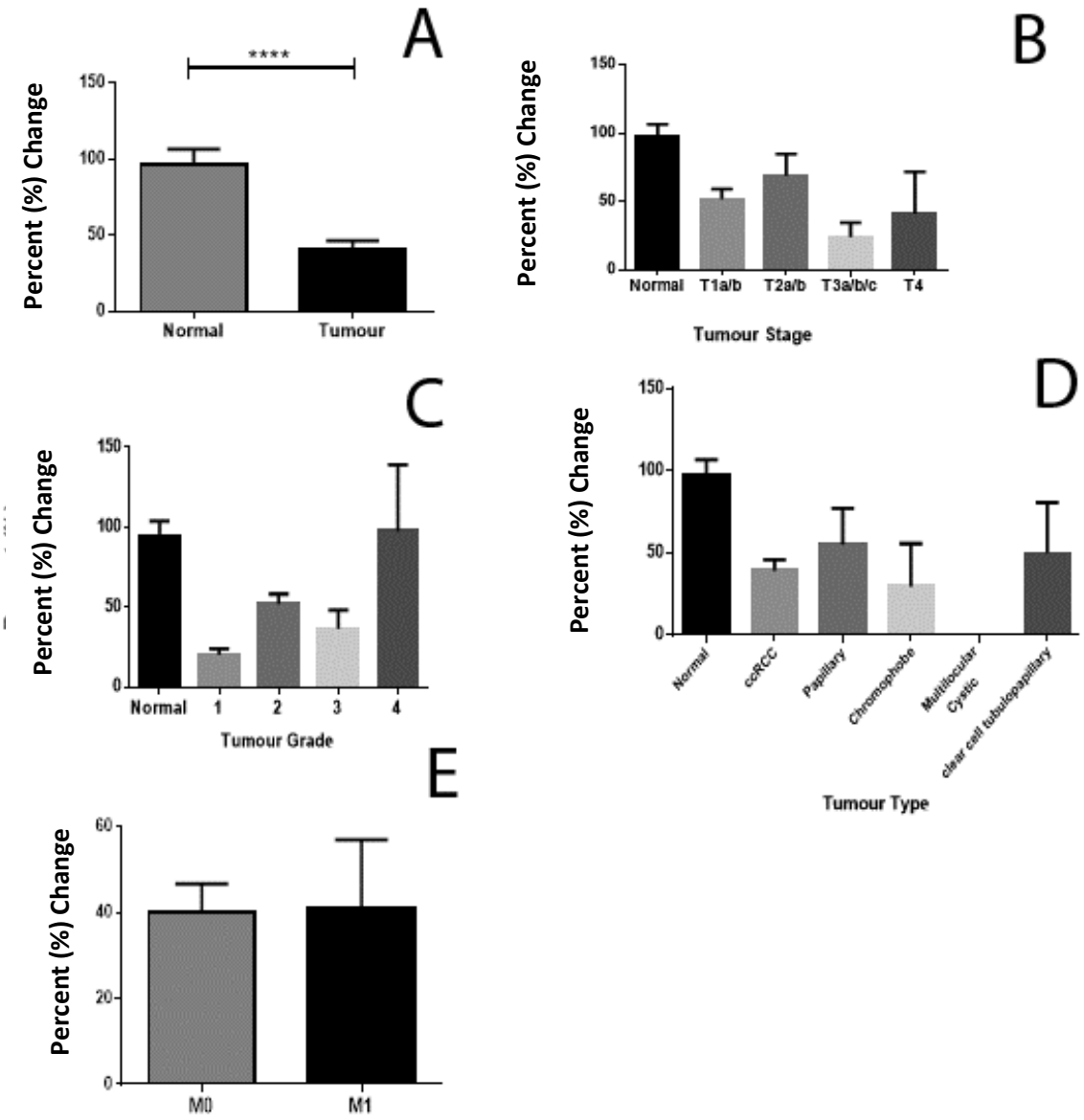

Figure 4.12: Membrane expression of p52

A. p52 membrane expression in tumour vs normal (****p<0.0001); **B**. p52 membrane expression in various tumour stages; **C**. p52 membrane expression in various tumour grades; **D**. p52 membrane expression in tumour subtypes; **E**. p52 membrane expression in M1 vs M0 stages.

4.3.4 NF-κB cRel subunit analysis

The cRel immunostaining was noted mainly as cytoplasmic with occasionally nuclear pattern as shown in Figure 4.13(A-D). RCC subtypes showed significantly lower overall, nuclear and membrane cRel expressions when compared to normal renal tissue (all $p < 0.0001$). (Figure 4.14A, 4.15A and 4.16A). There were no significant differences in cRel expression noted in clinical T stages, tumour subtypes grading and metastatic status. All tumour subtypes were noted to have minimal cRel immunostaining compared to normal renal tissue as shown in Figures 4.14D, 4.15D and 4.16D. There was also no significant correlation of cRel expression with cancer-specific survival.

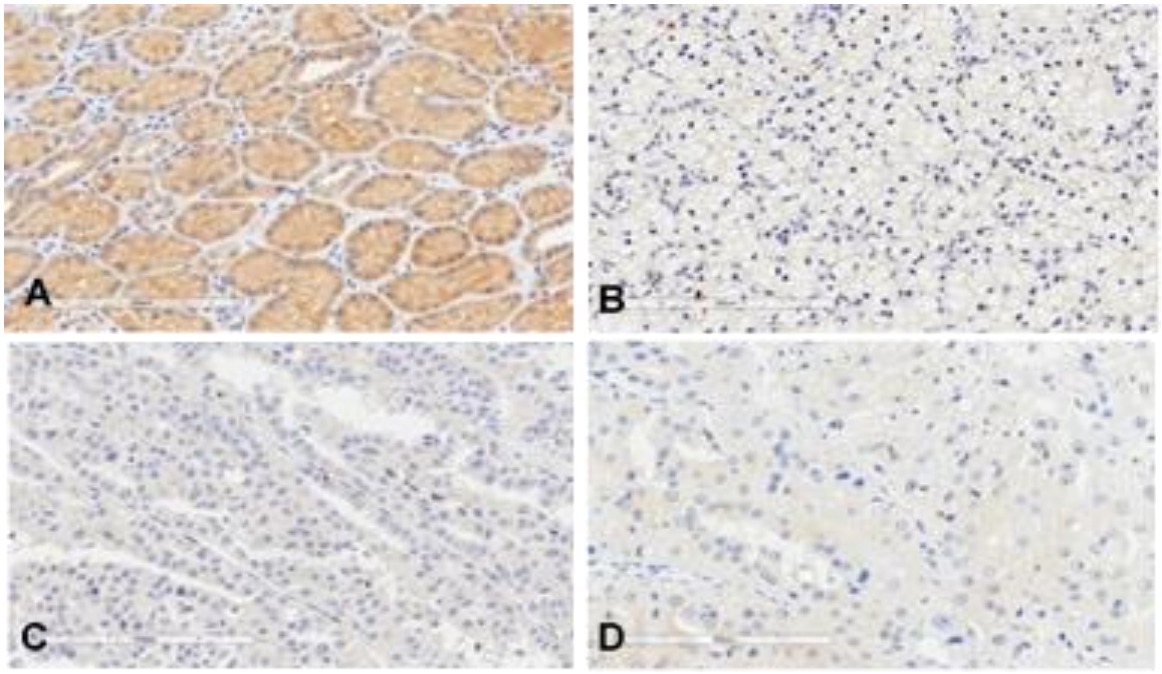

Figure 4.13: cRel immunostaining for renal tumours and normal kidney

A. Immunostaining of cRel in normal kidney; **B**. Immunostaining of cRel in ccRCC; **C**. Immunostaining of cRel in pRCC; **D**. Immunostaining of cRel in chRCC. Scale bar 200µm. (x20 Aperio magnification)

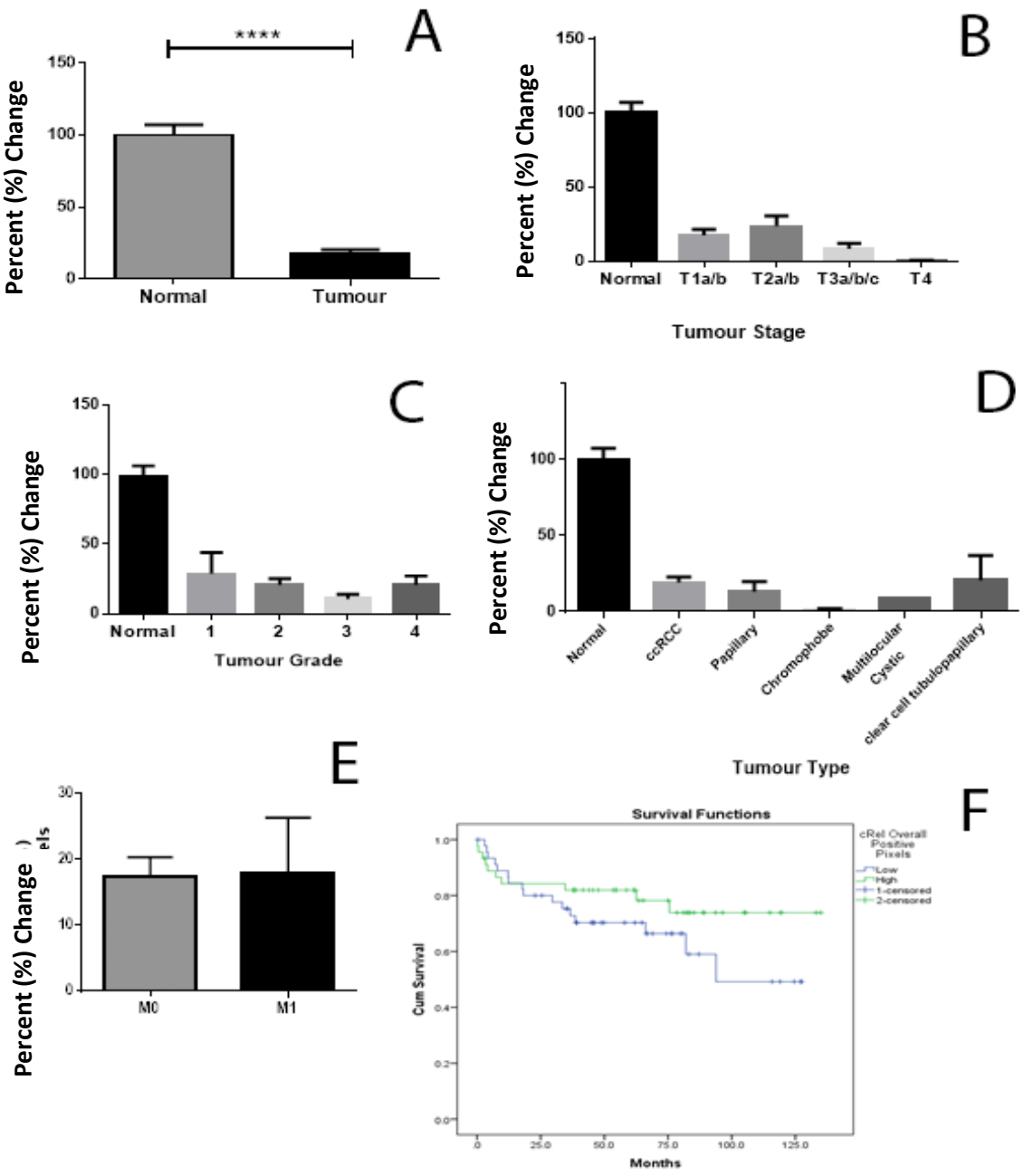

Figure 4.14: Overall positive pixel expression of cRel with survival analysis

A. cRel overall expression in tumour vs normal (****p<0.0001); **B**. cRel overall expression in various tumour stages; **C**. cRel overall expression in various tumour grades; **D**. cRel overall expression in tumour subtypes; **E**. cRel overall expression in M1 vs M0 stages; **F**. Kaplan Meier cancer specific survival analysis for cRel overall expression (**--- low, --- high**)

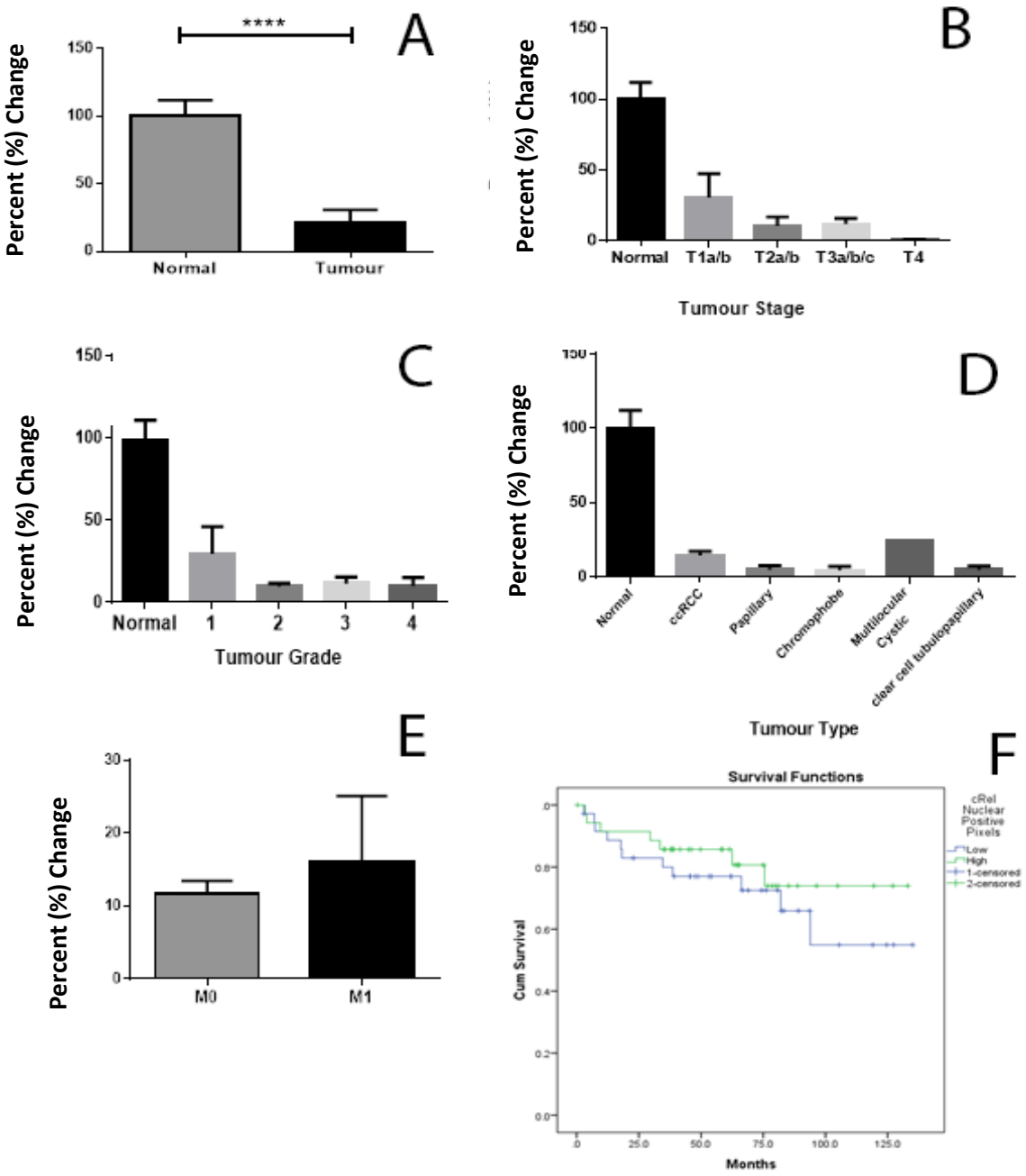

Figure 4.15: Nuclear expression of cRel with survival analysis

A. cRel nuclear expression in tumour vs normal (****p<0.0001); **B**. cRel nuclear expression in various tumour stages; **C**. cRel nuclear expression in various tumour grades; **D**. cRel nuclear expression in tumour subtypes; **E**. cRel nuclear expression in M1 vs M0 stages; **F**. Kaplan Meier cancer specific survival analysis for cRel nuclear expression. (**--- low, --- high**)

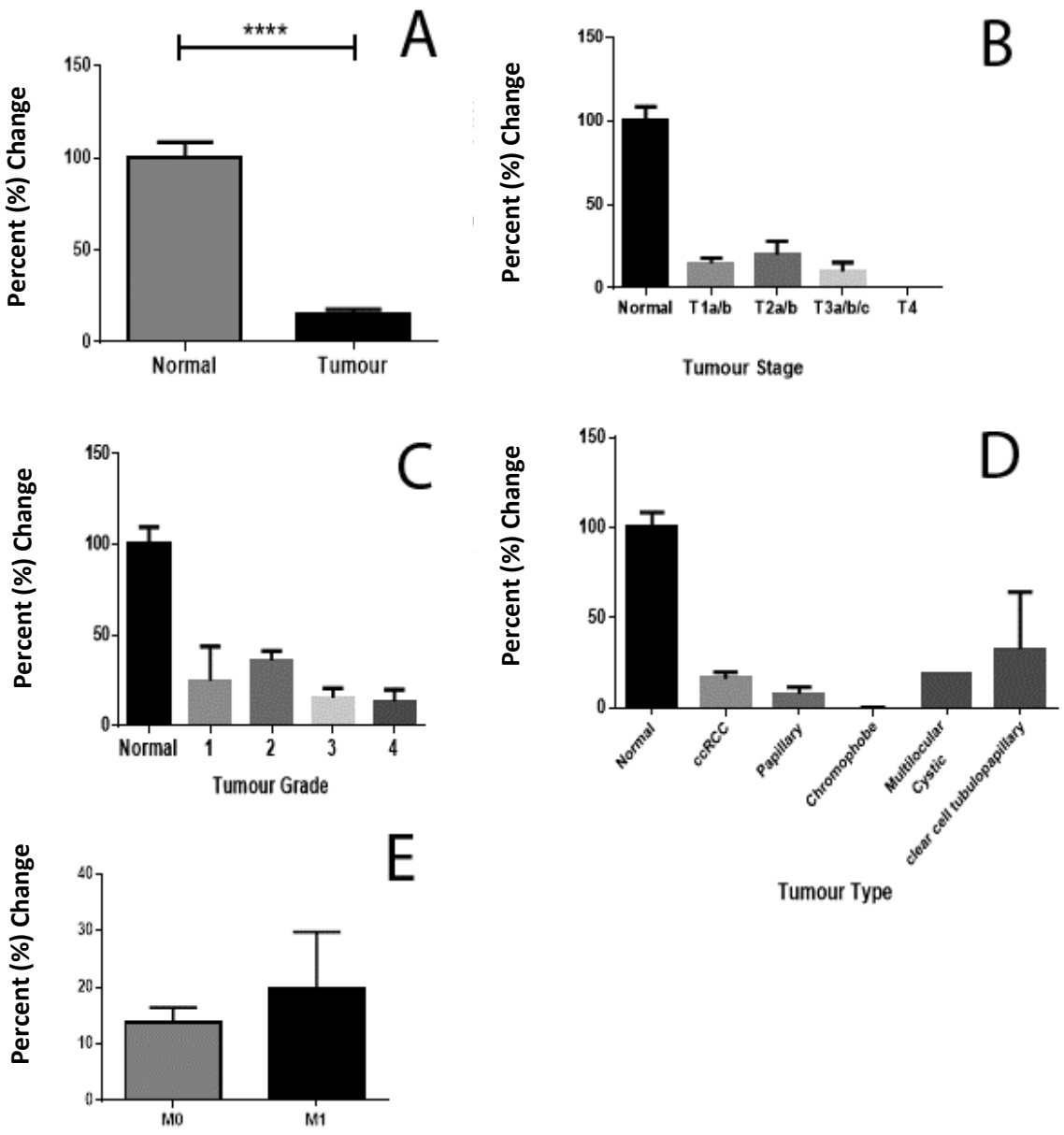

Figure 4.16: Membrane expression of cRel

A. cRel membrane expression in tumour vs normal (****p<0.0001); **B**. cRel membrane expression in various tumour stages; **C**. cRel membrane expression in various tumour grades; **D**. cRel membrane expression in tumour subtypes; **E**. cRel membrane expression in M1 vs M0 stages.

In summary, higher expression of p65 in RCC tumour tissue compared with normal kidney was seen in comparison with lower expressions of p50, p52 and cRel in RCC tumour tissue when compared to normal kidney tissue. Higher nuclear expression of p65, overall and nuclear expression of p52 and higher overall p50 were also correlated with worse cancer-specific survival in this cohort of RCC patients. (Table 4.2)

Table 4.2: Summary of IHC expression of NF-κB subunits in RCC tumours and normal kidney with correlation to cancer-specific survival.

	p65	**p50**	**p52**	**cRel**
RCC vs normal (overall and nuclear expresion)	⇧	⇩	⇩	⇩
Worse cancer-specific survival	nuclear expression ⇧ independent prognostic factor	overall expression ⇧ independent prognostic factor	overall & nuclear expression ⇧	–

4.4 DISCUSSION

The role of NF-κB subunits in RCC development and progression has not been well defined before. Here we have shown the expressions of four NF-κB subunits in human RCC tissue and described the association of p65, p50and p52 with cancer-specific survival.

NF-κB is found in its sequestered inactive form in the cytoplasm, where it is bound to the 43-kDa inhibitor protein IκB that covers the nuclear localization signal (NLS) region of the predominant p65/p50 dimer (Brasier 2006). In unstimulated cells, the NF-κB dimers are sequestered in the cytoplasm by a family of inhibitors, called IκBs (inhibitor of κB), which are proteins that contain multiple copies of the ARs. By virtue of their AR domains, the IκB proteins mask the NLS of NF-κB proteins, thereby keeping them sequestered in an inactive state (Karin and Ben-Neriah 2000). Activation of the NF-κB pathway is initiated by the signal-induced degradation of the evolutionarily conserved IκB proteins. This occurs primarily via the activation of IκB kinase (IKK) which is composed of a heterodimer of the catalytic IKKα and IKKβ subunits and a regulatory protein termed NEMO (NF-κB essential modulator), or IKKγ.

When activated, the IKK phosphorylates two serine residues located in an IκB regulatory domain. When phosphorylated on these serines (e.g., serines 32 and 36 in human IκBα), the IκB inhibitor molecules are modified by ubiquitination, which then leads them to be enzymatically degraded by a proteasome (Brasier 2006; Fusco et al. 2009; Gilmore 2006). With the degradation of IκB, the NF-κB complex is then freed to enter the nucleus where it can ‘turn on’ the expression of specific genes that have DNA-binding sites for NF-κB nearby. The activation of these genes by NF-κB then leads to the given physiological response, for example, an inflammatory or immune response, a cell survival response, or cellular

proliferation (Doyle and O'Neill 2006; Hayden et al. 2006). Aberrant regulation of NF-κB has been linked to certain conditions such as inflammatory and autoimmune diseases, septic shock, viral infections, improper immune responses and cancers (Haddad and Abdel-Karim 2011).

In anti-apoptotic pathways, NF-κB induces the expression of many anti-apoptotic genes of the Bcl-2 family including Bcl-XL, and Bcl-2 (Kurland et al. 2001; Lee et al. 1999; Viatour et al. 2003; Wang et al. 1999b). Matusan-Ilijas *et al* showed that p65 NF-κB signaling pathway may be involved in osteopontin-mediated ccRCC progression, partly by protecting tumour cells from apoptosis (Matusan-Ilijas et al. 2011).

Evidence of NF-κB-induced pro-angiogenic pathways includes VEGF, epidermal growth factor (EGF), interleukin-6 and interleukin-8 (Huang et al. 2001; Morais et al. 2009; Mukaida et al. 1994). Djordjevic *et al* showed there was significant association between cytoplasmic NK-κB/p65 staining and VEGF staining of diffuse pattern and that higher expression of VEGF in tumour cells, especially in clear cell RCC, is associated with NF-κB/65 activity (Djordjevic et al. 2008). Epidermal growth factor receptor (EGFR) gene has been shown to be upregulated in clear cell RCC and associated with osteopontin expression and NF-kB activation and signalling (Matusan-Ilijas et al. 2013). Recently, Du *et al* reported that downregulation of phospholipase Cε (PLCε) expression repressed growth and induced apoptosis in RCC cells by suppressing the nuclear factor kappa (NF-κB) signaling pathway which led to decreased VEGF expression (Du et al. 2014).

In addition, tumours with constitutive activation of NF-κB are generally resistant to chemo and radiotherapy and an inverse correlation between NF-κB activity and sensitivity of cancer cells to chemotherapy, due to up-regulation of multidrug resistance (MDR) genes, has been reported (Wang et al. 1999a). Recently, Zhu *et al* showed that oncogenic activation of p21-activated kinase 1 defines an important mechanism for maintaining stem-like phenotype and sunitinib resistance through NF-κB/IL-6 activation in RCC (Zhu et al. 2015).

Numerous NF-κB dependent mechanisms have been shown in the activation and promotion of RCC carcinogenesis. One of the well-studied mechanisms is the aberrant von Hippel Lindau (VHL) pathway which is the most commonidentified factor for the development of sporadic clear cell RCC. Loss of VHL tumour suppressor gene will lead to decreased VHL protein activity leading to accumulation of hypoxia inducible factor 1a which in turn increases angiogenic factors that promote unregulated neoangiogenesis, favouring tumour growth. The VHL gene is mutated in 34–56% and hyper-methylated in 19% of the sporadic ccRCC (George and Kaelin 2003; Lopez-Beltran et al. 2006). Moreover, the VHL syndrome (dominantly inherited cancer syndrome characterised by tumours of brain, eye, kidney, pancreas and adrenal) is also a predominant pre-disposing factor for the development of familial RCC. As VHL negatively regulates NF-κB; therefore in the absence of functional protein encoded by VHL gene (pVHL), NF-κB activation will be increased.

An *et al* have shown that VHL loss drives NF-κB activation by resulting in HIFα accumulation, which induces expression of transforming growth factor alpha, with consequent activation of an EGFR/phosphatidylinositol-3-OH kinase/protein kinase B (AKT)/IκB-kinase alpha/NF-κB signaling cascade. Thus, VHL expression reduces constitutive NF-κB activity (An and Rettig 2005; An et al. 2005). Qi *et al* also showed that

pVHL facilitates TNF-alpha-induced cytotoxicity in RCC cells, at least in part, through the down-regulation of NF-κB dependent anti-apoptotic pathway (Qi and Ohh 2003).

Therefore, increased activity of NF-κB in RCC will promote invasiveness and metastatic potential. Numerous studies have shown a positive association of increased NF-κB, in particular the p65 subunit, with higher tumour grade, increased tumour stage, larger tumour size, increased invasiveness and metastases (Kankaya et al. 2015; Oya et al. 2003; Ozbek et al. 2012). Interestingly, Meteoglu *et al* showed that there was no significant correlation between NF-κB p50 subunit with tumour grade, stage, age and sex (Meteoglu et al. 2008). With respect to RCC cancer overall survival, a recent meta-analysis on clear cell RCC gene expression identified a key NF-κB regulator (IKBKB) and established mediators of the NF-κB cell-survival and pro-inflammatory responses (MMP9, PSMB9, and SOD2), correlated with higher relative risk, poorer prognosis, and reduced overall patient survival (Peri et al. 2013). In comparison, some have reported that NF-κB was not a prognostic factor in RCC survival (Kankaya et al. 2015; Sourbier et al. 2007).

VHL loss also results in epithelial to mesenchymal transition (EMT). Tumour cells have reverted to EMT to enhance their invasive and metastatic potentials. Activation of NF-κB has been causally linked to an invasive phenotype and can directly or indirectly induce expression of transcription factors Snail, Slug, Twist, Zeb1, and Zeb2 which induce EMT program (Min et al. 2008). Pantuck *et al* provided evidence that in RCC, VHL loss induces an EMT that is largely dependent on HIFα-induced NF-κB pathway (Pantuck et al. 2010).

Due to the numerous target genes affected by NF-κB in promoting RCC carcinogenesis, research has tended to concentrate on targeting its pathway for therapeutic means. In the review by Morais *et al*, these therapeutic modalities include genetic or chemotherapeutic approaches. Most of the genetic approaches can be classified under three categories: over-expression of mutant IκB-α, RNA silencing (siRNA) or specific peptides; while chemotherapy approaches involved chemoinhibition of the NF-κB pathway (Morais et al. 2011). Other promising newer compounds targeting NF-κB in RCC include physalin F, chrysin and bortezomib (Rehman et al. 2013; Thapa et al. 2013; Wu et al. 2012).

Therefore, with the reliable evidence of the role of NF-κB in RCC tumourigenesis and its potential target for treatment; it is no doubt why there are still ongoing research into NF-κB family. Despite these extensive knowledge so far, little is known about the intimate interactions of the 5 subunits of NF-κB and their role in RCC. Therefore, this study explored the expression of all 5 subunits of NF-κB (p65, p50, p52, RelB and c-Rel) in human RCC tissue via IHC and studied the association of each subunit with clinico-pathological aspects of the RCC patients. As mentioned previously, RelB IHC staining was inadequate for interpretation and therefore only 4 subunits were described. To our knowledge, this is the first study that report the findings of various subunits NF-κB (p65, p50, p52 and c-Rel) in human RCC and also involving the largest series of human RCC tissue (n=96) with 76 ccRCC samples. The hypothesis is that these subunits interact and influence each other, leading to RCC tumourigenesis. Therefore, firstly we need to assess the expressions of these subunits in RCC.

The clinico-pathological data presented as above from the 96 patients are consistent with other published RCC data. RCC tend to occur more frequently in males. In this case the male to female ratio is 2.1:1. The most prevalent RCC subtype was ccRCC, as is well-published. The higher proportion of metastatic RCC patients at presentation and slightly larger median tumour size in this cohort of Malaysian patients is reflective of the delay by patients in seeking medical attention partly due to decreased health awareness, access to imaging facilities and reliance on traditional/local complimentary medicine. The median follow up assessment was approximately 4.5 years, during which 29% of patients succumbed to the disease. This highlights the lethal nature of metastatic RCC disease despite newer targeted molecular therapies which can prolong survival but fall short of providing cure.

In NF-κB p65 subunit analysis, overall, nuclear and membranous expression were higher in tumour when compared to normal, which concurs with other published results. The overall positive pixel expression of papillary RCC (pRCC) was noted to be highest among all subtypes. However, Sourbier *et al* presented that ccRCC had higher expression of NF-κB compared other subtypes (Sourbier et al. 2007). There was a slight trend for increasing overall expression of p65 with increasing tumour stages (T4 > T3 > T1/2) and metastatic cases (M1 > M0) but this showed no significant differences Importantly we were able to show that patients had worse cancer specific survival outcomes with increasing p65 nuclear expression (Figure 4.3F). On closer analysis, p65 nuclear expression was found to be an independent prognostic factor following multivariate analysis. However, p65 overall expression was found not to be an independent prognostic factor, which suggests that there must be other mechanisms or pathways that are involved at the nuclear microenvironment. A possible explanation for the link between nuclear p65 and poor survival outcome could be that activated NF-κB exerts its numerous downstream carcinogenesis effects only once

translocated into the nucleus where it binds with NF-κB target genes. One possible outcome is that cancer cells display better survival. Therefore NF-κB expression in nuclear compartments will be more influential in tumorigenesis and progression compared with the overall p65 expression.

Whilst p65 is the most studied subunit in the past, p50 is another subunit of NF-κB that has been studied singly or together with p65 in human RCC tissue (Meteoglu et al. 2008; Oya et al. 2003). In the current chapter, overall, nuclear and membrane expression of p50 were significantly lower in tumour when compared to normal kidney. This result is in contrast to results shown by Meteoglu *et al*; where their IHC p50 expression were higher in ccRCC than normal (n=40) (Meteoglu et al. 2008). Oya *et al* showed in their immunoblotting analysis that there was higher augmented p50 immunoblotting in tumour when compared to normal (n=45) and both increased p65 with p50 electrophoretic mobility shift assay correlated to higher stage and metastases (Oya et al. 2003).

These 2 studies were performed on only ccRCC tumour (all 40/40 cases in Meteoglu et al and 42/45 cases in Oya et al). Moreover, in Meteoglu et al (2008) there was weak or no p50 staining in 11/40 patients and 22/40 patients had cytoplasmic staining in the non-tumoral tubular cells. A possible explanation for differences seen between our results and other published data is that we investigated 5 different renal tumour subtypes and paired normal kidney tissue, an important comparison often omitted in many reports. However, our results also indicate that expression of p50 was not associated with tumour stage, grade or metastasis. Interestingly, increased overall expression of p50 led to worse cancer specific survival (p=0.005) (Figure 4.6F) and on multivariate analysis, was noted to be an independent prognostic indicator.

This is the first description of results for p52 and cRel NF-κB subunits IHC analysis in human RCC tissue. Similar to p50, overall expression, nuclear and membrane expressions of p52 and cRel were significantly lower in tumour when compared to normal renal tissue. There were no significant association between expression of these two subunits with tumour stage, grade and metastases. However, interestingly higher overall expression and nuclear expression of p52 were associated with worse survival prognosis (Figure 4.10F and 4.11F) and were independent prognostic factors in uni- and multi variate analyses. Based on these findings, it is worthwhile that these patients with increased expressions of p65 or p52 in their RCC tissue, will need closer radiological surveillance for early detection and opportunity for usage of targeted treatment if there was RCC progression.

These results of lower expressions of p50, p52 and cRel IHC analyses in RCC tumour compared to normal are difficult to explain. Unlike p65, RelB, and c-Rel, the p50 and p52 NF-κB subunits do not contain transactivation domains in their C terminal halves. Due to the presence of ARs in their C-terminal halves, p105/p50 and p100/p52 also function as IκB proteins. Nevertheless, the p50 and p52 NF-κB members play critical roles in modulating the specificity of NF-κB function. Although homodimers of p50 and p52 are, in general, repressors of the κB site of transcription, both p50 and p52 participate in target gene transactivation by forming heterodimers with p65, RelB, or c-Rel. In addition, p50 and p52 homodimers also bind to the nuclear protein Bcl-3, and such complexes can function as transcriptional activators (Brasier 2006; Fusco et al. 2009). Interestingly, NF-κB turns on the expression of its own repressor, IκBα. The newly synthesized IκBα then re-inhibits NF-κB and, thus, forms an auto feedback loop, which results in oscillating levels of NF-κB activity (Haddad et al. 2000; Li and Verma 2002). Therefore, we suggest that there are complex

interactions at the molecular and cellular level between these 5 subunits of NF-κB which will account for these results.

There were some limitations noted with this study: limited number of samples from the non ccRCC renal tumour subtypes; and the investigation was performed on an Asian population and may not reflect an Australasian cohort of patients' samples. With further time and funding, a multi-institutional, multi-national collaborative study on expression of NF-κB will be useful.

4.5 CONCLUSION

This chapter addressed Aim 2 of research theme which was to analyse the different molecular profiles of renal cancers via immunohistochemistry and morphometry techniques using selected biomarkers; and this case, NF-κB on renal tumour and normal tissue samples. From our knowledge, this is the first and largest series of IHC analysis on 4 subunits of NF-κB family in RCC human tissue. There is higher IHC expression of p65 (overall, nuclear and membrane) and lower IHC expression of p52, p50 and cRel (overall, nuclear and membrane) in RCC tumour compared to normal counterparts. Both higher nuclear expression of p65, overall and nuclear expressions of p52 and higher overall expression of p50 were associated with worse cancer specific survival; with higher nuclear p65 and overall p50 expression as independent prognostic factors in RCC.

From this chapter, the IHC expressions of NF-κB family, especially on the less studied subunits of p52, p50 and cRel, will aid in the further understanding of the molecular biology

and relationship between these subunits of NF-κB. This better understanding of the molecular profiles of NF-κB family in RCCs will encourage further research and pave the way for future targeted NF-κB subunit specific therapeutic pathways.

CHAPTER 5

IMMUNOHISTOCHEMICAL ANALYSES OF VARIOUS BIOMARKERS IN RENAL TUMOURS (ccRCC, chRCC and RO) AND IDENTIFICATION OF BIOMARKERS TO DIFFERENTIATE chRCC FROM RO

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Following the informative IHC results obtained for NFκB on the series of RCC specimens from Malaysia (Chapter 4), attention was now addressed to IHC of various biomarkers (CK7, Cav-1, S100A1 as established biomarkers; and Ob, its receptor ObR, and KIM-1 as novel biomarkers) on RCC specimens from an Australian cohort of patients using similar IHC methodology. In this chapter, IHC results obtained from these biomarkers were analysed from a perspective of achieving our research aim which was to identify biomarkers that would aid in the differentiation of chRCC from RO. Following the results gained from the meta-analysis (chapter 3), we investigated the differential IHC ability of three biomarkers, CK7, Cav-1 and S100A1 on our cohort of patients. In addition, we also investigated novel biomarkers, Ob and ObR to assess their differential expressions if any between chRCC and RO; and the biomarker KIM-1 that have not been specifically assessed to compare chRCC and RO in the past.

5.1 INTRODUCTION

Currently there is no "gold standard" for IHC biomarkers that can aid in the differentiation of chRCC and RO, with pathologists relying on the histological features on routine H&E. Therefore, since there exists considerable overlap in the histological features, especially eosinophilic variants of chRCC from RO, general pathologists who are relying on histology

and may be inexperienced uropathologists will face difficulties in discerning the two entities (Tan et al. 2013). As discussed in the meta-analysis of chapter 3, numerous IHC biomarkers have been employed to aid in distinguishing cases of chRCC from RO where overlapping morphological features present a diagnostic dilemma. Three biomarkers analysed in this chapter were chosen from extensive literature review. Following the meta-analysis, CK7, Cav-1 and S100A1 were chosen because of their apparent usefulness in differentiating the two renal tumour entities. As novel biomarkers, Ob, ObR and KIM-1 were selected based on literature that indicates KIM-1 expression is increased in ccRCC but not chRCC and RO (Han et al. 2005; Zhang et al. 2014); and the significant association of obesity as a relative risk factor for development of RCC (Ljungberg et al. 2011). Since leptin is an adipokine that is produced by abundant adipocytes in obese patients, it was important to study the relationship of Ob and ObR in differentiation of chRCC and RO, and in malignancies such as ccRCC. Following the IHC results of these biomarkers, it is hoped that in future these results can be replicated in pre-operative diagnostic pathways via plasma, urine or biopsy tissue.

5.2 MATERIALS AND METHODS

The materials and methods employed in this Chapter have been described in Chapter 2. Archived human renal tumour slides with paired normal kidney were obtained from Aquesta Pathology. The renal tumour slides were obtained from a dedicated uropathology centre, Aquesta Pathology with experienced uropathologists who specialise in genitourinary malignancy. IHC analyses for various biomarkers were performed with help from Mr. Clay Winterford at QIMR-Berghofer Histology Facility and Dr. David Small at CKDR in TRI. Positive and negative controls were routinely stained with each batch of IHC. The relevant ethics approvals have also been discussed in Chapter 2 and are included in Appendix 4.

5.3 CLINICAL CHARACTERISTICS OF RCC NEPHRECTOMY SPECIMENS

Seventy-five formalin-fixed, paraffin-embedded human renal tumour specimens were obtained from Aquesta Pathology. These were from 75 patients who underwent RCC tumour nephrectomy at various centres in Brisbane from 2009 to 2014. Each of the sections from these blocks had some non-cancerous kidney as well as the tumour. Ratio of males:females was 1.9:1, with median age of 64 years (range 18-88), in concordance with more RCC in males than females, and most patients being in the 50s to 60s age group. The median renal tumour size from this series was 3.8cm (range 1.2 – 18). From this case series, there were 30 ccRCC, 30 chRCC and 15 RO. Although the ultimate aim was identifying IHC biomarkers that differentiated chRCC and RO, ccRCC was included for completeness as this subtype of RCC is the most common, constituting about 70-80% of all RCC. The low number (15 cases) of RO analysed in this study was because RO account for approximately only 5% of all adult renal tumours (Kawaguchi et al. 2011). Among the ccRCC cases, 63.3% were Fuhrman grade 2, 20% grade 3 and 16.7% grade 4. The histopathological diagnoses were made by experienced uropathologists in Aquesta Pathology. Although it is recognised that the system for grading RCC has been modified in the past 2 years (Delahunt et al. 2014), these samples were graded using the older Fuhrman grading system, and these grades will be utilised for these specimens.

One third of the patients underwent partial nephrectomy (25 out of 75 patients). The majority of the patients were in stage T1 (62.7%), and the rest were in T2 (9.3%), T3 (26.7%) and T4 (1.3%). The trend of patients presenting with smaller confined tumours in T1 stage is due to

increasing detection rates for incidental renal tumours from widespread availability of radiological imaging; similar with other published series (Duchene et al. 2003). There were only 2 patients who presented with metastatic disease and underwent cytoreductive nephrectomy subsequently. These results are summarised in Table 5.1

Table 5.1:- Clinicopathological characteristics of the cohort of RCC patients

Patients	75		
Period	2009 – 2014		
Gender	49 Male : 26 Female		
Median age (years)	64 (18-88)		
Median size (cm)	3.8 (1.2-18)		
Nephrectomy	Partial = 25 (33.3%) Radical = 50 (66.7%)		
Subtype	30 ccRCC	30 chRCC	15 RO
T stage	ccRCC	chRCC	RO
T1 = 47 (62.7%)	20(66.7%)	15(50%)	12(80%)
T2 7 (9.3%)	0	5(16.7%)	2(13.3%)
T3 = 20 (26.7%)	9(30%)	10(33.3%)	1(6.7%)
T4= 1 (1.3%)	1(3.3%)	0	0
M1 stage	2 (2.67%)		
Fuhrman (ccRCC)			
Grade 2	63.3%		
Grade 3	20%		
Grade 4	16.7%		

Tumour (T), Metastases (M),

5.4 RESULTS OF IMMUNOHISTOCHEMISTRY AND MORPHOMETRY

5.4.1 Cytokeratin 7 (CK7)

CK7 is a low molecular weight keratin, belonging to a large family of structural polypeptides that are the fundamental markers of epithelial differentiation. The CKs found in simple epithelia (CK7, CK8, CK18 and CK19) are widely expressed in normal kidney and renal neoplasms (Skinnider et al. 2005). In the present study, normal renal cortical tissue adjacent to the tumours showed positivity for CK7 in the cytoplasm of distal tubular cells (identified by structure). There was strong membranous and cytoplasmic expression of CK7 in chRCC, with minimal or no staining in ccRCC and RO, as seen in Figure 5.1. In chRCC, there was intense cytoplasmic immunostaining with characteristic strong peripheral membrane staining. Based on the IHC characteristics, overall positive pixel expression was analysed using Aperio ImageScope.

5.4.1.1 Morphometry of CK7 (positive pixels) and overall expression

The overall positive pixel expression in tumour (ccRCC, chRCC and RO included) was lower compared to normal renal tissue (Figure 5.2A). When compared separately, ccRCC and RO had significantly lower overall positive pixel expression compared to normal tissue ($p<0.0001$ and $p=0.002$ respectively) (Figure 5.2B, 5.2D). However, there was no significant difference between the overall expression of CK7 in chRCC compared to normal renal tissue (Figure 5.2C). Therefore, the expression of CK7 immunostaining was higher in chRCC when compared to RO and ccRCC (Figure 5.2E). Importantly, there was significantly higher expression of CK7 in chRCC in compared with RO ($p=0.03$) as shown in Figure 5.2F. This significantly different expression pattern of CK7 in both chRCC and RO provide a useful and efficient IHC biomarker that can aid in differentiating the two entities.

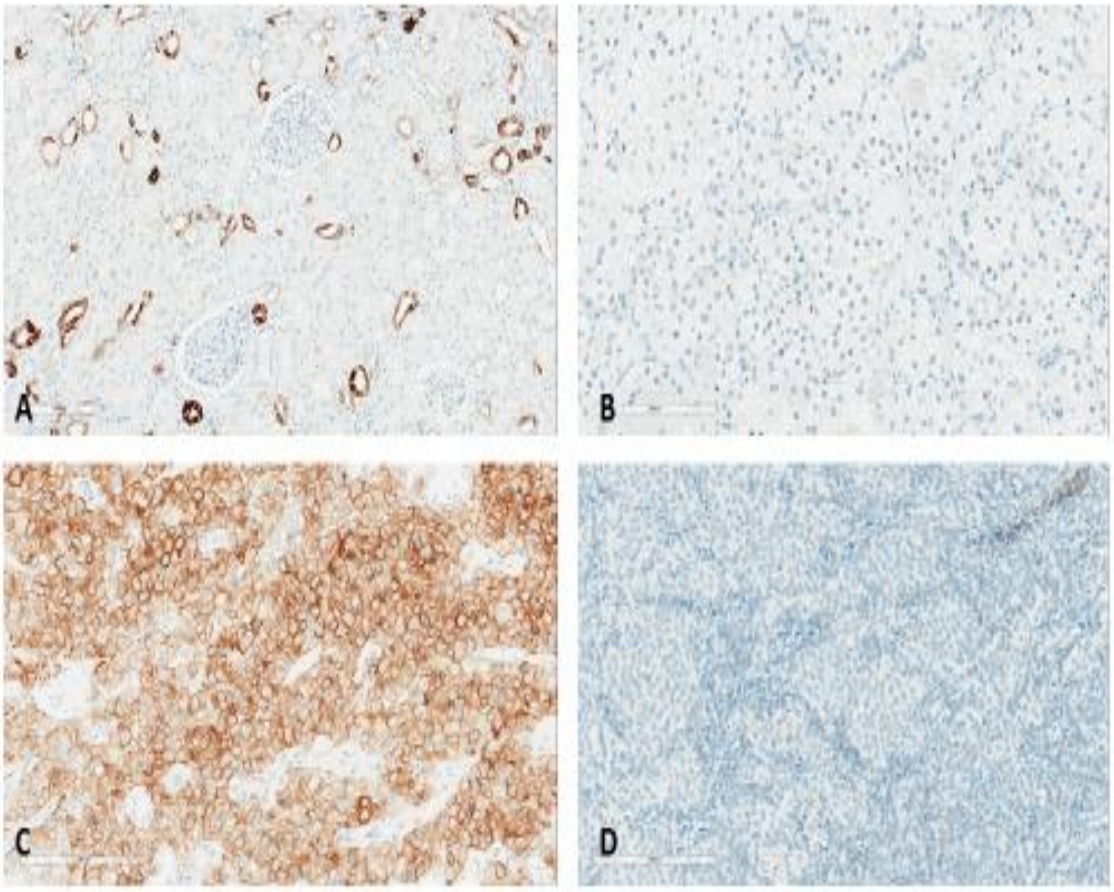

Figure 5.1: CK7 immunohistochemistry

A. Immunostaining in normal renal cortical tissue shows CK7 positivity in the vessels and some tubular epithelium; **B**. In clear cell RCC, minimal CK7 IHC is visible; **C**. CK7 IHC is strong in chRCC; **D**. RO was clear of CK7 staining. Scale bar 200µm. (x20 Aperio magnification)

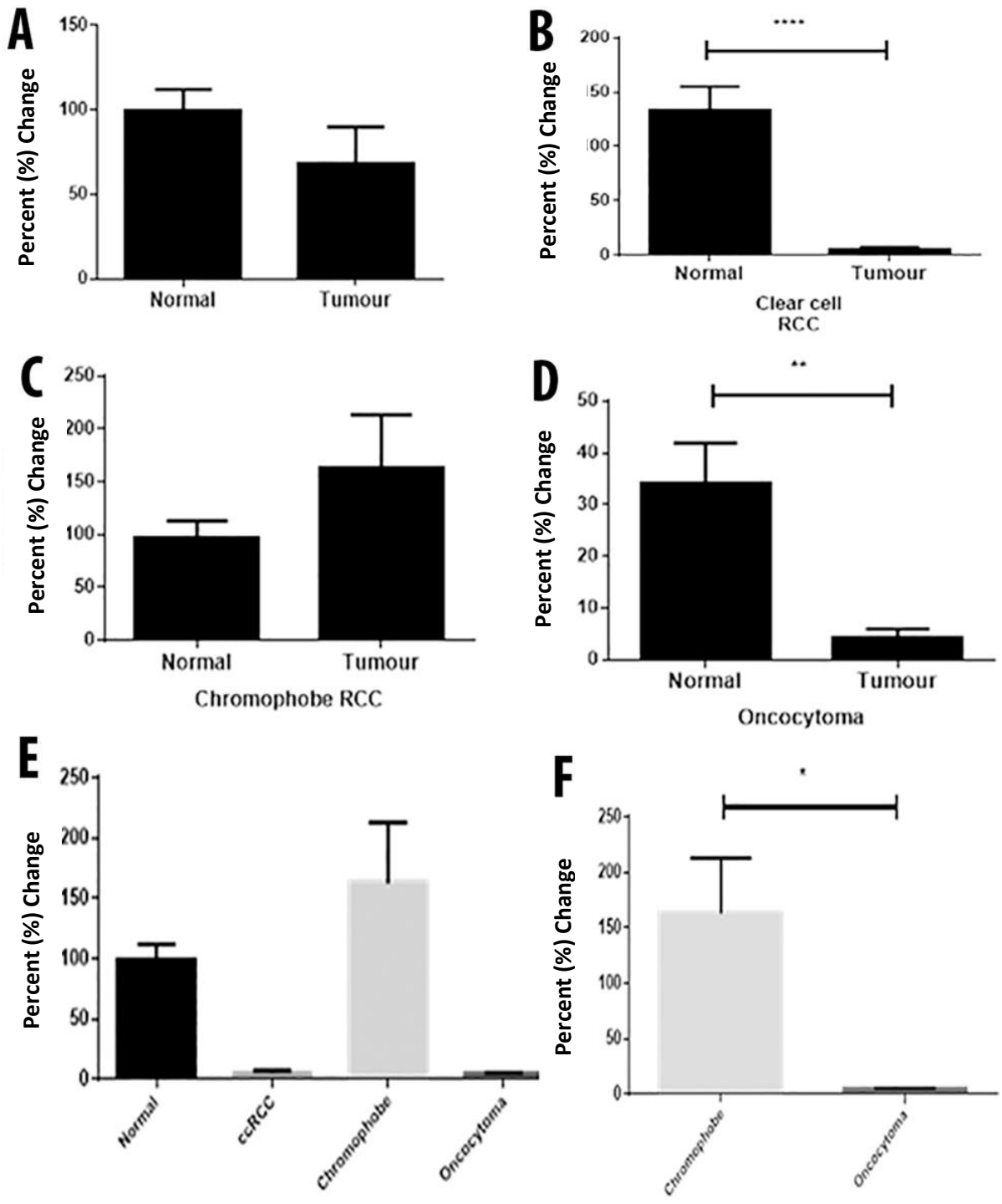

Figure 5.2: Expression of CK7 in renal tumours and matched normal renal tissue

A. Expression of CK7 in normal vs tumour; **B**. Decreased expression of CK7 in ccRCC vs normal kidney (****p<0.0001); **C**. Increased expression of chRCC vs normal kidney; **D**. Decreased expression of CK7 in RO vs normal kidney (**p=0.002); **E**. Expression of CK7 in tumour subtypes; **F**. Significantly increased expression of CK7 in chRCC vs RO (*p=0.03).

5.4.2 Caveolin-1

Cav-1 is a membrane protein present in most cells. Cav-1, a 24kDa membrane protein, is a major component of membrane caveolae. Functionally, Cav-1 serves important roles in macromolecular transcytosis, endocytosis of pathogens, lipid metabolism and cellular signal transduction (Cohen et al. 2004). In non-neoplastic renal tissue, there was minimal basolateral membrane and cytoplasmic staining in distal convoluted tubules, along with staining of vascular endothelial cells. The immunostaining patterns of Cav-1 were mainly membranous in ccRCC, diffuse cytoplasmic in chRCC and patchy cytoplasmic in RO, as shown in Figure 5.3A-D. On closer inspection, there was a distinguishing staining pattern observed in chRCC where there was diffuse cytoplasmic with peripheral membranous enhancement and a perinuclear halo; compared to patchy granular cytoplasmic staining in RO (Figure 5.3C, D). This distinctly different Cav-1 immunostaining pattern between chRCC and RO may prove to be useful in separating the two tumour subtypes. Based on the IHC staining patterns, overall and membrane expressions were analysed on Aperio ImagScope.

5.4.2.1 Morphometry of Cav-1 (positive pixels) and overall expression

All ccRCC, chRCC and RO had significantly higher overall expression of Cav-1 compared to normal renal cortical tissue (Figure 5.4A). Individually, ccRCC Cav-1 expression was significantly higher compared to normal kidney ($p=0.01$, Figure 5.4B); chRCC recorded similarly higher expression ($p<0.0001$, Figure 5.4C), with RO also having higher immunostaining compared to paired normal kidney ($p=0.003$, Figure 5.4D). As demonstrated in Figure 5.4E, ccRCC had higher expression compared to chRCC, which had higher expression than RO. There was very little difference in overall Cav-1 expression in chRCC

versus RO. However as shown in Figure 5.3, the useful discriminatory feature lies in the different staining patterns between chRCC and RO.

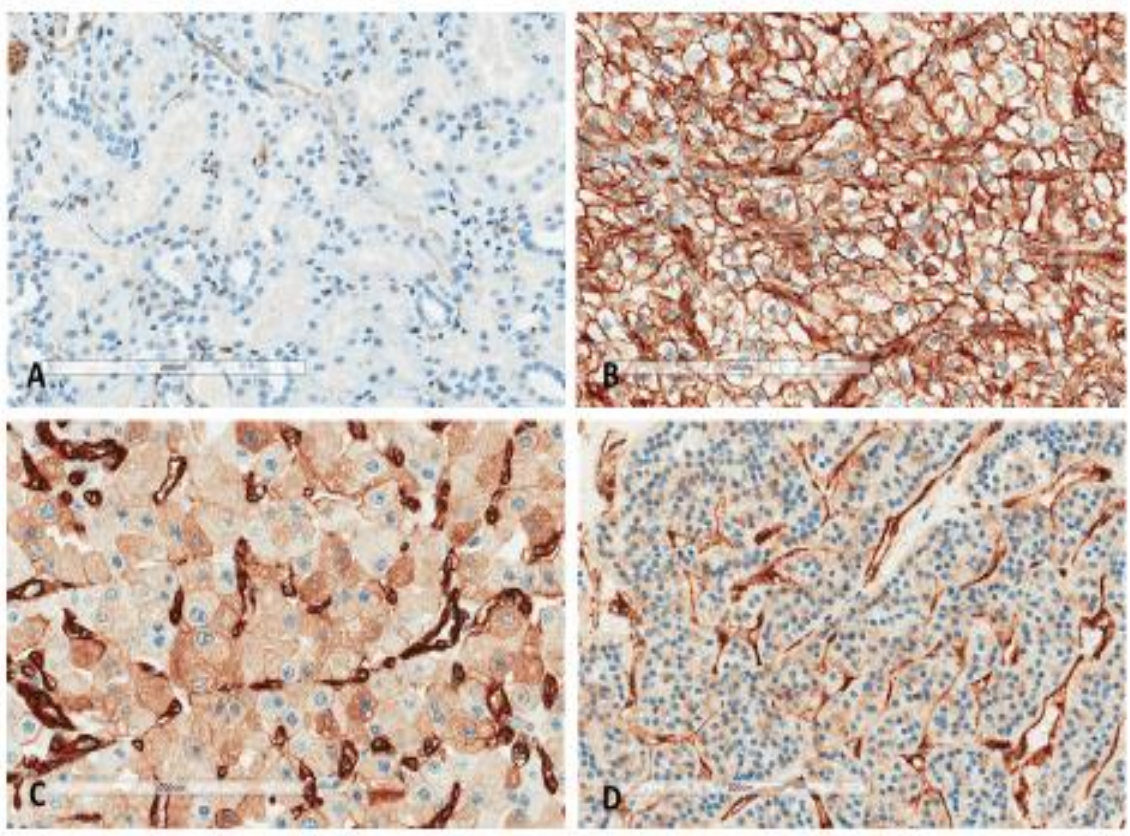

Figure 5.3: Caveolin-1 immunohistochemistry

A. Immunostaining of Cav-1 in normal renal cortical tissue localised to cytoplasm of distal convoluted cells and vascular endothelial cells. Proximal tubular epithelial cells were clear of Cav-1; **B**. In clear cell RCC, Cav-1 staining was mainly membranous; **C**. Strong diffuse Cav-1 cytoplasmic staining with peripheral enhancement and a perinuclear halo was noted in chRCC; **D**. Patchy granular cytoplasmic staining was seen in RO. Scale bar 200µm. (x20 Aperio magnification)

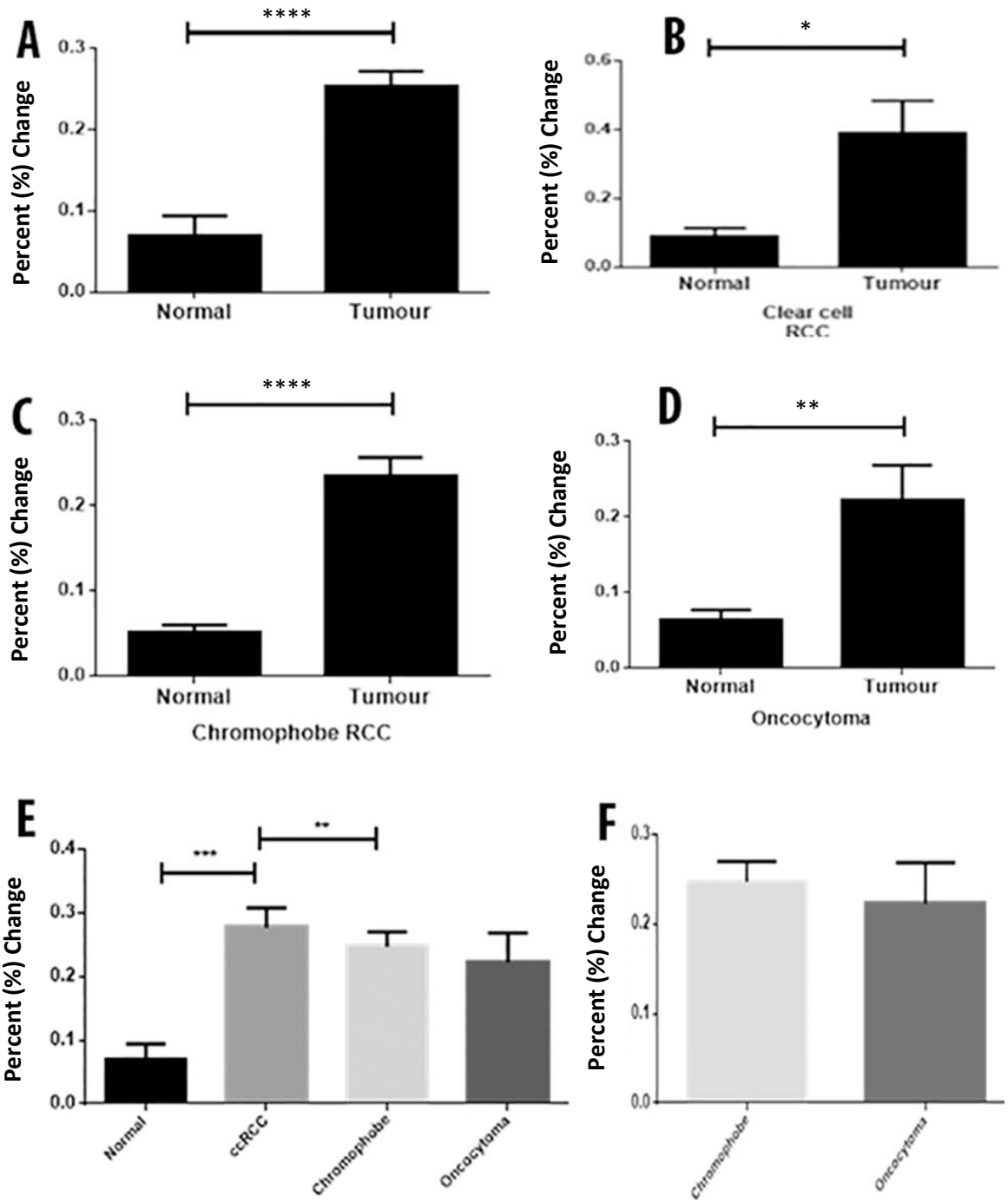

Figure 5.4: Expression of Cav-1 in renal tumours and matched normal renal tissue

A. Increased expression of Cav-1 in tumour vs normal tissue (****p<0.0001); **B.** Increased overall Cav-1 expression in ccRCC vs normal kidney (*p=0.01); **C.** Increased overall Cav-1 expression of chRCC vs normal kidney (****p<0.0001); **D.** Increased overall Cav-1 expression in RO vs normal kidney (**p=0.003); **E.** Expression of Cav-1 in tumour subtypes; **F.** Overall expression of Cav-1 in chRCC vs RO.

5.4.2.2 Caveolin 1 membrane expression

Since there was notable membranous enhancement in ccRCC and chRCC, the membranous immunostaining of Cav-1 was analysed quantitatively using Aperio ImageScope, asking the question "are there any differences in the membranous expression of Cav-1 in ccRCC, chRCC and RO?" Membranous expression of all tumours (ccRCC, chRCC and RO) was significantly higher when compared to normal renal cortical tissue ($p<0.0001$, $p<0.0001$ and $p=0.003$ respectively) as shown in Figures 5.5 A-D. Membranous expression was highest in ccRCC followed by chRCC then RO (Figure 5.5E). However, despite a higher membranous Cav-1 expression in chRCC compared to RO, it was not statistically significant ($p=0.1$) as shown in Figure 5.5F.

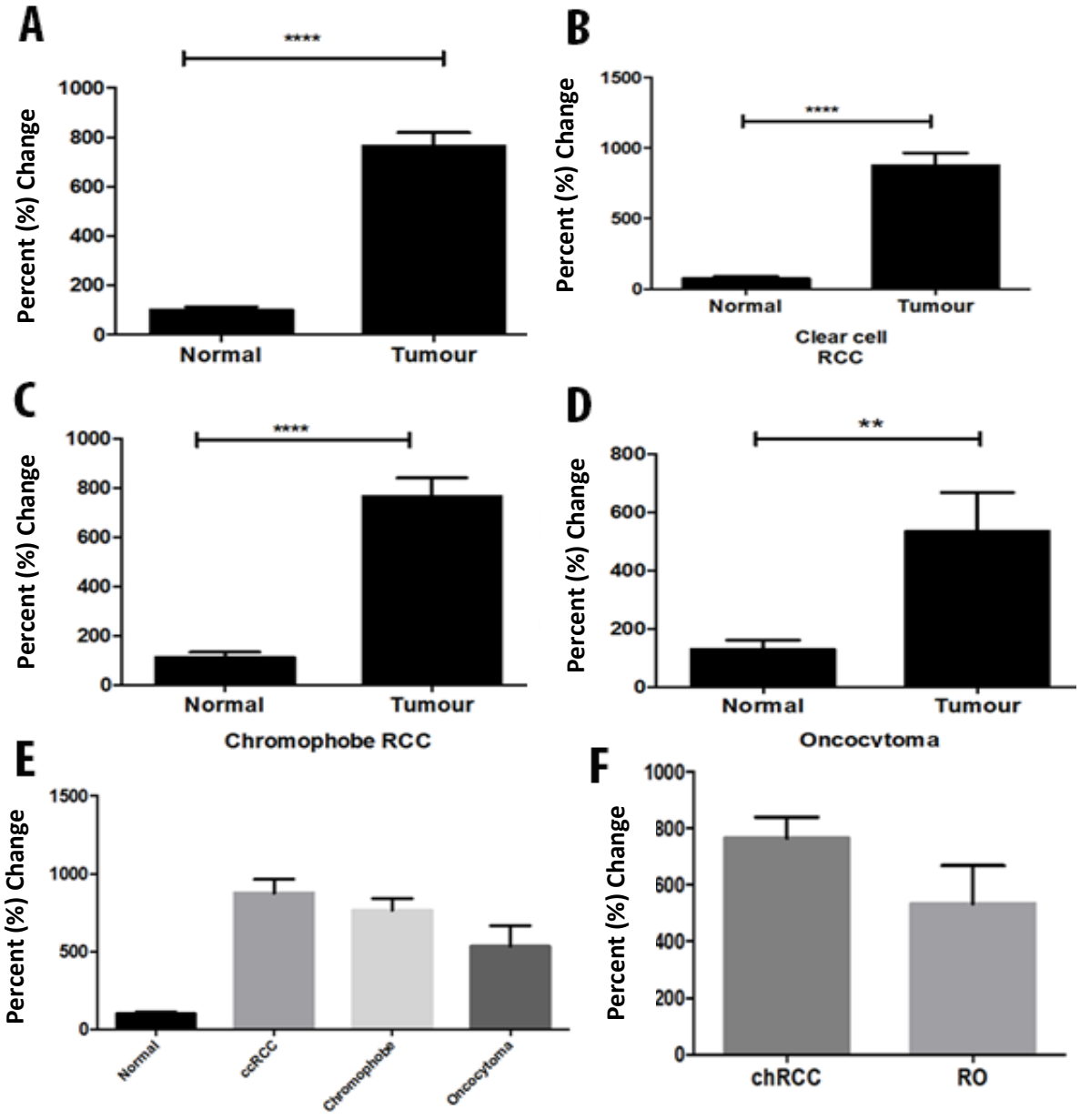

Figure 5.5: Expression of Cav-1 (membranous) in tumours and matched normal renal tissue

A. Increased membranous expression of Cav-1 in tumour vs normal kidney(****p<0.0001); **B**. Increased membranous Cav-1 expression in ccRCC vs normal kidney(****p<0.0001); **C**. Increased membranous Cav-1 expression of chRCC vs normal kidney(****p<0.0001); **D.** Increased membranous Cav-1 expression in RO vs normal kidney(**p=0.003); **E**. Expression of Cav-1(membranous) in tumour subtypes; **F**. Expression of Cav-1 (membranous) in chRCC vs RO.

5.4.3 Leptin (Ob)

Leptin is a hormone made by adipose cells that helps to regulate energy balance by inhibiting hunger. Obesity is a known risk factor for RCC and leptin is increased in obesity. Studies have strongly suggested that leptin plays a role in carcinogenesis through cell proliferation, angiogenesis, apoptotic inhibition and proinflammatory effects (Housa et al. 2006; Renehan et al. 2008; Tilg and Moschen 2006). Ob was used as the alias for leptin as the Ob(Lep) gene codes for the human leptin protein. From the associated risk of obesity with the development of RCC and previous studies that correlated serum leptin and its receptor to RCC progression (Horiguchi et al. 2006), we investigated the expression of Ob and ObR in RCC. CcRCC tumour cells have "clear" cytoplasm due to its abundant lipids and glycogen. Therefore, we postulated that there might be differences in expression of these biomarkers in RCC subtypes, since other subtypes (ie chRCC and RO) do not share the same abundant lipids in their cytoplasm. Therefore we studied the IHC of both leptin (Ob antibody) and its receptor (ObR antibody) in our human renal tumour tissues. Based on the IHC Ob staining characteristics, overall, nuclear and membrane expression were analysed on Aperio ImageScope.

5.4.3.1 IHC showing Ob positive pixel and overall expression

IHC of Ob revealed mainly nuclear staining with some cytoplasmic expression in adjacent normal renal parenchyma and in ccRCC. In chRCC, the staining was mainly cytoplasmic with minimal or none in nuclear regions, in contrast to RO where the staining was more diffuse in the cytoplasm and prominent nuclear staining patterns were seen (Figure 5.6 A-D).

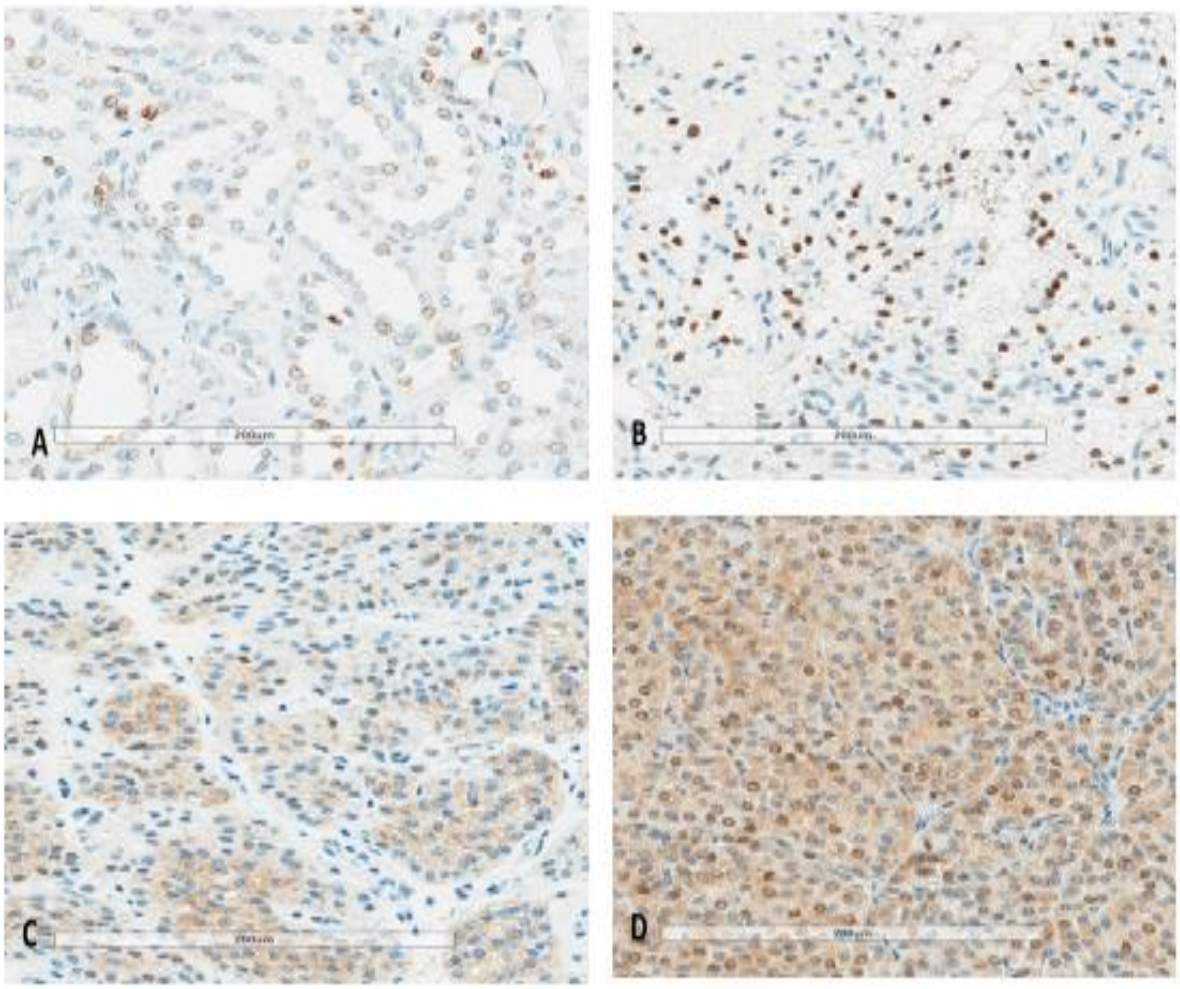

Figure 5.6: Immunostaining of leptin (Ob)

A. Immunostaining of Ob is demonstrated in normal renal cortical tissue and shows nuclear and some cytoplasmic staining; **B**. In clear cell RCC, nuclear Ob staining was prominent; **C**. Ob IHC was localised to cytoplasm in chRCC, with minimal staining; **D**. Diffuse cytoplasmic and nuclear staining of Ob was seen in RO. Scale bar 200µm. (x20 Aperio magnification)

5.4.3.2 Morphometry of IHC showing overall Ob expression

The expression of Ob in renal tumour tissue was higher than normal tissue (Figure 5.7A). In ccRCC, chRCC and RO, there was significantly increased overall expression of Ob compared to normal kidney (p=0.01, p=0.01, p=0.03 respectively) as shown in Figures 5.7B-D. When compared to each tumour subtype, there was a trend of highest to lowest expression of Ob noted in RO followed by chRCC then ccRCC (Figure 5.7E). There was no significant difference in the overall expression of Ob inRO compared to chRCC (p=0.16) (Figure 5.7F).

5.4.3.3 IHC showing Ob nuclear expression

When nuclear expression of Ob was analysed, there was no marked difference between tumour and normal tissue. For ccRCC, there was higher expression in the tumours compared to normal tissue, but the difference did not reach significance. In RO, the Ob nuclear expression was similar in tumour and normal kidney. However in chRCC, there was significantly higher Ob nuclear expression in normal compared to chRCC tumour cells (Figure 5.8A-D). Importantly, there was significantly higher expression of Ob in nuclear regions of RO in contrast to chRCC, where there was minimal/absent nuclear staining (p=0.02) (Figure 5.8E, F). This useful differential nuclear expression between chRCC and RO can aid in the diagnosis of one subtype from the other.

5.4.3.4 Morphometry of IHC showing membrane expression of Ob

Membrane expression of Ob was increased in ccRCC and RO, but lesser in chRCC compared to normal (Figure 5.9A-D). When compared across the board, membrane expression of Ob was highest in RO, followed by ccRCC and chRCC. Although there was an increase in Ob membrane expression in RO compared to chRCC, it was not statistically significant (p=0.13) as shown in Figure 5.9E and 5.9F.

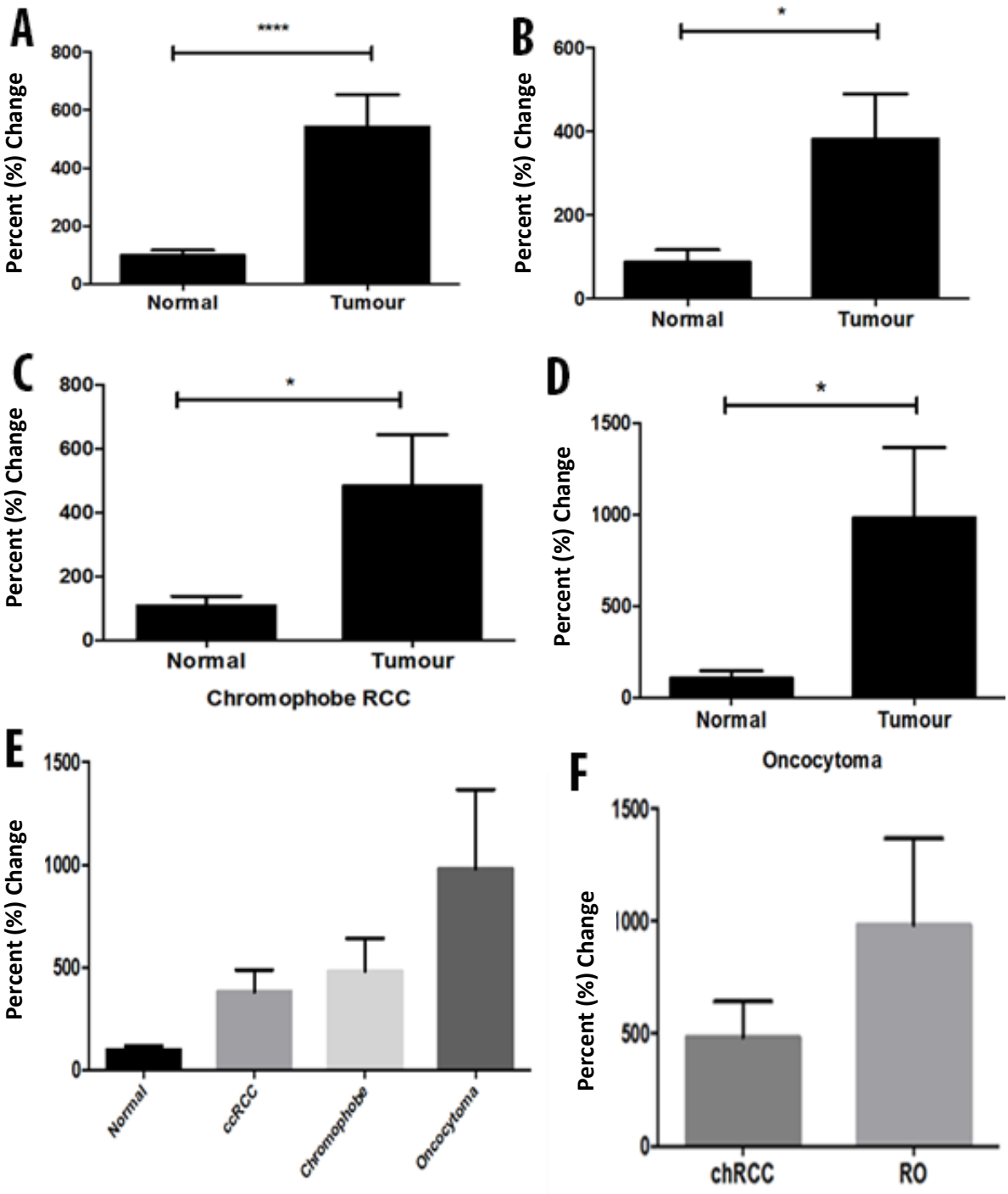

Figure 5.7: Ob overall expression in renal tumours and matched normal renal tissue

A. There was increased overall expression of Ob in tumour vs normal kidney (****p<0.0001); **B**. Increased overall Ob expression in ccRCC vs normal kidney (*p=0.01); **C**. Increased overall Ob expression of chRCC vs normal kidney (*p=0.01); **D**. Increased overall Ob expression in RO vs normal kidney (*p=0.03); **E**. Expression of Ob in is shown in tumour subtypes; **F**. Expression of Ob was higher in RO vs chRCC (not significant).

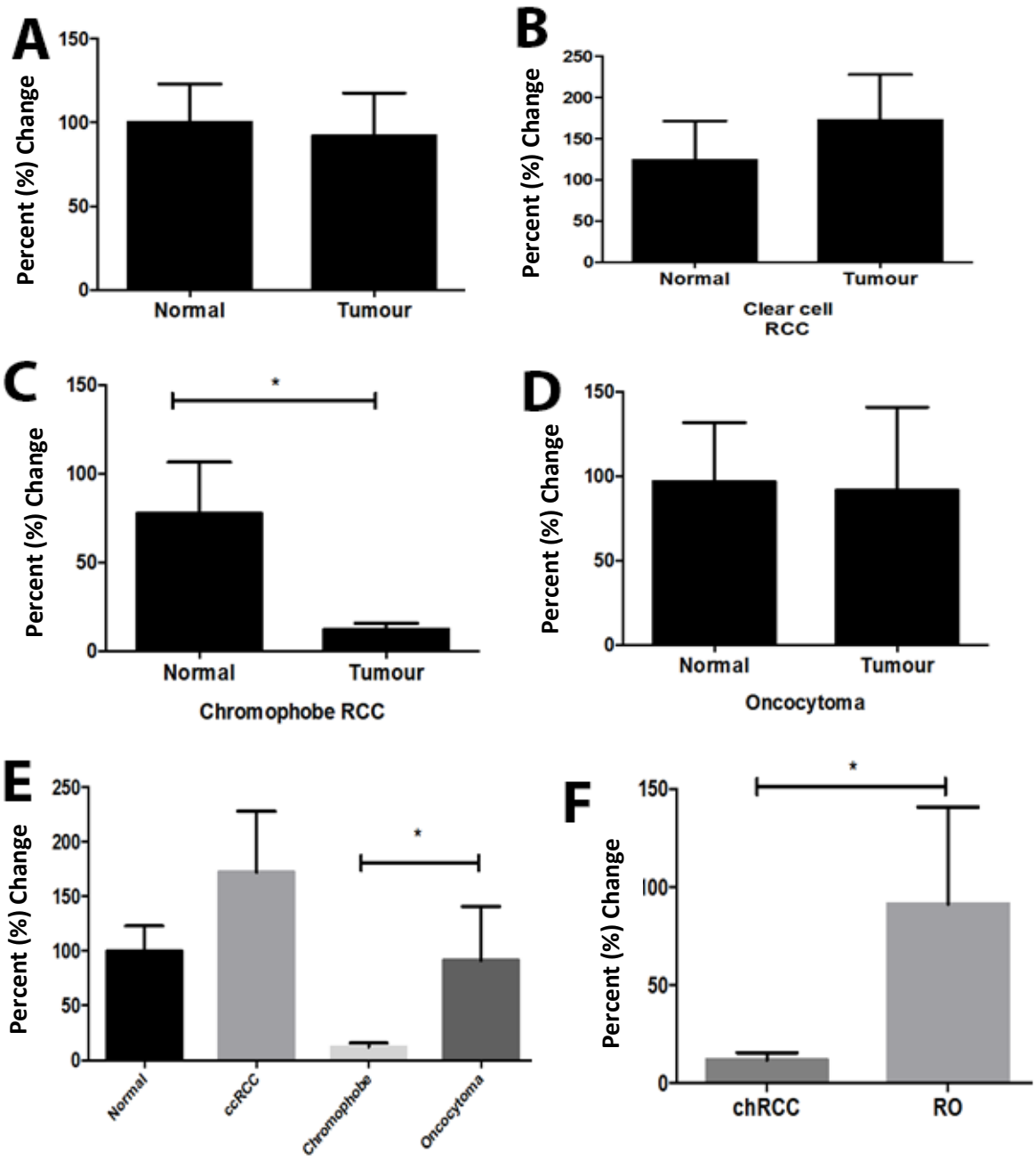

Figure 5.8: Ob nuclear expression in renal tumours and matched normal renal tissue

A. Nuclear expression of Ob in tumour vs normal kidney; **B**. Increased Ob nuclear expression in ccRCC vs normal kidney; **C**. Minimal Ob nuclear expression of chRCC vs normal kidney (*p=0.03); **D**. Ob nuclear expression in RO vs normal kidney; **E**. Expression of Ob nuclear in tumour subtypes; **F**. Increased expression of nuclear Ob in RO vs chRCC (*p=0.02).

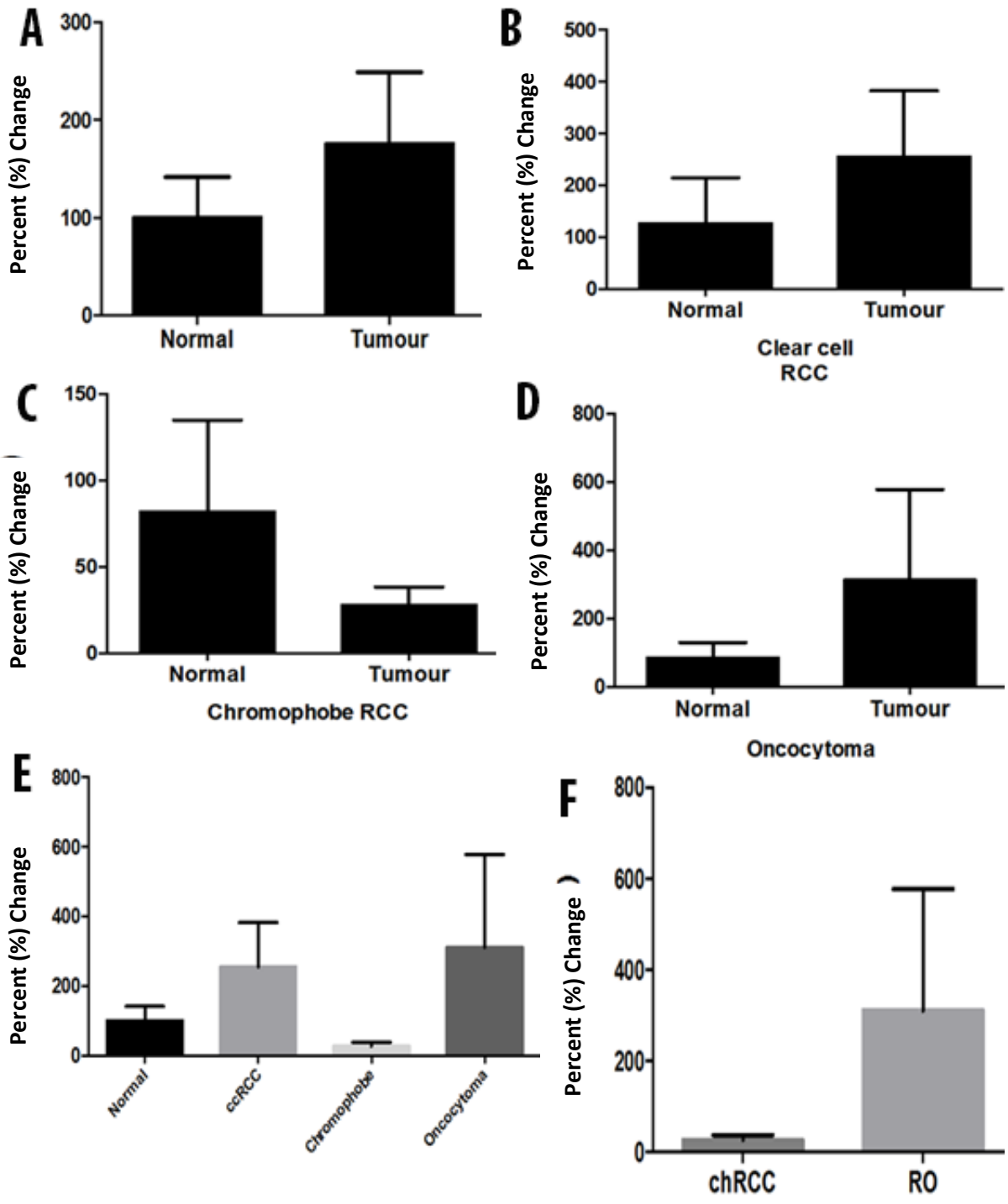

Figure 5.9: Ob membrane expression in renal tumours and matched normal renal tissue

A. Membrane expression of Ob in tumour vs normal; **B**. Increased Ob membrane expression in ccRCC vs normal; **C**. Minimal Ob membrane expression of chRCC vs normal; **D**. Ob membrane expression in RO vs normal; **E**. Expression of Ob membrane in tumour subtypes; **F**. Increased expression of Ob membrane in RO vs chRCC (not significant).

5.4.4 Leptin receptor (ObR)

Ob acts through its receptor ObR, a single-transmembrane-domain receptor of the cytokine receptor family. Previous study have shown that increased leptin/ObR signalling may promote renal cancer cell invasion and metastasis (Horiguchi et al. 2006a). For IHC, there was cytoplasmic staining of ObR in the proximal convoluted tubular cells of non-neoplastic renal cortical tissue. In ccRCC, both cytoplasmic and nuclear staining were evident. The cells in chRCC had variable mild cytoplasmic stains with no nuclear staining at all. In comparison, in RO, there was more intense diffuse cytoplasmic and nuclear staining. The examples of these sections are shown in Figures 5.10A-D. Overall, nuclear and membrane expression were analysed on Aperio ImageScope based on the IHC staining patterns of ObR.

5.4.4.1 IHC and overall expression patterns of ObR

Generally all tumours had stronger ObR expression than normal renal tissue ($p<0.0001$) as depicted in Figure 5.11A. The overall expression patterns of ObR in ccRCC, chRCC and RO were all significantly elevated compared with to normal kidney tissue ($p=0.005$, $p=0.02$, $p=0.05$, respectively) as shown in Figures 5.11B-D. RO had the strongest expression followed by similar but lowerlevels of intensity in ccRCC and chRCC. RO recorded a higher ObR overall expression compared to chRCC, but the difference was not significant, $p=0.23$ (Figures 5.11E, F).

5.4.4.2 Nuclear expression patterns of ObR

All tumours recorded stronger ObR nuclear immunostaining compared to normal kidney tissue (Figure 5.12A-D), however, the differences were not significant. ccRCC had the highest nuclear staining followed by RO then chRCC. There was no difference in ObR nuclear expression between RO and chRCC ($p=0.72$) as shown in Figures 5.12E, F.

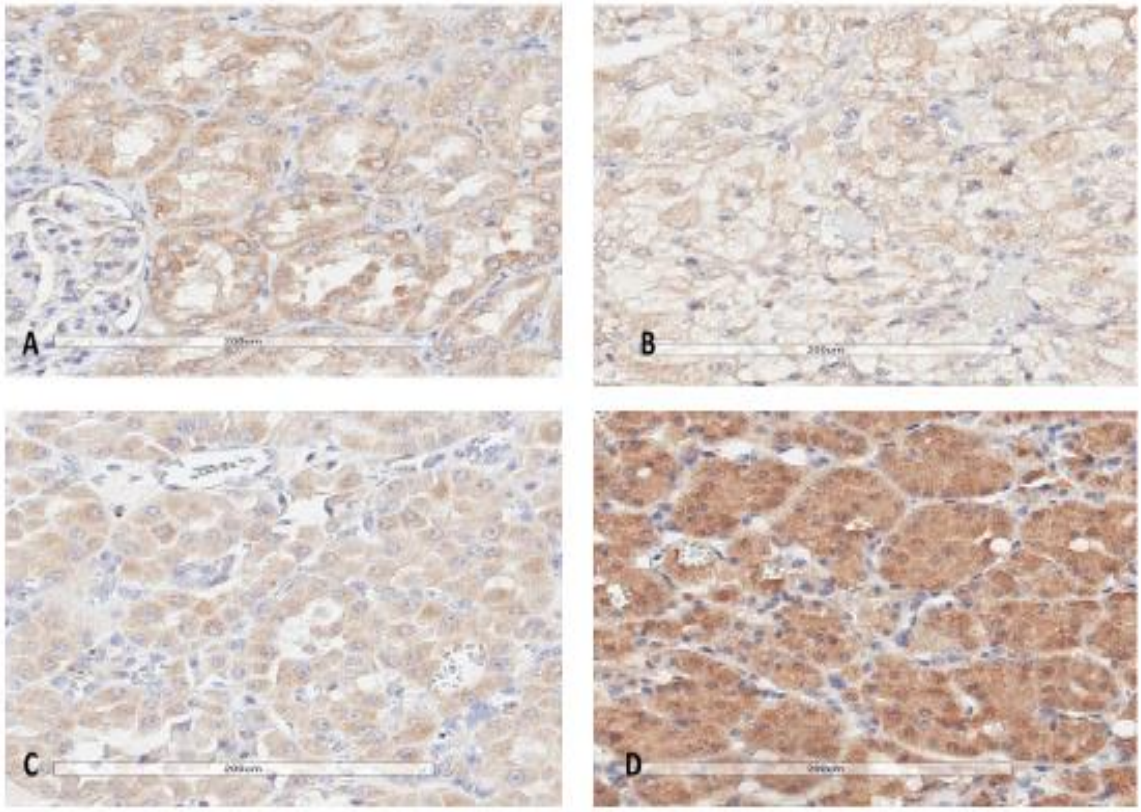

Figure 5.10: ObR immunostaining in renal tumour and matched normal renal cortical tissue

A. Immunostaining of ObR in normal renal cortical tissue showing cytoplasmic staining; **B**. In clear cell RCC, nuclear and cytoplasmic ObR staining was visible; **C**. ObR staining was minimal in cytoplasm with no nuclear staining in chRCC; **D**. Diffuse cytoplasmic and nuclear staining of ObR in RO. Scale bar 200µm. (x20 Aperio magnification)

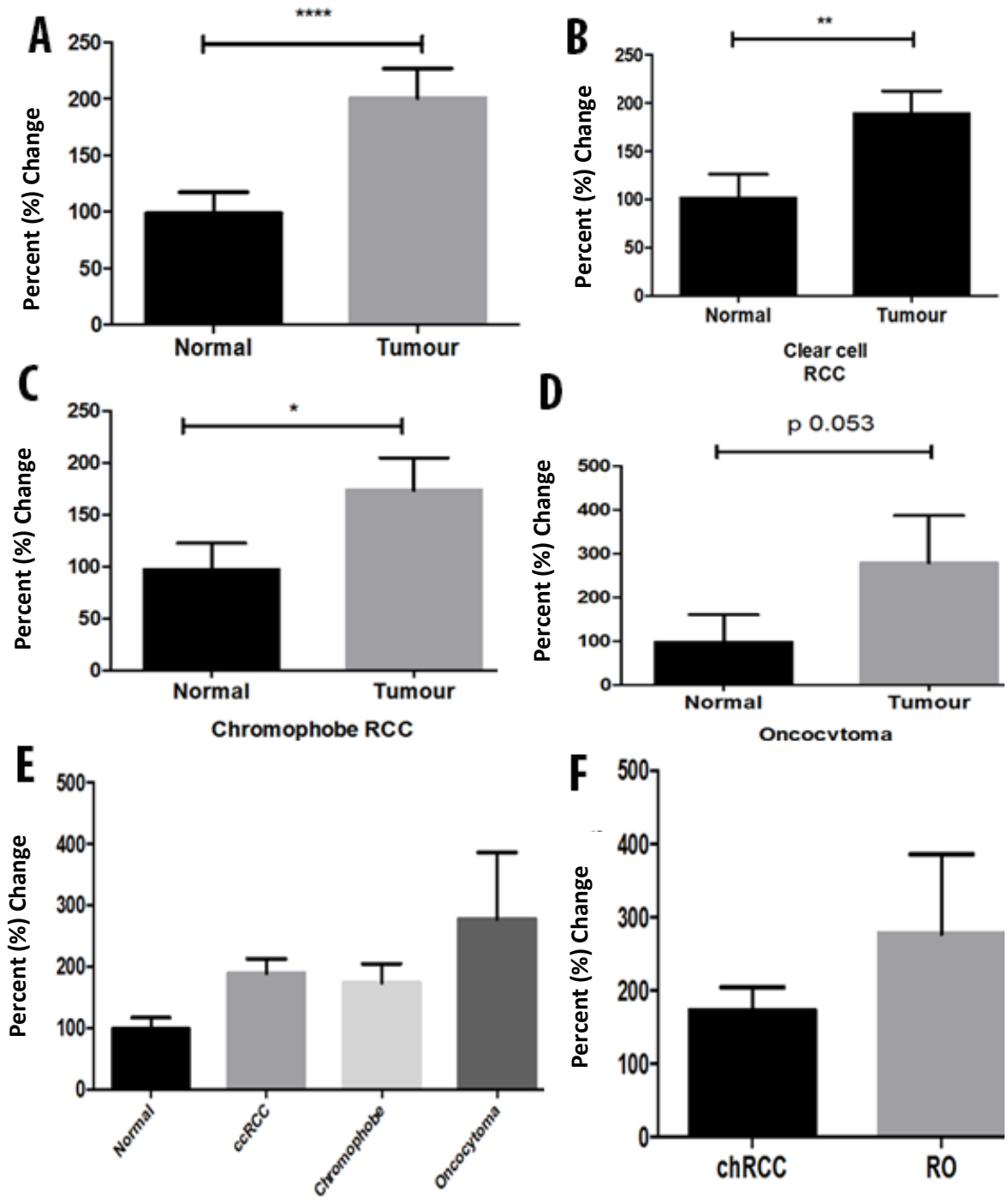

Figure 5.11: ObR expression patterns in renal tumours and matched normal renal tissue

A. Increased expression of ObR overall in tumour vs normal kidney (****p<0.0001); **B.** Increased ObR overall expression in ccRCC vs normal kidney (**p=0.005); **C.** Increased ObR overall expression of chRCC vs normal kidney (*p=0.02); **D.** ObR overall expression in RO vs normal kidney; **E.** Expression of ObR in tumour subtypes; **F.** Expression of overall ObR in RO vs chRCC.

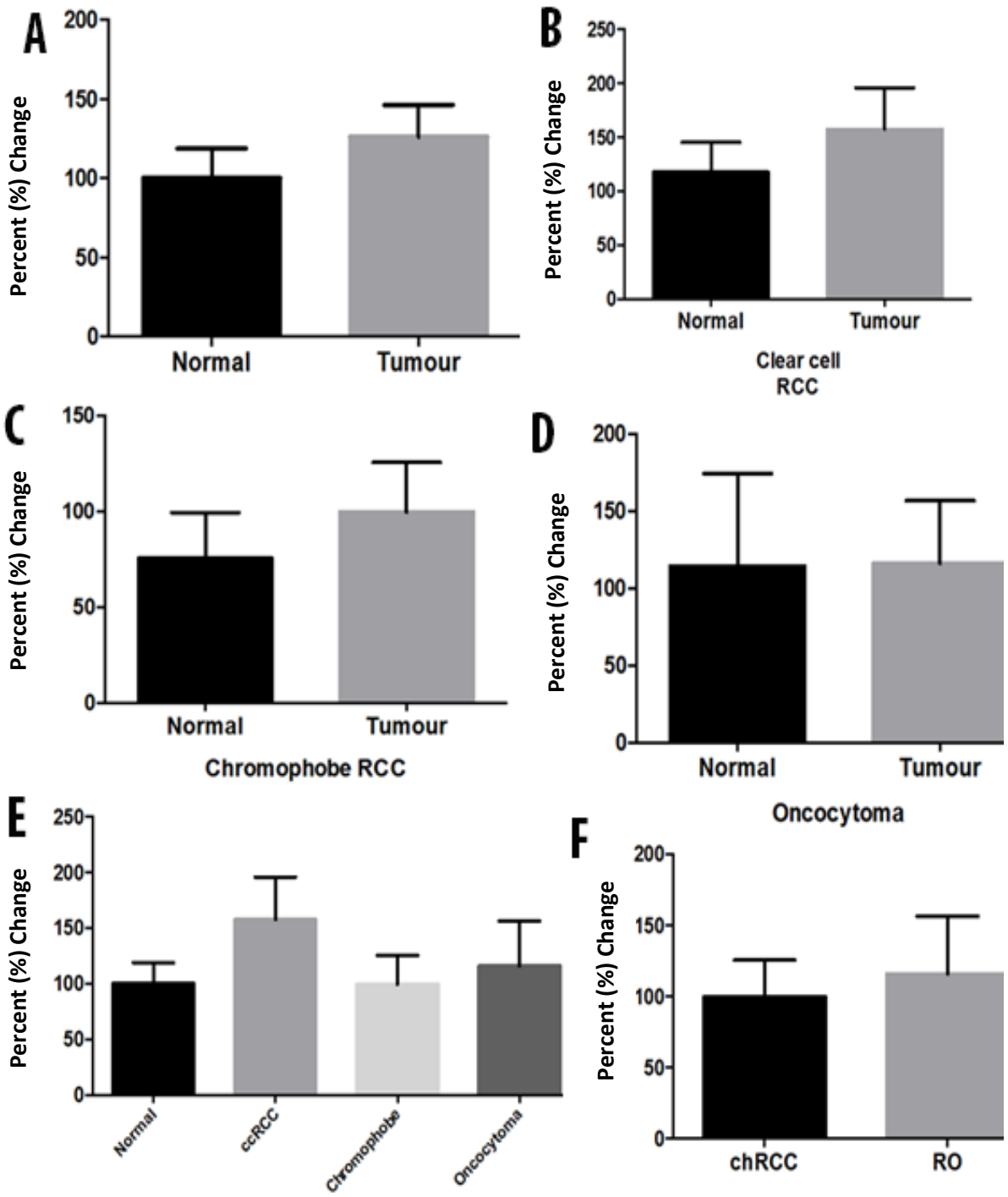

Figure 5.12: ObR nuclear expression in renal tumours and matched normal renal tissue

A. Expression of ObR nuclear in tumour vs normal kidney; **B.** ObR nuclear expression in ccRCC vs normal kidney; **C.** ObR nuclear expression of chRCC vs normal kidney; **D.** ObR nuclear expression in RO vs normal kidney; **E.** Expression of ObR nuclear in tumour subtypes; **F.** Expression of ObR nuclear in RO vs chRCC.

5.4.4.3 Membrane expression of ObR

Morphometry of membrane IHC of ObR revealed tumours collectively had more intense membranous staining compared to normal kidney tissue; except for chRCC which had lower expression (Figures 5.13 A-D). Oncocytoma had strongest membranous staining compared to ccRCC and chRCC. The difference in ObR membranous expression between RO and chRCC was not significant p=0.08 (Figure 5.13E, F).

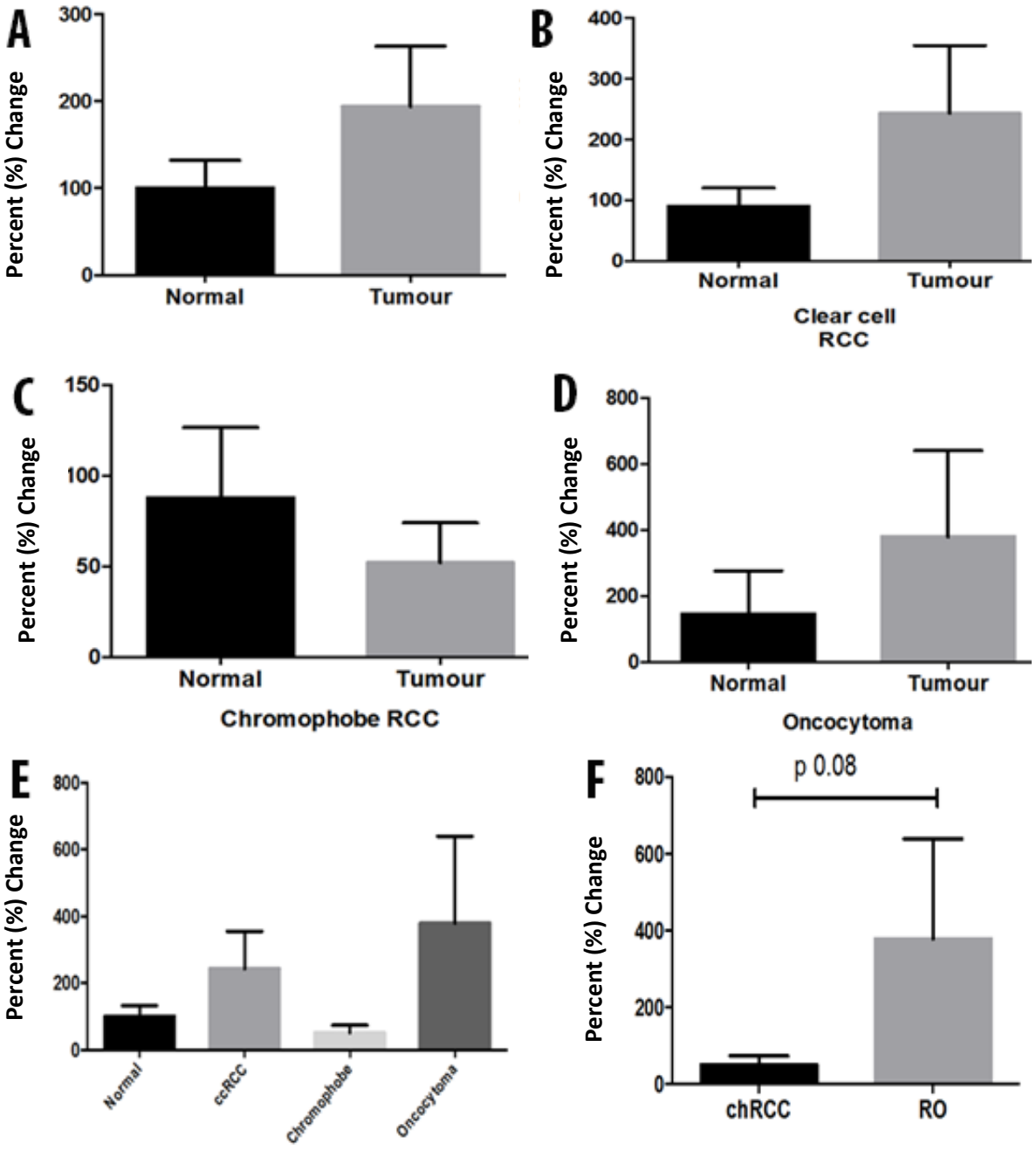

Figure 5.13: ObR membrane expression in renal tumours and matched normal renal tissue

A. Expression of ObR membrane in tumour vs normal kidney; **B**. ObR membrane expression in ccRCC vs normal kidney; **C**. ObR membrane expression of chRCC vs normal kidney; **D**. ObR membrane expression in RO vs normal kidney; **E**. Expression of ObR membrane in tumour subtypes; **F**. Expression of ObR nuclear in RO vs chRCC.

5.4.5 Kidney injury molecule-1

KIM-1 is a type 1 transmembrane protein, with an immunoglobulin and mucin domain, whose expression is markedly up-regulated in the proximal tubule following kidney injuries. : KIM-1 has been investigated widely and has been found to be a useful biomarker in acute and chronic kidney injuries and also in RCC (Bonventre 2014). The utility of KIM-1 has not gained widespread clinical usage despite its promising published research results in acute and chronic kidney injury studies (Liangos et al. 2007, van Timmeren et al. 2007). However, there was increased expression of KIM-1 noted in renal tumours compared with normal kidney, mainly in ccRCC (Lin et al. 2007; Zhang et al. 2014). We wanted to investigate further if there was any differential expression in various renal tumour subtypes apart from ccRCC and to assess if there was any usefulness in differentiating chRCC from RO. Overall positive pixel expression of KIM-1 was analysed on Aperio ImageScope.

5.4.5.1 IHC of kidney injury molecule-1

In adjacent non neoplastic renal parenchyma, mainly cytoplasmic and some nuclear immunostaining of KIM-1 was noted in the proximal tubular cells (Figure 5.14 A). In the majority of ccRCC, there was intense diffuse cytoplasmic and membranous immunostaining (Figure 5.14B). There was also moderate cytoplasmic and nuclear staining in 12 out of 15 (80%) slides of RO (Figure 5.14D). The adjacent normal renal tissue near the RO tumours expressed cytoplasmic and nuclear immunostaining. There was minimal focal cytoplasmic and occasional nuclear expression of KIM-1 in 7 out of 30 (23.3%) chRCC slides (Figure 5.14C). In some chRCC tumour cells which did not express any immunostaining, the adjacent normal renal tissue did reveal positive immunostaining in cytoplasm of the tubular cells.

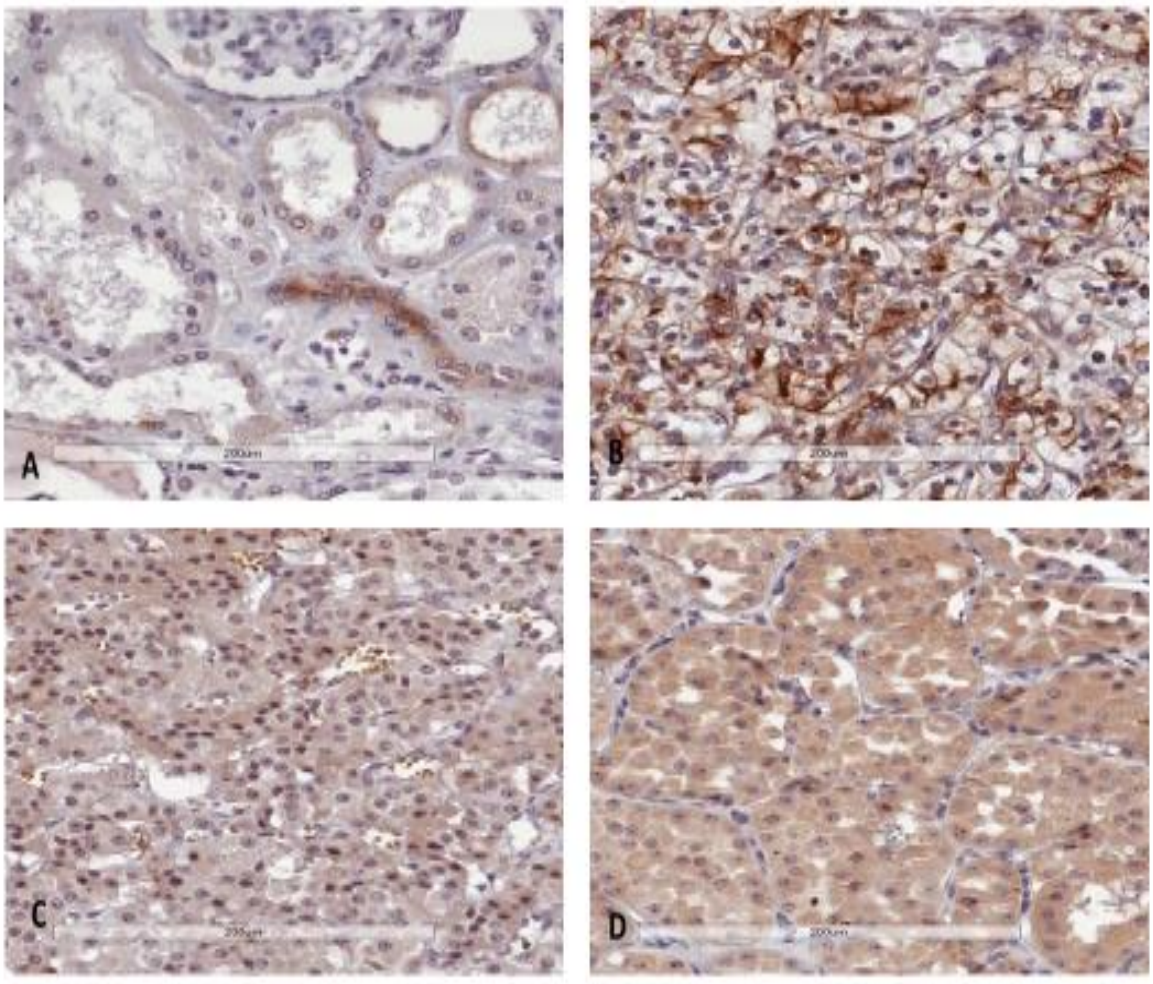

Figure 5.14: KIM-1 immunostaining in normal renal tissue and tumour tissue

A. Immunostaining of KIM-1 in adjacent normal renal cortical tissue showing mainly cytoplasmic and some nuclear staining; **B**. In clear cell RCC, there was intense membranous and cytoplasmic KIM-1 staining; **C**. There was focal cytoplasmic and occasional nuclear KIM-1 staining in chRCC; **D**. Moderate cytoplasmic and nuclear staining of KIM-1 in RO. Scale bar 200µm. (x20 Aperio magnification)

5.4.5.2 Morphometry of IHC for kidney injury molecule-1

All tumour subtypes recorded higher KIM-1 overall expression compared to normal kidney tissue ($p<0.0001$) (Figure 5.15A). In ccRCC, KIM-1 expression was markedly elevated when compared to its normal counterpart ($p<0.0001$) as shown in Figure 5.15B. As in ccRCC, RO tissue also recorded significantly higher levels compared to normal tissue, with $p=0.001$ as shown in Figure 5.15D. There was only a minimal increase (with no significant difference) in KIM-1 expression in chRCC compared with normal tissue (Figure 5.15C). When the tumours were compared, both RO and ccRCC had almost similarly-elevated expression of KIM-1 (Figure 5.15E), but there was a significantly higher expression of KIM-1 expressions in RO compared with chRCC ($p=0.002$). The difference between RO and chRCC could help in differentiating these two difficult-to-separate histological entities (Figure 5.15F).

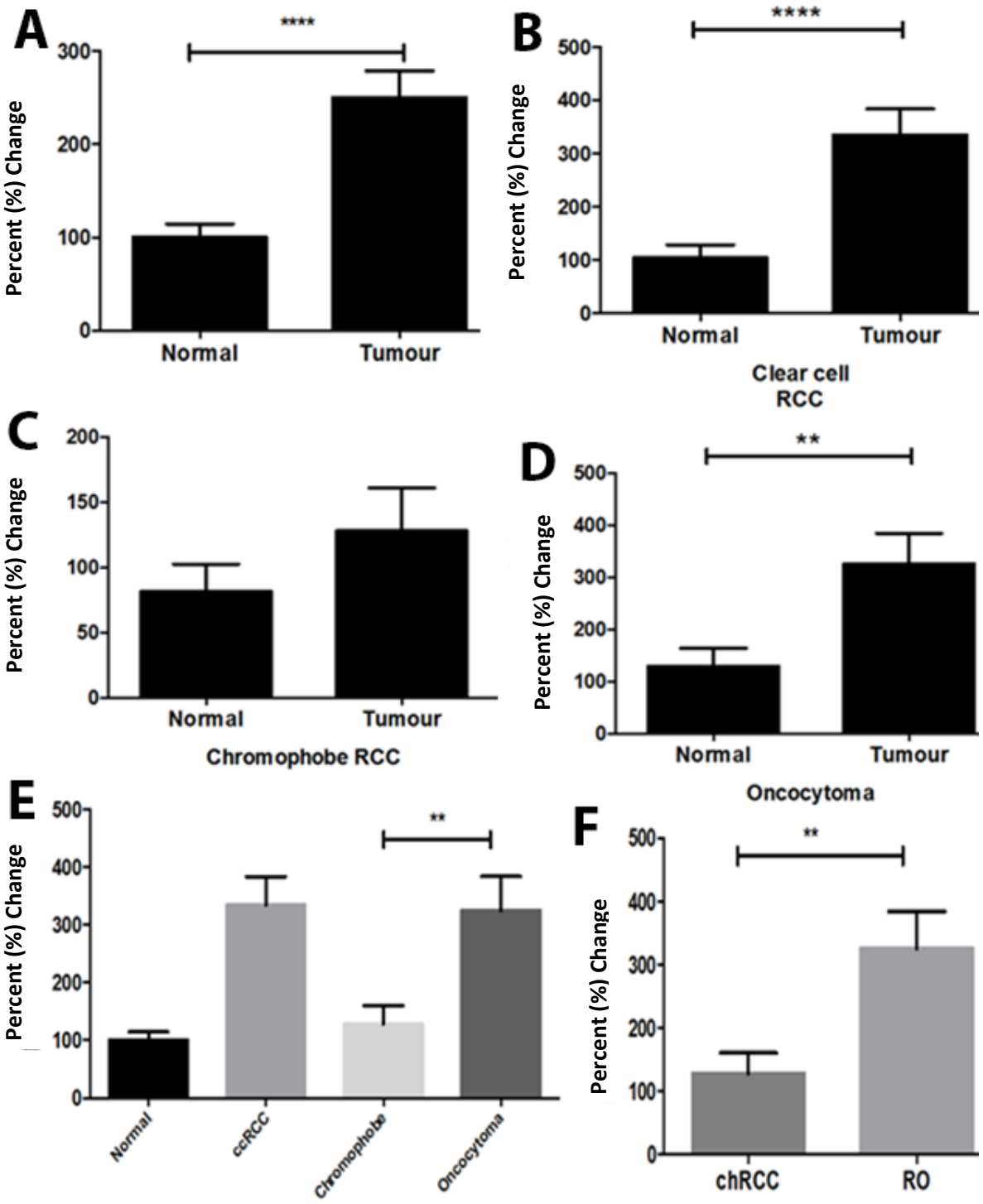

Figure 5.15: KIM-1 expression in renal tumours and matched normal renal tissue

A. Increased KIM-1 expression in tumour vs normal kidney (****p<0.0001); **B**. Increased KIM-1 expression in ccRCC vs normal kidney (****p<0.0001); **C.** KIM-1 expression of chRCC vs normal kidney; **D**. Increased KIM-1 expression in RO vs normal kidney (**p=0.001); **E**. Expression of KIM-1 in tumour subtypes; **F**. Increased expression of KIM-1 in RO vs chRCC (**p=0.002).

5.4.6 S100 calcium-binding protein α1 (S100A1)

S100A1 is a member of the S100 family of calcium binding molecules, most of which are clustered on chromosome 1q21, and expressed in RCC (Teratani et al. 2002). Importantly, these proteins are involved in cell cycle progression and cell differentiation (Li et al. 2007) and therefore implicated in tumorigenesis, a basis for its investigation in renal tumour subtypes. Based on the IHC staining characteristics of S100A1, overall and nuclear expression were analysed in Aperio ImageScope.

5.4.6.1 IHC of S100A1

From our IHC study, S100A1 stained the cytoplasm of proximal and distal tubular cells in nearby normal renal tissue. In ccRCC, there was both cytoplasmic and membranous immunostaining noted. There was patchy cytoplasmic staining noted in chRCC while in RO, there was intense and diffuse cytoplasmic and nuclear staining (Figures 5.16 A-D). Overall and nuclear expression were analysed on Aperio ImageScope based on the IHC staining patterns of S100A1.

5.4.6.2 Overall expression patterns of S100A1

In the analyses of overall expression, all tumours recorded a higher expression of S100A1 compared to normal as shown in Figure 5.17A. Both ccRCC and chRCC had higher expression to normal (not significantly); but in RO, there was significantly higher expression of S100A1 compared to normal with $p=0.02$ (Figures 5.17 B-D). However, there was no significant difference in expression between RO and chRCC. (Figure 5.17E, F).

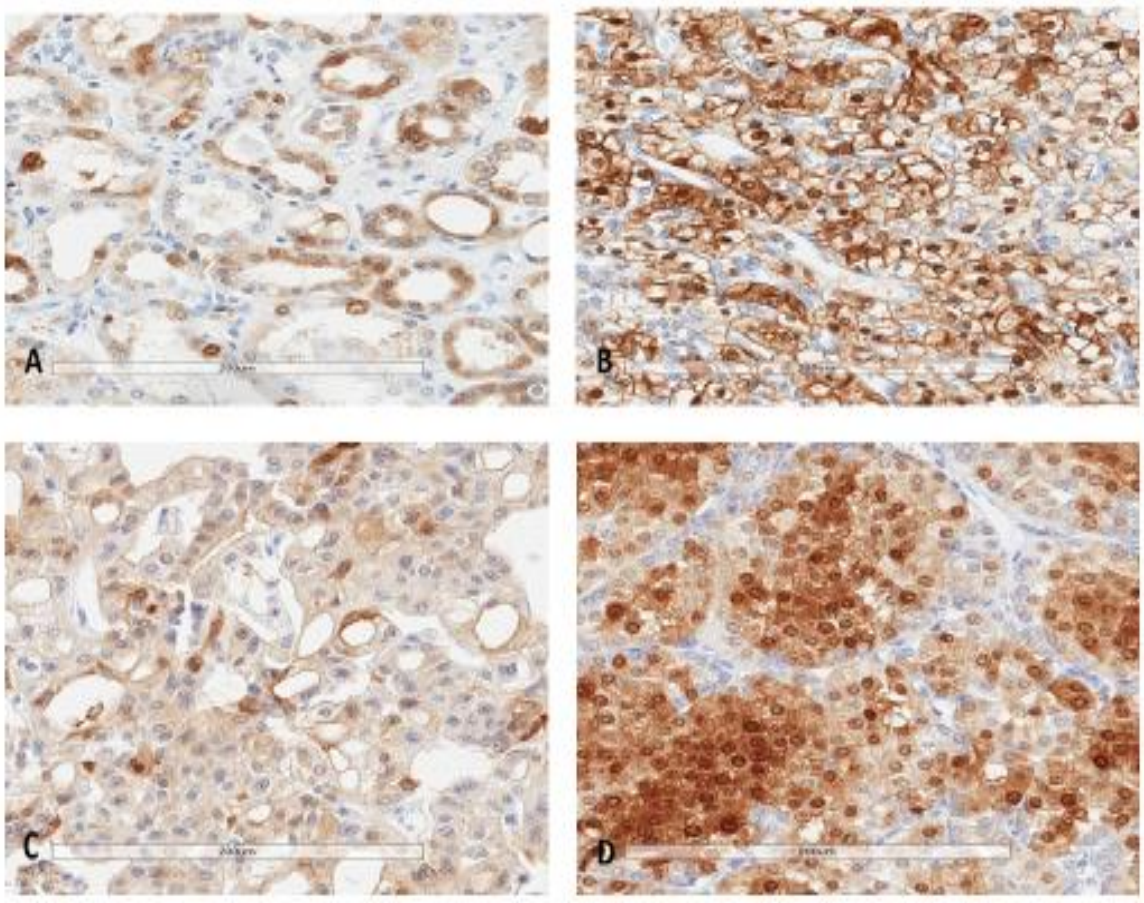

Figure 5.16: S100A1 immunostaining in normal renal cortical tissue and renal tumour tissue

A. Immunostaining of S100A1 in adjacent normal renal cortical tissue showing mainly cytoplasmic staining; **B**. In clear cell RCC, there was membranous and cytoplasmic S100A1 staining; **C**. There was patchy cytoplasmic S100A1 staining in chRCC; **D**. Intense and diffuse cytoplasmic and nuclear staining of S100A1 in RO. Scale bar 200µm. (x20Aperio magnification)

5.4.6.3 IHC showing S100A1 nuclear expression

When nuclear expression of S100A1 was analysed, there was no marked difference between tumour and normal tissue. For ccRCC, there was higher expression in the tumours compared to normal tissue, but the difference did not reach significance. In RO, the S100A1 nuclear expression was slightly higher in tumour than normal kidney. However in chRCC, there was higher S100A1 nuclear expression in normal compared to chRCC tumour cells (Figure 5.18A-D). There was no difference in the expression of S100A1 in nuclear regions of RO in contrast to chRCC (p=0.06) (Figure 5.18E, F).

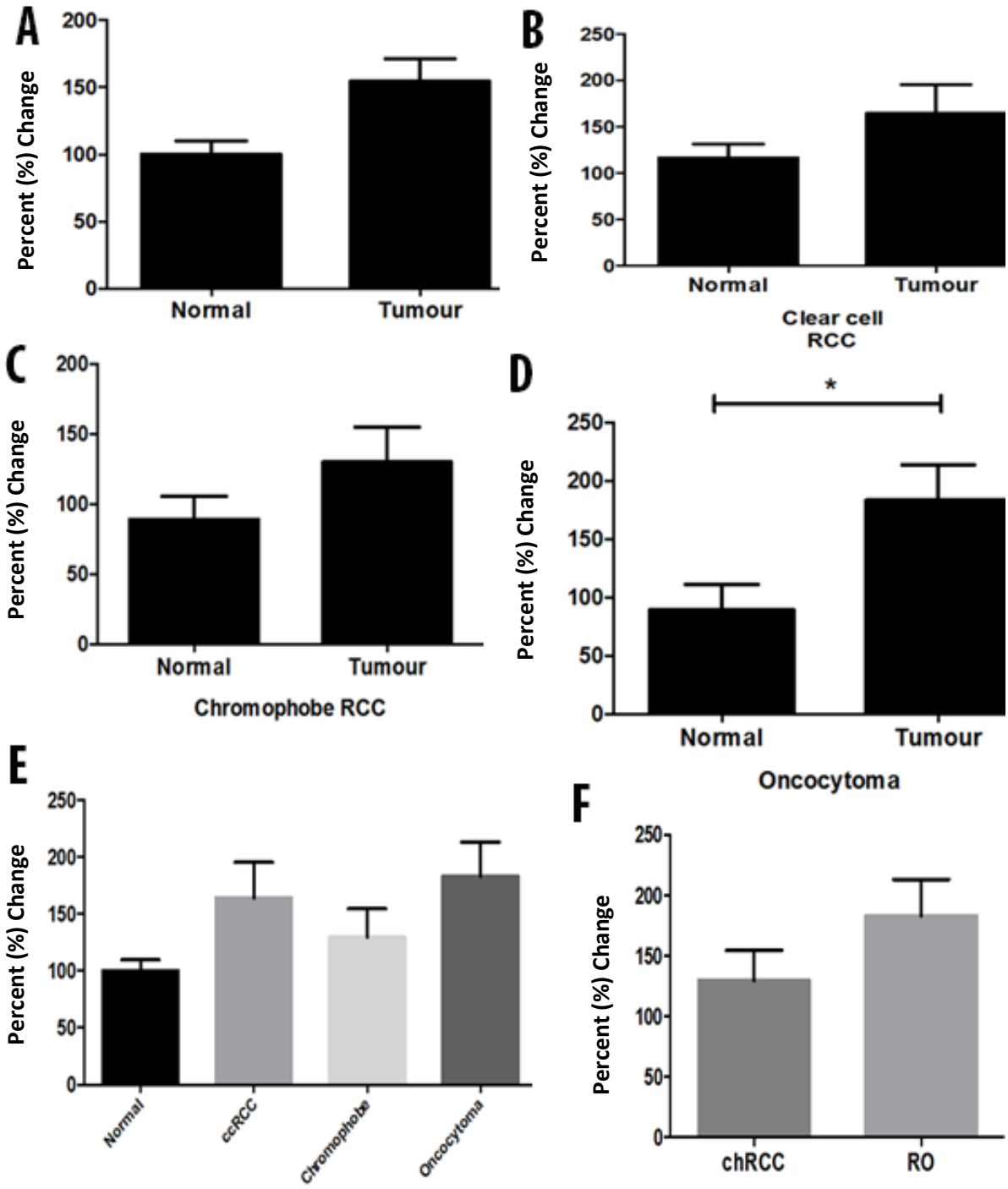

Figure 5.17: S100A1 expression in renal tumours and matched normal renal tissue

A. S100A1 expression in tumour vs normal kidney; **B**. S100A1 expression in ccRCC vs normal kidney; **C**. S100A1 expression of chRCC vs normal kidney; **D**. Increased S100A1 expression in RO vs normal kidney (*p=0.02); **E**. Expression of S100A1 in tumour subtypes; **F**. Expression of S100A1 in RO vs chRCC (not significant).

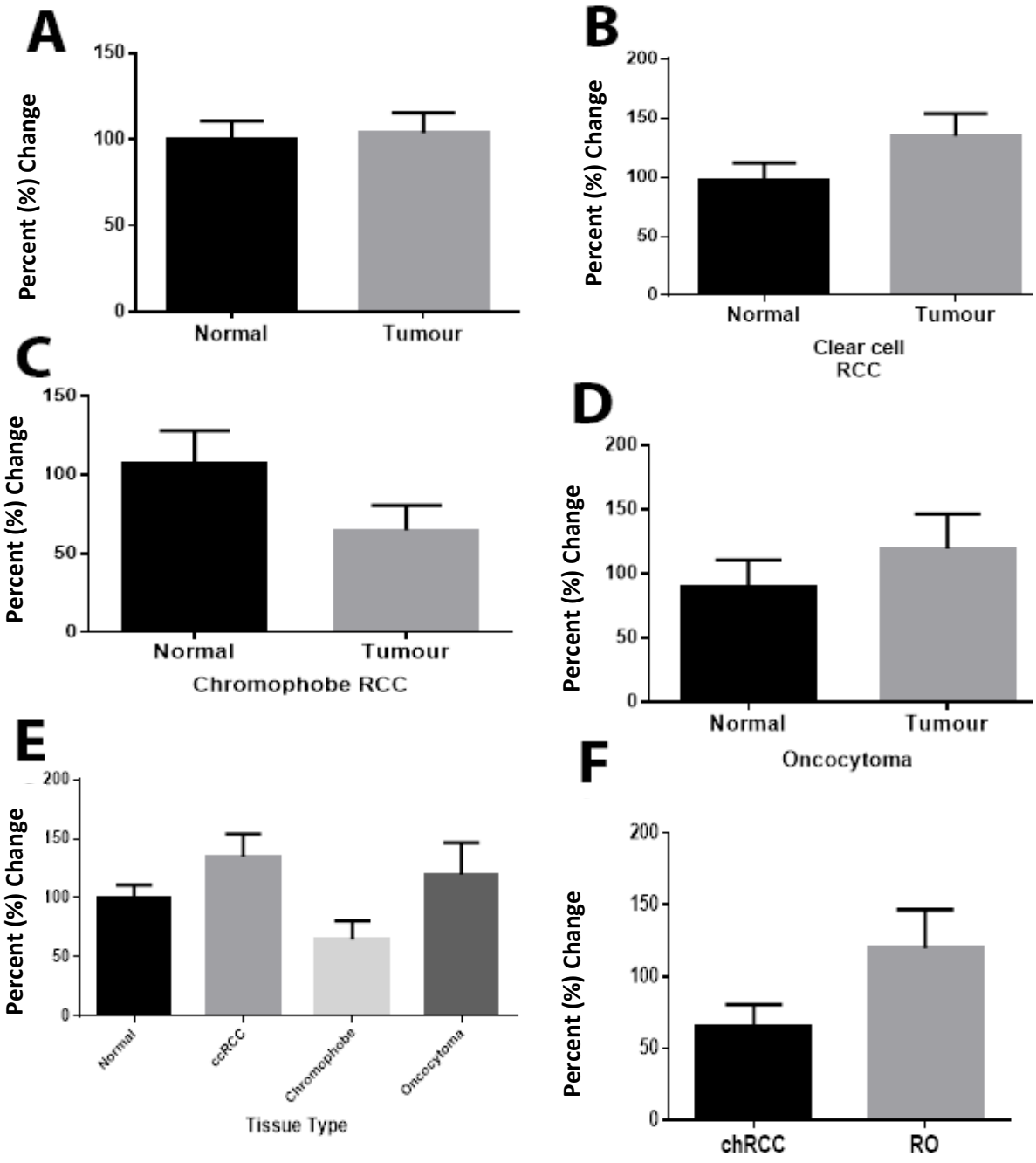

Figure 5.18: S100A1 nuclear expression in renal tumours and matched normal renal tissue

A. S100A1 expression in tumour vs normal kidney; **B**. S100A1 expression in ccRCC vs normal kidney; **C**. S100A1 expression of chRCC vs normal kidney; **D**. Increased S100A1 expression in RO vs normal kidney; **E**. Expression of S100A1 in tumour subtypes; **F**. Expression of S100A1 in RO vs chRCC (p=0.06)

5.5 DISCUSSION

Histopathological diagnosis of renal tumour subtypes poses a significant diagnostic dilemma when the morphological characteristics of tumour subtypes overlap; especially eosinophilic variants of chRCC from RO and eosinophilic variants of ccRCC (Liu et al. 2007). Obviously, the distinction for RO from chRCC will dictate different management pathways as RO is benign while chRCC is a malignant subtype which will require further surveillance. Another important distinction will be chRCC from ccRCC as chRCC have a more favourable prognosis than ccRCC (Gelb 1997).

Traditionally, Hale colloidal iron staining has been used to distinguish chRCC from the other mimics. However, the reproducibility of Hale colloidal iron staining is technically-difficult, due to variations in pH. Results are hard to interpret (Leroy et al. 2000) and its reproducibility in various laboratories is not consistent. Ultrastructurally, chRCC has numerous cytoplasmic microvesicles and RO on the other hand has abundant giant mitochondria (Cochand-Priollet et al. 1997) but electron microscopy facilities are not readily available, and this technique is not clinically practical in an era when cost and time must always be considered.

Therefore utility of IHC remains the most readily accessible and efficient method of distinguishing RO and chRCC. In Chapter 3, our systematic review and meta-analysis of IHC demonstrated that there are numerous biomarkers which have been investigated to aid in the histological differentiation between the two entities. From this meta-analysis, we selected to analyse and validate some of the most apparently-efficient IHC biomarkers (CK7, S100A1 and Cav-1) on Australian cohort of patients. Leptin, leptin receptor and KIM-1 were the other IHC biomarkers that we selected to investigate.

5.5.1 Cytokeratin 7

Cytokeratins are important markers of epithelial differentiation. They consist of at least 20 distinct molecules, the expression of which depends on cell type and differentiation status, making them useful in differential diagnosis of many epithelial tumours (Teratani et al. 2002). As a result CK7 has been widely investigated as a biomarker in renal neoplasms, including the distinction of chRCC from other mimicking renal tumours (eg RO, eosinophilic variant of ccRCC).

In the current study, CK7 immunostaining was seen in cytoplasm of normal distal tubular cells. This is consistent with published reports where CK7 staining in normal kidney was expressed in distal tubules and collecting ducts (Mertz et al. 2008). There was minimal CK7 staining in ccRCC and RO in our study; with diffuse cytoplasmic and peripheral membranous enhancement in chRCC. This is in concordance with other previous works, where in chRCC, there was diffuse cytoplasmic with peripheral enhancement expression while only weak patchy sporadic expression was reported in RO (Bing et al. 2013; Mathers et al. 2002). In addition, the CK7 expression was weak or absent in most of our ccRCC. These strong expression of chRCC as compared to weak or absent expression in RO and ccRCC are consistent with previous published results (Geramizadeh et al. 2008; Kuroda et al. 2004; Mazal et al. 2005; Yasir et al. 2012). The exact reason behind these expression differences in these 3 subtypes of renal tumours remains to be defined or understood.

The strong and enhanced peripheral membranous immunostaining noted in our chRCC cases was consistent with other reports as mentioned above. This may reflect the peripheral distribution of intermediate filaments within the tumour cells. Abundant cytoplasmic

microvesicles in chRCC may push the intermediate filaments aside in the peripheral area of the cytoplasm, because chRCC has more abundant cytoplasmic microvesicles (Latham et al. 1999).

The overall expression of chRCC was highest amongst the 3 tumour subtypes and was significantly higher compared to RO. This differential IHC result between positively-stained chRCC versus poorly-stained RO on our Australian cohort of patients provides further validation to other published results (Adley et al. 2006a; Leroy et al. 2000; Liu et al. 2007; Mathers et al. 2002). However, there were also some authors who had different results where RO had also prominent CK7 expression when compared to chRCC (Taki et al. 1999; Wu et al. 2002). Reasons for these disparate results could be due to small numbers of chRCC and RO being used in some studies, difficult histological interpretation of IHC and inaccurate initial diagnoses of the cases.

Nevertheless, the CK7 IHC study provided similar results as revealed by our meta-analysis where CK7 has been identified as the most studied IHC biomarker in the differentiation between chRCC and RO (Ng et al. 2016). From this meta-analysis, we also recommended CK7 as part of our panel of IHC biomarkers than can be useful in differentiating chRCC from RO. Other authors have also recommended CK7 as part of their panel of IHC biomarkers for this purpose and these include: CK7, s100A1, claudin 8 panel (Kim et al. 2009); CK7 and EpCAM panel (Liu et al. 2007) and CK7, KIT, PAX2 panel (Memeo et al. 2007).

The association of CK7 with RCC tumourigenesis or progression needs further evaluation. One proposed mechanism includes metalloproteinase. It is possible that the clinical behaviour and better prognosis of chRCC in contrast to other RCCs could be related to the association

of CK7 with absence of membrane type 1 matrix metalloproteinase (MT1-MMP). MMPs are zinc-dependent endopeptidases, which are largely involved in tissue remodelling, degradation of the extracellular matrix and basal membranes leading to tumour invasion and progression (Nagase and Woessner 1999). One study showed the absence of MT1-MMP in CK7-positive ccRCCs, suggesting that any good prognosis of CK7-expressing ccRCC can be partially explained by absence of MT1-MMP expression (Mertz et al. 2008).

5.5.2 Caveolin-1

Caveolae are morphologically identifiable plasma membrane invaginations that were identified first in the 1950s by electron microscopic examination. They constitute a membrane system that is essential for normal cellular functions. Caveolae are specialized lipid raft microdomains forming 50 to 100 nm flask-shaped vesicular invaginations of the plasma membrane, which serve as a scaffold for signalling molecules related to cell adhesion, growth and survival (Anderson 1998). Caveolins are functionally and structurally highly conserved, and they initiate caveolae formation from raft derived components. Cav-1 is involved in the regulation of numerous signalling cascades, including receptor and non-receptor tyrosine kinases such as epidermal growth factor, Neu and the Src family tyrosine kinases, protein kinase C, heterotrimeric G-protein α-subunits and endothelial nitric oxide synthase (Okamoto et al. 1998).

Some studies have demonstrated that Cav-1 acts as a tumour suppressor protein, inhibiting the functional signalling activity of several proto-oncogenes and, consequently, disrupting the process of cellular transformation (Cohen et al. 2004). Expression of Cav-1 has been studied in various types of tumours; and previous authors have published results in RCC (Carrion et al. 2003; Garcia and Li 2006; Mete et al. 2005).

From our study, there was minimal staining of Cav-1 noted in distal tubules and more pronounced staining of endothelial cells in normal renal tissue. This is reflective of previous studies where Cav-1 was localised to distal tubular cells, collecting ducts, parietal cells of Bowman's capsule, endothelial and smooth muscle cells (Breton et al. 1998). All 3 tumours (ccRCC, chRCC and RO) recorded significantly higher overall and membranous expression of Cav-1 compared to normal renal tissue.

There was prominent membranous staining of ccRCC. In chRCC, intense diffuse cytoplasmic staining with peripheral membranous enhancement and a distinctive perinuclear halo was noted; while in ROs there was patchy cytoplasmic staining. Membranous expression of Cav-1 was highest in ccRCC followed by chRCC and RO. These staining patterns of the 3 tumours were similar to reports published by Tamaskar et al, where ccRCC were noted to have predominantly membranous expression while chRCC and RO had cytoplasmic expression (Tamaskar et al. 2007). Similarly Mete et al also recorded a difference in staining patterns between chRCC (diffuse and peripheral cytoplasmic) and RO (diffuse cytoplasmic) (Mete et al. 2005). The observations by previous authors strengthen our findings of differences in staining patterns noted in our chRCC and RO. The differential IHC Cav-1 staining pattern between the two entities will aid in the important differentiation of the two tumours.

Although increased overall and membranous expression of Cav-1 was noted in chRCC as compared to RO, these were not statistically significant. Other published results have also shown that Cav-1 expression was higher in the majority of chRCC versus focal positivity in the minority of RO (Garcia and Li 2006; Lee et al. 2011). However, one contrasting report had RO with increased cytoplasmic staining and lower expression in chRCC (Carrion et al.

2003). Nonetheless, the different staining patterns may be beneficial in differentiation between chRCC and RO.

The significance of Cav-1 over-expression in RCC has been linked to higher tumour grades, venous invasion, lymph node metastases, tumour progression and poorer prognosis (Horiguchi et al. 2004). It is well known that ccRCC have a more aggressive malignant nature and therefore, as expected, the highest expression of Cav-1 was noted in ccRCC compared to chRCC (less aggressive but malignant) and benign RO. A meta-analysis also recently reported the association of Cav-1 levels with cancer-specific survival in renal cancers with a hazard ratio of 1.98 (Liu et al. 2015).

The mechanisms by which Cav-1 exerts its tumourigenesis include enhancement of VEGF secretion, thereby stimulating angiogenesis (Li et al. 2009); and interaction with phospho-ERK-1/2 to promote tumour survival and growth (Campbell et al. 2013). Also in RCC, Cav-1 may serve as a 'gatekeeper' for activation of the hypoxia-inducible factor (HIF) pathway HIF is a downstream effector molecule of mTOR that accumulates in RCC in response to the loss of function of VHL and promotes angiogenesis, vascular invasion and chemoresistance (Patel et al. 2006). Cav-1 has also been identified as a molecular target of bortezomib in advanced RCC clinical trials (Kondagunta et al. 2004). From the previous studies above, it appeared that Cav-1 can be utilised as diagnostic tool, prognostic indicator and also a possible therapeutic target in RCC. In our study, there was no difference in the overall or membranous expression between RO and chRCC. However, there was distinctive difference in the staining characteristics, with chRCC displaying diffuse staining in the peripheral cytoplasmic regions and a perinuclear halo devoid of staining compared to the patchy granular staining in RO. This difference in staining patterns may be useful in in distinguishing chRCC from RO.

5.5.3 Leptin and leptin receptor

Obesity is considered a risk factor in many cancers, including renal cancers. The World Cancer Research Fund has estimated that 24% of incident kidney cancer cases in the United States can be attributed to adiposity (Ljungberg et al. 2011). With increasing obesity, there are raised serum Ob levels. Ob may act as a mitogenic promoter in renal tumourigenesis. Ob is a 16-kDa adipokine that is produced mainly, but not exclusively, by white adipose tissue. Others sites of production include the placenta, intestine, stomach, ovaries, bone marrow, brain, pituitary, liver, mammary epithelial cells and skeletal muscle. Ob levels are positively correlated with white adipose tissue mass, and are therefore increased in obesity. Its synthesis is influenced by insulin, tumour necrosis factor alpha (TNF-α), glucocorticoids, sex hormones and prostaglandins (Paz-Filho et al. 2011). Its expression is also stimulated by hypoxia (commonly found in solid tumours), through HIF-1 (Garofalo and Surmacz 2006).

The main role of Ob is to regulate energy homeostasis by controlling energy intake and energy expenditure, through its action on the arcuate nucleus of the hypothalamus. It has additional effects in the endocrine and immune systems, including reproduction, glucose homeostasis, bone formation, tissue remodelling, and inflammation (Boguszewski et al. 2010; Kelesidis et al. 2010). Ob exerts its action through binding to the extracellular domains of leptin receptor (2 major isoforms): Ob-Ra (short form found in most cells) and Ob-Rb (long form found in hypothalamus, adipocytes, lungs and kidney) (Fantuzzi and Faggioni 2000).

Ob binds to its receptor and activates different signalling pathways, such as the JAK/STAT (Janus Kinase/Signal Transducer and Activator of Transcription), MAPK (mitogen-activated protein kinase), PI3K/Akt (phosphatidylinositol 3-kinase/protein-kinase B), AMPK (5' AMP-

activated protein kinase) and IRS (insulin receptor substrate) pathways, which affect cell proliferation and survival (Fruhbeck 2006). Ob is a pleiotropic hormone, being mitogenic, anti-apoptotic, pro-angiogenic, and pro-inflammatory in various cellular systems (Paz-Filho et al. 2011).

Studies associating Ob and renal cancer are scarce. However, there is contradiction between the epidemiological and the molecular findings regarding the role of Ob in the pathogenesis of kidney cancer. In a case-control study that included 70 patients with RCC, serum Ob was inversely associated with cancer risk (OR: 0.53, CI: 0.28-0.99, p=0.05), which the authors attributed to the pro-immunogenic effects of Ob (Spyridopoulos et al. 2009). Conversely, higher serum Ob was an independent predictor of progression-free survival, and along with increased expression of ObR in renal tumour tissue, were also associated with tumour specimen venous invasion (Horiguchi et al. 2006a).

ObR is present in human RCC cell lines (Caki-1, ACHN, 769P, A498, SKRC44 and SKRC49)and in the murine renal cancer cell line Renca. (Horiguchi et al. 2006b). In the murine cell, Ob induces invasiveness. In another in vitro study, Ob increased the proliferation and mobility capabilities of Caki renal carcinoma cells by up-regulating the expression of the JAK/STAT3 and ERK1/2 signalling pathways (Li et al. 2008). Ob also induces collagen gel invasion of non-tumorigenic kidney MDCK epithelial cells through PI3K-, Rho-, and Rac-dependent signalling pathways (Attoub et al. 2000). Ob's effects on lymphangiogenesis, mediated by Akt and ERK1/2, and on lipid and protein biosynthesis, mediated by acyl-coenzyme A: cholesterol acyl transferase (ACAT), may explain the roles of Ob in the pathogenesis and in the phenotype of renal cancer (Drabkin and Gemmill 2010). Ob has also been linked to mTOR activation that links nutrient signalling to cell growth, proliferation and

cancer (Dann et al. 2007). Furthermore, Ob together with other cytokines (IL-6, TNF) potently activates STAT3 signalling processes which include cell survival and proliferation in renal neoplasia (Horiguchi et al. 2002).

Despite all the above studies on Ob and ObR, there was surprising paucity in the research into IHC of Ob and ObR on human renal tumour tissue; as most studies have concentrated on serum leptin and adiponectin levels instead. There was only one study by Horiguchi et al. which only analysed ObR expression (and not Ob expression) in 57 human renal tumour specimens (39 ccRCC, 18 others) and their corresponding serum Ob levels (Horiguchi et al. 2006a). Therefore, we believe our study which examined IHC of Ob and ObR in a cohort of 75 human renal tumour specimens is the largest to date, and the first to characterise and compare the IHC staining of Ob and ObR in less-studied subtypes of renal tumours (chRCC and RO), together with the most common ccRCC.

In the normal renal tissue of the present study, Ob and ObR expression was noted mainly in the cytoplasm, with minimal nuclear regions of proximal and distal tubular cells and vascular endothelial cells. In ccRCC, there was moderate cytoplasmic, membranous and nuclear Ob and ObR expression. In chRCC, there was moderate cytoplasmic and nuclear Ob and ObR expression. In contrast, RO had intense diffuse cytoplasmic and nuclear expression. Horiguchi *et al* showed that ObR expression was predominantly cytoplasmic and membranous in tumour tissues (39 ccRCC, 18 others); with 10/38 ccRCC and 12/18 others having higher staining intensity than the staining intensity of vascular endothelial cells (Horiguchi et al. 2006a).

As described above, Ob and ObR IHC was significantly higher in tumour than in normal kidney tissue. Overall expression of Ob and ObR was highest in RO compared to chRCC and ccRCC. On closer scrutiny of overall, nuclear and membrane intensities of Ob and ObR in comparing chRCC and RO, Ob nuclear expression in RO had significantly higher intensity compared to chRCC. This important finding may prove to be helpful in the distinction between chRCC and RO. Ob could be added to the existing panel of useful IHC biomarkers. The exact reason or mechanism behind the more intense nuclear staining in RO compared to chRCC is yet to be determined. This is the first study to investigate Ob and ObR expression in chRCC and RO. Further studies investigating the role and mechanistic pathway for leptin and its receptor in RCC tumourigenesis are required. One possible explanation for high RO expression lies in the abundance of mitochondria in RO compared to scanty presence in chRCC. In breast cancer cell lines, Ob has been shown to improve mitochondrial biogenesis and dynamics with an amelioration of oxidative stress and higher mitochondrial ATP production, leading to tumoral growth (Blanquer-Rosselló et al. 2015). Nevertheless, this initial IHC result on Ob and ObR in ccRCC, chRCC and RO should enhance further studies in this respect on larger human renal tumour samples and subtypes.

5.5.4 Kidney injury molecule-1

KIM-1 was identified as the most highly upregulated protein in the proximal tubule of the kidney after acute or chronic insults (Bonventre 2014). KIM-1 (also known as T cell immunoglobulin domain and mucin domain protein 1 and hepatitis A virus cellular receptor 1) is a type-1 membrane glycoprotein which contains an extracellular immunoglobulin- and mucin-like domain, with N- and O-glycosylation sites. It has a transmembrane domain and short intracellular domain with intracellular tyrosine phosphorylation sites. The ectodomain is heavily glycosylated and stable and appears in the urine after injury (Bonventre 2014).

The main functions of KIM-1 include: 1) As a phosphatidylserine receptor, it recognises apoptotic cells and directs them to lysosomes (Ichimura et al. 2008); 2) As a receptor for oxidized lipoproteins; and 3) As a unique first molecule that, although not also present on myeloid cells, can transform kidney proximal epithelial cells into semi-professional phagocytes to enhance clearance of dead cells (Bonventre 2014). Therefore KIM-1 has an important role in mounting an immune response in acute kidney injury. KIM-1 is approved by the United States Food and Drug Administration and the European Medicines Agency for preclinical assessment of nephrotoxicity and, on a case-by-case basis, for clinical evaluation.

In renal cancers, KIM-1 has been shown to be expressed in various RCC, especially ccRCC and papillary RCC, both of proximal tubular origin; and urinary KIM-1 is also a good biomarker for RCC detection (Han et al. 2005; Zhang et al 2014). The authors concluded that KIM-1 expression occurs with dedifferentiation of the proximal tubule epithelial cell, which is also a property of RCC cells that are derived from the proximal tubule (Bonventre 2014). One proposed mechanism in ccRCC tumourigenesis involved KIM-1 inducing IL-6 expression which activates STAT-3/HIF-1A axis in ccRCC derived cell lines and thus promotes expression of growth and angiogenic factors on tumour, and likely in non-tumour-associated cells that would help tumour growth and metastasis (Cuadros et al. 2014).

In the present study, minimal cytoplasmic staining of KIM-1 was noted in proximal tubular cells in normal renal tissue adjacent to the tumours. This result is similar to other published reports where KIM-1 expression was noted in adjacent normal tubular cells, irrespective of whether or not RCC tumour cells were negative or positive (Cuadros et al. 2014; Han et al. 2005). The authors argued that KIM-1 expression in normal adjacent tubular cells was due to

tubular injury from compression by adjacent tumour cells or adjacent cells undergoing early stage of cancer transformation (Han et al. 2005); and probably related to an endogenous condition of individuals at risk of developing these renal tumours (Cuadros et al. 2014).

In the renal tumour tissue, diffuse intense cytoplasmic and membranous KIM-1 immunostaining was noted in the majority of ccRCC. Previous reports have also showed strong expression of KIM-1 in ccRCC (Han et al. 2005; Lin et al. 2007; Zhang et al. 2014). There was also diffuse cytoplasmic and nuclear expression in RO, whereas there was minimal or none in chRCC. Interestingly, the overall expression of KIM-1 in RO was significantly increased compared to chRCC. This is a novel result and may prove to be an important distinguishing feature of KIM-1 in separating chRCC from RO.

From the present study, the majority of RO show overexpression of KIM-1 whereas majority of chRCC were negative. The poor expression of KIM-1 in chRCC concurred with other previous IHC results where the majority of chRCC were negative for KIM-1. However, our findings of KIM-1 expression in 80% of RO was certainly different to previous reports where RO were all negative for KIM-1 immunostaining (Han et al. 2005; Zhang et al. 2014) or only (4/41) 9.75% of ROs expressed KIM-1 (Lin et al. 2007). The reason behind our unique increased expression of KIM-1 in RO with minimal/absent expression in chRCC from the Australian cohort of patients remains uncertain. There were notable differences between our study which analysed renal tumour sections of 30 chRCC and 15 RO as compared to IHC on tissue microarray sections (25 chRCC and 25 RO) in Zhang et al (2014). In Han et al (2005), there was positive staining on tumour slides of 1/6 chRCC and 0/8 other renal tumours (which included oncocytoma, angiomyolipoma and transitional cell carcinoma of renal pelvis). In Lin et al (2007), they noted KIM-1 expression in 9.75% of 15 RO and none in 16

chRCC tissue microarray samples. So our results of increased KIM-1 in RO compared to minimal in chRCC support the expression pattern seen in the report by Lin and colleagues. Nevertheless, the increased expression of KIM-1 in RO compared to chRCC in this initial study can be further validated with larger national and international studies. Studies involving larger cohorts of RCC subtypes, analysing KIM-1 immunostaining, and measuring urinary KIM-1 pre and post-nephrectomy may allow correlation with the urinary and tissue levels to assess and validate the utility of KIM-1 in RCC.

Urinary KIM-1 levels have also been closely associated with renal cancers, especially ccRCC and papillary RCC. In the first study, Han et al showed that in all 5 RCC patients with detectable prenephrectomy urinary KIM-1, there was either complete disappearance or marked reduction after nephrectomy. They concluded that the cleaved ectodomain of KIM-1 can be detected in the urine of patients with RCC and may serve as a new biomarker for early detection of RCC (Han et al. 2005). Following this, another study reported significant reduction in urinary KIM-1/urinary creatinine after nephrectomy in the KIM-1 positive group (8 ccRCC and 4 papillary RCC), suggesting that urinary KIM-1 may serve as a surrogate biomarker for kidney cancer and a non-invasive pre-operative measure to evaluate the malignant potential of renal masses (Zhang et al. 2014). Therefore, urinary KIM-1 can be used as a non-invasive diagnostic screening tool for patients at risk of RCCs and also serve as a prognostic surveillance investigation following RCC nephrectomy.

In summary, there might be differences in the urinary KIM-1 levels in all renal tumour subtypes; in particular inpatients with RO having increased urinary KIM-1 levels compared to patients with chRCC. A small study which compared urinary KIM-1 between renal tumour subtypes (24 ccRCC, 4 pRCC and 3 chRCC) and controls (which included 3 oncocytomas, 3

benign lesions and 9 non-functioning kidneys) failed to reveal any differences among the groups (Shalabi et al. 2013). However, as can be seen, there were relatively small numbers of chRCC and RO in that study. Therefore future studies investigating larger samples to compare urinary KIM-1 levels in renal tumour subtypes might be useful.

5.5.5 S100 calcium-binding protein A1

A member of calcium-binding proteins, S100A1, has been found in renal cell neoplasms. This protein is a member of the S100 family, the largest subgroup of the EF-hand proteins (Schafer and Heizmann 1996). S100A1 has been reported to be involved in different biological activities such as transduction of intracellular calcium signalling, cytoskeleton-mediated interactions, as well as cell cycle progression and cell differentiation (Li et al. 2005). Therefore it has been studied in a variety of tumours, including renal cancers.

From the present study, S100A1 immunostaining was noted in nuclei and cytoplasmic regions of proximal tubular cells and collecting ducts in adjacent non neoplastic renal parenchyma. This is similar to previous published results (Rocca et al. 2007). In ccRCC, strong S100A1 immunostaining in cytoplasmic and membranous regions of tumour cells was noted, while there was only patchy minimal cytoplasmic expression in chRCC and strong diffuse cytoplasmic and nuclear staining in RO. In comparing the recent studies examining IHC expression of S100A1 in renal neoplasms, ccRCC was found to have expression in 66–73% of cases and 67–94% of pRCC. The highest level of expression was identified in RO, with 92–93% of cases demonstrating reactivity with S100A1 compared to 0–6% of chRCC, which have been found to be negative (Kim et al. 2009; Li et al. 2007; Rocca et al. 2007). Recently, Kuroda et al reported that IHC cytoplasmic expression of S100A1 was 100% of ROs compared to only 30% of chRCC (Kuroda et al. 2011).

The IHC results of the present study were in concordance with the differential immunostaining of S100A1 in RO when compared to chRCC. There were apparent higher overall and nuclear expressions of S100A1 in RO over chRCC in our cohort, but this unfortunately did not reach statistical significance. This is perhaps related to the small RO sample size of 15 cases. Nevertheless, the majority of the RO in the study expressed diffusely intense cytoplasmic and nuclear staining of S100A1 compared with minimal patchy cytoplasmic expression in chRCC, similar to other published reports. Following our meta-analysis as well, the pooled OR of RO compared to chRCC for S100A1 staining is 100. Therefore, S100A1 is another reproducible IHC biomarker from a panel of IHC biomarkers,that can differentiate RO from chRCC (Ng et al. 2016). Other authors have suggested a panel of CK7, S100A1 and claudin 8 (Kim et al. 2009) and the utility of cluster analysis of S100A1 and CK7 (Carvalho et al. 2011) which could discriminate the two entities. Recently, Conner et al reported the usefulness of S100A1 IHC in fine needle aspirates and core needle biopsies which showed positivity in 80% of ROs versus 8% in chRCC (Conner et al. 2015); which could provide valuable distinction between the two entities in selective groups of patients with indeterminate small renal masses.

The main limitation in this research into IHC biomarkers was the sample size of RO. A larger number of RO, for example, n= 30, may have strengthened the power of statistical analysis. However, incidence of RO is low, only 3-5% of all renal tumours (both benign and malignant). We wanted renal tumour slides from approximately the same range of years (2012-2015), from one dedicated uropathology centre. Larger multi-institutional studies investigating larger case numbers of chRCC and RO may provide stronger statistical comparisons of the biomarkers.

5.5.6 Summary of IHC results

Various IHC biomarkers that could differentiate chRCC from RO were investigated. Most of the IHC staining patterns of the various biomarkers matched other previous published reports. In the present study, not only qualitative analysis of the expression and their differences in staining patterns and locations, but also quantitative expressions analysis (overall, membrane and nuclear) via morphometry using Aperio Imagescope, was presented.

The results gained from our study are summarised in Table 5.2, with the significant results highlighted in **red and bold**. In summary, chRCC had higher CK7 overall expression intensity compared to RO and a difference in Cav-1 staining patterns between the two subtypes was recorded. RO recorded higher Ob nuclear expression and higher KIM-1 overall expression than chRCC.

5.5.7 Clinicopathological data of Renal Tumour Biobank.

As mentioned in Chapter 2, serum, urine and renal tumour and normal tissues have been collected from patients undergoing nephrectomy for suspected renal tumours and stored in the Renal Tumour Biobank. The clinicopathological data from these patients have also been collected to provide a comprehensive database. As seen in Table 2.1, 202 patients have been recruited and samples obtained. The ratio of male to female was 1.67 : 1, mean age of 57 years, mean body mass index (BMI) of 28.9. Almost two thirds of patients had hypertension in their past medical history, with a mean eGFR of 72.7 ml/min/1.73m^2. From this cohort, high BMI and hypertension are 2 of the risk factors known to be associated with RCC risk.

Mean tumour size was 4.5cm and not surprisingly most patients presented at clinical stage T1 (78.2%), followed by T2 (7.9%), T3(8.5%), T4(2.9%) and 5 patients (2.5%) underwent cytoreductive nephrectomy due to M1 disease at presentation. The vast majority of patients seen at early T1 stage support the rise in early detection of incidental small renal masses due to increasing usage and availability of radiological scans.

The majority of the renal tumours were ccRCC (64.8%), pRCC (10.9%), chRCC (9.9%), oncocytoma (4.5%), multilocular cystic RCC (2.5%), clear cell tubulopapillary RCC (2%) and others (benign and malignant) (5.4%). These proportions of renal tumour subtypes are typical of the representation of renal tumour pathology. With the ongoing collection of samples, these proportions of renal tumours will increase and provide a large comprehensive bank of samples for future research.

Currently, two research projects from our group have started using data and samples collected in the Renal Tumour Biobank:

(1) The development of a comprehensive clinical assessment tool that can be used to stratify patients into risk groups for developing adverse renal functional outcomes post-nephrectomy. The aim of the project is to evaluate the risk of CKD progression in patients following tumour nephrectomy, through evaluation of blood, urine, tissue and clinical data on a short and long term follow up basis.

(2) Identification of distinct metabolic changes that occur amongst RCC subtypes using magnetic resonance spectroscopy (MRS). The aim is to characterise metabolic patterns that occur across tumour, normal, urine and serum samples that are capable of accurately differentiate renal tumour subtypes. The results analysed will be

translated to clinical trials to assess feasibility of non-invasive MRS to accurately diagnose benign from malignant renal lesions, especially small renal masses.

Table 5.2 Summary of IHC biomarkers in differentiation of chRCC and RO

Biomarker	ChRCC	RO	p value
Cytokeratin 7 Overall expression	+++	**-**	**0.03**
Caveolin-1			
Overall expression	++	+	ns
Membrane expression	++	+	ns
	Diffuse cytoplasmic, peripheral enhancement, perinuclear halo	**Patchy cytoplasmic**	
Leptin			
Overall expression	+	++	ns
Membrane expression	+	++	ns
Nuclear expression	**+**	**+++**	**0.02**
Leptin receptor			
Overall expression	+	++	ns
Membrane expression	+	++	0.08
Nuclear expression	+	++	ns
Kidney injury molecule-1 Overall expression	**-**	**+**	**0.002**
S100A1			
Overall expression	+	++	ns
Nuclear expression	+	++	0.06

Red bold = biomarkers with significant results which can differentiate chRCC and RO

CHAPTER 6

CONCLUSION AND FUTURE DIRECTIONS

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6.1 OVERVIEW

The incidence of renal tumours is on the rise in the last decade, largely due to diagnoses of incidental renal masses arising from widespread availability of radiological imaging. Despite advances in radiological imaging and improved techniques of renal lesion biopsy, accurate diagnosis often eludes clinicians and final pathological diagnoses are only made post-operatively. A significant proportion of these renal lesions are benign, thus subjecting patients to unnecessary surgery and significant nephron loss. Accurate preoperative diagnostic non-invasive molecular biomarkers which can accurately distinguish benign from malignant renal tumours can potentially reduce unnecessary surgery, preserve nephron mass and subsequently reduce development of chronic renal insufficiency with its associated cardiovascular mortality.

Another difficult diagnostic dilemma following surgery is the histopathological analyses of certain subtypes of renal tumours where morphological features overlap. The distinction of malignant chRCC from benign RO is one such diagnostic dilemma that can pose significant difficulties to pathologists as histological, morphological and histochemical features often overlap between the two entities. Accurate diagnosis of the pathological specimens is crucial and dictates further surveillance and potential management for malignant chRCC as compared to benign RO cases, where an expectant approach is sufficient. Therefore novel and reproducible effective biomarkers which can aid in the differential diagnoses of chRCC from RO are needed.

RCC still remain a lethal disease as 30% of patients still present with metastases and 30-40% will eventually die from their cancer due to tumour recurrence and progression (Lam et al. 2008). In the last two decades, molecular targeted therapies like tyrosine kinase inhibitors and mTOR inhibitors have revolutionalised the management of metastatic RCC. Nevertheless, despite the numerous adverse effects of these agents, there is modest improvement in overall survival, but ultimately all patients succumb to this disease. Therefore newer therapeutic targets are required to achieve the utopian curative stage.

Further characterisation of molecular signatures for renal tumour subtypes will help solve some of the diagnostic and therapeutic issues mentioned above. This will in turn lead to improved treatment algorithms with reduction of overtreatment of benign/indolent renal lesions leading to efficient management of healthcare costs. Therefore identification of such biomarkers which can aid in the differentiation of chRCC and RO is crucial and forms the basis of this research project. The relevance of this research result can then be translated to provide useful interventions into clinical urological day to day management of renal tumours.

Gene expression profiling techniques have provided valuable information of the differential gene expression profiles of various renal tumour subtypes (Higgins et al. 2003; Takahashi et al. 2003; Tan et al. 2004). In two studies investigating the differential gene expression profiles between chRCC and RO, Rohan et al (2006) identified 5 target genes (AP1M2, MAL2, PROM2, PRSS8, and FLJ20171) that had differential expression patterns; and Yusenko et al (2009) identified CD82 and S100A1 as valuable markers for chRCC as well as AQP6 for RO, but found that these genes were expressed at the protein level in other types of

kidney cancers albeit at a low frequency and low intensity, and that none of the selected genes marked exclusively one type of kidney cancer. Nevertheless these gene expression profiling studies may lead to the discovery of useful biomarkers that can be used with IHC. In this research, we have concentrated on protein as the translated molecule and the technique used more-commonly by diagnostic pathologists, that of IHC, to assess useful biomarkers in differentiating chRCC from RO.

This PhD research is centred upon the hypothesis that there are distinct differences in the molecular signatures between renal cancers that can be exploited to distinguish between malignant chRCC and benign RO phenotypes. The aims of this research included: 1) identification of panel of IHC biomarkers which can effectively differentiate chRCC from RO through a comprehensive meta-analysis approach; 2) assessment of the different molecular profiles of renal cancers via immunohistochemistry and morphometry techniques using selected biomarkers on renal tumour and normal tissue samples; 3) analyses of IHC biomarkers that are useful in differentiating chRCC from RO via IHC and Aperio Imagescope morphometry techniques; and 4) creation of comprehensive Renal Tumour Biobank from nephrectomy specimens. I believe I have managed to provide some valuable recommendations in regards to the utility of unique molecular signatures of chRCC and RO, which can be translated into urological clinical practice.

6.2 SUMMARY OF MAJOR RESULTS

6.2.1 Meta-analysis

The clinical diagnostic dilemma and difficult histopathological differentiation of RO from chRCC still persist. This systematic review and meta-analysis has revealed numerous IHC

biomarkers that have been investigated and regularly used across laboratories to aid in differentiating chRCC and RO. PubMed database was used to identify relevant literature. The primary end point was comparison of positive immunostaining of the biomarkers in chRCC and RO, with extracted data used to calculate OR and 95% CI and statistical I^2 test of heterogeneity for multiple studies. This meta-analysis has provided us with a panel of the ten most relevant IHC biomarkers that may help to discriminate the two entities. This panel of biomarkers includes amylase α1A, Wnt-5a, FXYD2, ARPP, CD63, TGFβ1, CK7, S100A1, caveolin-1 and claudin-7. From these results, we studied the IHC expressions of CK7, Cav-1 and S100A1 in our laboratory. Further large international collaborative studies are needed to validate the clinical usefulness and reproducibility of these IHC biomarkers.

6.2.2 NF-κB

NF-κB importantly affects target genes involved in immunity, cellular proliferation, pro- or anti-apoptotic functions and carcinogenesis. In addition, NF-κB is unique in RCC as it regulates all important aspects of RCC biology that pose challenge to conventional therapy: resistance to apoptosis; angiogenesis; and multi-drug resistance (Morais et al. 2011). NF-κB IHC analyses on a cohort of RCC patients provided interesting molecular NF-κB signatures. Most studies in the past have focussed on p65 and p50 subunits of NF-κB in human RCC tissue (Kankaya et al. 2015; Meteoglu et al. 2008; Oya et al. 2003; Ozbek et al. 2012). From our knowledge, this is the largest and most comprehensive series of IHC analyses on the subunits of NF-κB family in human RCC tissue. There was higher IHC expression of p65 (overall, nuclear and membrane) and lower IHC expression of p52, p50 and cRel (overall, nuclear and membrane) in RCC tumour compared to normal counterparts. Higher p65 nuclear, p50 overall and p52 overall and nuclear expressions were associated with worse

cancer specific survival; with higher p65 nuclear and p50 overall expressions shown to be independent prognostic factors in RCC survival. These results have provided us with new insights on the molecular profiles of NF-κB subunits in RCC tumourigenesis. This better understanding will encourage more research into the NF-κB family and pave way for future targeted NF-κB subunit specific therapeutic pathways.

6.2.3 IHC results of various biomarkers

The analyses of various IHC biomarkers in our human RCC and adjacent matched normal renal tissue and Aperio morphometry (overall, membrane and nuclear expression) assessment included CK7, Cav-1, Ob, ObR, KIM-1 and S100A1. Biomarkers CK7, Cav-1 and S100A1 were selected based on the meta-analysis, while Ob, ObR and KIM-1 were novel biomarkers chosen to assess their ability in differentiating chRCC from RO. Most of results from this study for CK7, Cav-1 and S100A1 matched the previous published reports. However, the positive IHC results that could aid in the differentiation of chRCC from RO include: higher CK7 overall expression in chRCC compared to RO; higher Ob nuclear expression; higher KIM-1 overall expression in RO compared to chRCC; and diffuse cytoplasmic staining with peripheral enhancement and perinuclear halo Cav-1 pattern in chRCC compared to patchy cytoplasmic staining pattern in RO. Interestingly, 2 new findings were reported of increased Ob nuclear expression and KIM-1 overall expression in RO over chRCC. These new findings need to be validated with larger samples in future but, potentially, they could be used as differential IHC biomarkers in the differentiation of the two tumour subtypes.

6.3 FUTURE DIRECTIONS

Future directions based on the results of this thesis have been discussed to some extent in each of the original research chapters. From the meta-analysis results, our findings recommended a panel 10 IHC biomarkers (amylase α1A, Wnt-5a, FXYD2, ARPP, CD63, TGFβ1, CK7, S100A1, Cav-1 and claudin-7) that have demonstrated their ability to differentiate chRCC and RO. It is hoped that further international large-scale studies will be performed on these 10 biomarkers in the future to further consolidate or affirm the reproducibility of similar results in differentiation of RO from chRCC. Hopefully, there will also be further more specific novel biomarkers that can be discovered in this respect.

In addition, further studies can delve into the correlation between sera and urine levels of these biomarkers in relation to these two renal tumour subtypes. Therefore, non-invasive serum or urine levels of these biomarkers can also be investigated to assess if they can discriminate a benign lesion like RO from malignant chRCCs or from other more aggressive RCCs.

With respect to NF-κB, there is extensive research in targeting the NF-κB pathway for therapeutic purposes, especially in metastatic RCC. Recently, a study showed that targeting the phospholipase Cε (PLCε)/NF-κB/VEGF pathway may be a potential therapeutic strategy for preventing RCC progression (Du et al. 2014). Another study also indicated that p65 NF-κB signalling pathway may be involved in osteopontin-mediated ccRCC progression, partly by anti-apoptotic effect; thus both molecules can be potential targets of therapeutic intervention in ccRCC (Matusan-Ilijas et al. 2011).

Our study showed the unique molecular expressions of various subunits of NF-κB (p65, p50, p52 and cRel), but further studies are needed to build upon targeting these subunits pathways (either singly or in combination) as potential therapeutic pathways. These studies could concentrate particularly on the roles of p65, p50 and p52 subunits, as these were shown to affect cancer specific survival outcomes in our cohort of patients. Based on these findings, it is worthwhile that these patients with increased expression of p65, p50 and p52 in their RCC tissue, will need closer surveillance and more aggressive targeted treatment if there was RCC progression. From another diagnostic perspective, further larger international studies could also assess the role of various NF-κB subunits in the differentiation of renal tumour subtypes, especially chRCC and RO.

As shown in Chapter 5, where a panel of selected biomarkers was investigated, the results of IHC for CK7 concur with other previous published results that there was increased expression of CK7 in chRCC as opposed to only minimal patchy expression in RO. It has proven to be a reliable discriminatory IHC biomarker for chRCC from RO. Further work should focus on pathophysiology responsible for the increased expression of CK7 in chRCC compared to minimal or patchy in RO as both tumours originate from intercalated cells of collecting duct, which also expresses CK7. One study proposed that most biomarkers that are expressed in the collecting duct system may show decreased expression or disappear in many RO because cell-to-cell interactions of the majority of RO decrease during tumorigenesis (Ohe et al. 2012). In addition, the association of expression of CK7 in chRCCs with cancer specific survival should also be investigated. Perhaps expression intensity of CK7 could be a prognostic predictor for the smaller aggressive group of malignant chRCC phenotype which

will metastasise, unlike the majority of chRCC cases where risk of metastasis is low. Also, there is paucity of research investigating the utility of CK7 as a therapeutic target in RCC management.

In the case of Cav-1, a different unique staining pattern of chRCC compared to RO was noted as the distinguishing feature of this biomarker. In fact, its role as a differential IHC biomarker with more intense positivity in chRCC over RO has been discussed in several previous studies. Also of note is the association of increased levels of Cav-1 with poorer prognosis in RCC, suggesting its use as not only a diagnostic but a prognostic biomarker (Campbell et al. 2013; Joo et al. 2004; Liu et al. 2015). Therefore further research should also focus not only on its diagnostic function but also as a prognostic biomarker in serum and/or urine of RCC patients.

One of the novel and interesting findings concerned Ob expression. Increased Ob nuclear expression in RO as compared to chRCC was identified. Further work should validate this finding on a larger scale and also investigate the mechanism behind this observed differential expression. As mentioned in Chapter 5 section 5.9.3, previous studies have investigated serum adipokines (leptin and adinopectin) and their relationship with obesity and RCC. In the study by Horigochi *et al*, serum leptin levels were higher in one group of renal tumours (granular cell carcinoma and papillary) compared to ccRCC (Horiguchi et al. 2006a). Perhaps, future study should focus on the serum Ob levels in a large cohort of RCC patients including chRCCs and ROs; as there might be differences in the serum levels of Ob in these various renal tumour subtypes. Furthermore, future studies should assess the association of serum leptin and Ob nuclear expression in RCC patients with respect to RCC progression and

cancer specific survival. Therefore, with funding support, we are planning to validate the IHC of Ob and ObR, and analyse the role and mechanistic pathway of Ob in obesity and RCC pathogenesis. We plan to characterise Ob and ObR amongst the RCC subtypes available in our Renal Tumour Biobank and correlate these with the serum leptin from these patients stored in the Biobank.

KIM-1 immunostaining expression in RO was noted to be increased compared to chRCC in our study. This result is in contrast to most reported studies (Han et al. 2005; Zhang et al. 2014). Nevertheless, we believe future larger international studies should be able to address this issue. Furthermore, urinary KIM-1 levels of patients with chRCCs and ROs should be investigated to assess if there are any significant differences. If there are reproducible significant different levels of urinary KIM-1, these can be correlated with the expression of KIM-1 in the RCC tissue. Urinary KIM-1 levels of patients with various renal tumours need to be further assessed to see whether there are any differences between tumour subtypes, (especially benign versus malignant phenotypes) and also if there are any changes in levels post resection of tumours. The utility of urinary KIM-1 can then be an invaluable non-invasive diagnostic tool in workup of indeterminate renal lesions and also a surveillance technique in RCC patients following treatment. Since KIM-1 has been shown to be a useful urinary biomarker, we are planning to utilise the urine and renal tumour tissue samples stored in the Renal Tumour Biobank to compare the various urinary levels of these patients with different renal tumour subtypes and correlate with KIM-1 IHC expression in the tissue samples. We hypothesise that different renal tumour subtypes will have varying levels of urinary KIM-1 pre and post operatively.

One hypothesis is that different renal tumour subtypes will have different ratios of serum leptin to urinary KIM-1 levels. Future research from our laboratory will compare serum leptin and urinary KIM-1 from our stored samples and correlating them to the histology. This will hopefully pave way to non-invasive investigation of renal tumour subtypes, especially chRCC and RO, so that in the future, patients with radiological diagnoses of indeterminant small renal mass may only require serum leptin and urinary KIM-1 analyses to denote the renal tumour subtype, both benign and malignant.

Following from this panel of results on the differentiation of chRCCs and ROs so far, a worthwhile study in near future that we will explore in our lab will be to analyse the efficacy of IHC on these panel of CK7, Cav-1, Ob, S100A1 and KIM-1 in ex vivo tissue core biopsy obtained from our nephrectomy renal tumour samples. One study has shown the improvement in diagnostic accuracy for 4 major renal tumour subtypes (ccRCC, pRCC, chRCC and RO) from following the utilisation of panel of IHC stains (CAIX, CD117, AMACR, CK7, and CD10) in ex vivo tissue core biopsy from renal tumours (Al-Ahmadie et al. 2011), however, that study did not fully qualify the distinction between chRCC from RO. It will be interesting to apply our panel of CK7, Cav-1, Ob, S100A1 and KIM-1 IHC and assess the diagnostic implications on tissue core biopsies. If this study on ex vivo tissue core biopsy is successful in differentiating renal tumour subtypes, especially chRCC from RO, then this will improve the diagnostic classification of renal tumours on needle biopsy. We have also started collecting ex vivo tissue core biopsies (18G) from the renal tumour immediately following nephrectomy; and will perform IHC from our panel (CK7, Cav-1, Ob, KIM-1, S100A1) to assess their diagnostic accuracies in differentiating renal tumour subtypes especially chRCC from RO. Therefore, hopefully future diagnostic accuracies of renal mass biopsies can further be enhanced from our panel of IHC biomarkers.

Another future study planned will be the assessment of serum Ob and urinary KIM-1 levels in RCC and its association with their respective IHC expressions in RCC patients with various subtypes. Serum Ob and urinary KIM-1 levels might correlate well with their tumour IHC results, thus leading to the utility of sera and urine analyses instead of tissue analyses. Perhaps a non-invasive diagnostic algorithm incorporating serum leptin and urinary KIM-1 levels could be predictive of renal tumour subtypes. If successful, this will definitely improve the sensitivity and specificity of diagnostic tests when combined with radiological characteristics of renal tumours and help clinicians discern benign from malignant renal lesions.

Last but not least, the comprehensive creation and management of the Renal Tumour Biobank in CKDR in TRI will serve as invaluable source of clinicopathological data and serum, urine, renal tumour and normal renal tissue samples for further research projects. As mentioned in Chapter 5, serum, urine and tissue samples from the Biobank are being analysed with MRS to characterise molecular fingerprints of various renal tumours. Hopefully these results can be translated to clinical urological practice into distinguishing benign from malignant renal tumours. Numerous and important clinical and longitudinal follow up data can be obtained and analysed in this cohort of renal tumour patients. Furthermore, approved research projects locally and internationally involving renal tumours in the future can utilise the stored patient samples from the Biobank. Hopefully this future research work into RCC will further enhance our discovery and knowledge in this humbling disease of renal cancers.

6.4 CONCLUSION

The increasing detection of asymptomatic incidental renal tumours due to the widespread use of high resolution abdominal imaging for other indications is providing a significant clinical challenge. There is emerging concern regarding overdiagnosis, unnecessary treatment and treatment related harm including CKD. Current clinical practice dictates treatment of all solid lesions on the presumption they are malignant and contemporary surgical series continue to report significant numbers of unnecessary surgical and ablative procedures for benign and low malignant potential lesions. Advances in imaging and renal lesion biopsy have not provided sufficient certainty in the preoperative diagnosis of indolent lesions to arrest this trend. There is an urgent need for ongoing development of molecular biomarkers that accurately distinguish benign and low malignant potential lesions.

The application of these biomarkers to preoperative functional imaging techniques, urine and serum assessment and renal mass biopsy will ultimately result in the reliable characterization of lesions with no or limited malignant potential such as oncocytoma and chromophobe RCC. This will have a major clinical impact in reducing unnecessary intervention and treatment related harm.

The results gained from this PhD research have provided insight to the expressions of various IHC biomarkers in different renal tumour subtypes. In addition, discriminatory IHC biomarkers have been shown to be useful in the differentiation of chRCC from RO. Further research can be built upon these results and will hopefully encourage the development of better diagnostic and therapeutic pathways for patients with renal tumours.

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APPENDIX 1

Please review the publication: Ng KL, Rajandram R, Morais C, Yap NY, Samaratunga H, Gobe GC, Wood ST. 2014. Differentiation of oncocytoma from chromophobe renal cell carcinoma (RCC):- can novel biomarkers help solve an old problem? Journal of Clinical Pathology 67:97-104 at the hyperlink:- DOI: 10.1136/jclinpath-2013-201895

APPENDIX 2

Enquiries to: Metro South
Human Research Ethics Committee
Phone: 07 3443 8049
Fax: 07 3443 8003
HREC Ref: HREC/05/QPAH/95
E-mail: PAH_Ethics_Research@health.qld.gov.au
Amendment AM08

Professor Judith Clements
Queensland University of Technology
60 Musk Avenue
Kelvin Grove QLD
4059

Dear Professor Clements

HREC Reference number: HREC/05/QPAH/95
Protocol title: Australian Prostate Cancer Collaboration (APCC) Bio-Resource

The Office of the Metro South Human Research Ethics Committee noted and approved the following:-

Document	Version	Date
Notification of Amendment – Addition of Associate Investigators: 1. Dr Keng Lim Ng 2. Prof. Glenda Gobe 3. Dr Chris Morais 4. Mr David Small	N/A	16 April 2013
Investigator CVs: 1. Dr Keng Lim Ng 2. Prof. Glenda Gobe 3. Dr Chris Morais 4. Mr David Small	N/A	N/A

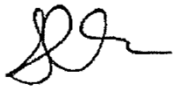
The Metro South Hospital and Health Service HREC is constituted and operates in accordance with the National Health and Medical Research Council's *"National Statement on Ethical Conduct in Human Research (2007), NHMRC and Universities Australia Australian Code for the Responsible Conduct of Research (2007)* and the "*CPMP/ICH Note for Guidance on Good Clinical Practice".*

It should be noted that all requirements of the original approval still apply.

A copy of this letter should be forwarded to {insert relevant} Research Governance Office(r).

If you have any queries please do not hesitate to contact the Human Research Ethics Committee office on +617 3443 8049.

Yours sincerely,



Sonia Hancock
HREC Coordinator
Metro South Hospital and Health Service
Human Research Ethics Committee (EC00167)
Centres for Health Research
Princess Alexandra Hospital
Woolloongabba QLD 4102

29/9/13

~~C.c. «Name and address of Research Assistant»~~

GREENSLOPES
PRIVATE HOSPITAL

Greenslopes Private Hospital
ABN 36 003 184 889
Newdegate Street
Greenslopes Qld 4120
Telephone (07) 3394 7111
Facsimile (07) 3394 7322
www.ramsayhealth.com.au

21 May 2013

Dr Keng Lim Ng
Urology Research Fellow
Department of Urology
Princess Alexandra Hospital
Woolloongabba QLD 4102

Dear Dr Keng Lim Ng

Protocol 13/23
Urological Diseases and Urological Cancer Research Now and in the Future

The following protocol documents were initially considered by the Greenslopes Research and Ethics Committee at the meeting held on Monday 13 May 2013.

- Cover email dated 1st May 2013
- Application form dated 1st May 2013

The Committee reviewed these documents and required further information.

On Tuesday 21st May, the chair reviewed the following documents and has agreed to grant full approval to conduct this study at Greenslopes Private Hospital.

- Your cover letter dated 16 May 2012
- Revised application form, including a clause stating that any future new research will require ethics approval at that time.
- Revised Participant Information and Consent forms on Greenslopes Private Hospital letterhead.

The Greenslopes Research and Ethics Committee is constituted and functions in accordance with the National Statement on Ethical Conduct in Human Research (2007).

Greenslopes Research and Ethics Committee continuing approval is subject to the following conditions being met:

1. **Conditions**
   - The Greenslopes Research and Ethics Committee will be notified, giving reasons, if the project is discontinued at a site before the expected date of completion.
   - The Coordinating Investigator will provide an annual report to the Greenslopes Research and Ethics Committee and at completion of the study in the specified format.
   - It is important that you inform the Ethics Committee immediately of any problems which arise during the course of the project which may have implications relating to the ethics of continuing the project in its present form.
   - Approval is conditional upon the commencement of the project within twelve months of the date of approval being granted. If the project does not commence within this time limit then a new protocol will require to be submitted to the Greenslopes Research and Ethics Committee.

RAMSAY
HEALTH CARE

- The Ethics Committee is to be advised when the project is completed.
- All Visiting Medical Officers are advised to check with their Medical Defence Organisation re personal indemnity for any research work about to be undertaken.
- Any public recruitment information, publicity or press releases are to be approved by the committee before release.

2. **Reporting**
   - An annual report is required to be submitted to the Ethics Secretary in a timely manner. A review questionnaire will be circulated to you annually to keep the Ethics Committee informed of the progress of the project.
   - The Coordinating Investigator will immediately report anything which might warrant review of ethical approval of the project in the specified format, including any unforeseen events that might affect continued ethical acceptability of the project.
   - Serious Adverse Events must be notified to the Committee. In addition, the Coordinating Investigator must provide a summary of the adverse events, in the specified format, including a comment as to suspected causality and whether changes are required to the Participant Information Sheet and Consent Form. In the case of Serious Adverse Events occurring at the local site, a full report is required from the Principal Investigator, including duration of treatment and outcome of event.

3. **Amendments**
   Amendments to the protocol should be forwarded to the Ethics Secretary for consideration at a committee meeting.

A copy of this letter should be presented when required as official confirmation of the approval of the Greenslopes Private Hospital Research Ethics Committee.

The Greenslopes Research and Ethics Committee wish you every success in your research.

Yours sincerely

Dr Jim Houston
Chair
Greenslopes Research and Ethics Committee

Enquiries to: Metro South Hospital and Health Service Human Research Ethics Committee
Phone: 07 3443 8049
Fax: 07 3176 7667
HREC Ref: HREC/12/QPAH/125
E-mail: Ethicsresearch.pah@health.qld.gov.au
Amendment AM03

Dr David Alan Vesey
Centre for Kidney Disease Research
Building 33
Princess Alexandra Hospital
Woolloongabba QLD 4102

Dear Dr Vesey

HREC Reference number: HREC/12/QPAH/125
Project title: Utilisation of fresh human kidney tissue for research into kidney disease

Thank you for submitting information regarding the above study. I am pleased to advise that the Metro South Hospital and Health Service Human Research Ethics Office noted and approved the following:-

Document	Version	Date
Notification of Amendment: • Addition of Dr Michael Ng as an Associate Investigator • Addition of collection of perirenal adipose tissue		11 June 2014
Protocol	2	26 June 2014
Participant Information and Consent Form	3.0	26 June 2014
Investigator CV: Dr Michael Ng		

The Metro South Hospital and Health Service HREC is constituted and operates in accordance with the National Health and Medical Research Council's *"National Statement on Ethical Conduct in Human Research (2007), NHMRC and Universities Australia Australian Code for the Responsible Conduct of Research (2007)* and the "*CPMP/ICH Note for Guidance on Good Clinical Practice".*

This will be ratified by the HREC at its 5th August 2014 meeting.

Please provide a copy of this approval letter to the Research Governance Office.

It should be noted that all requirements of the original approval still apply. Please continue to provide at least annual progress reports until the study has been completed.

If you have any queries please do not hesitate to contact the Human Research Ethics Committee office on +617 3443 8049.

Yours sincerely,

A/Prof Richard Roylance
Chair
Metro South Hospital and Health Service
Human Research Ethics Committee (EC00167)
Centres for Health Research
Princess Alexandra Hospital
Woolloongabba QLD 4102

11/7/14

THE UNIVERSITY OF QUEENSLAND

Institutional Human Research Ethics Approval

Project Title:	Utilisation Of Fresh Human Kidney Tissue For Research Into Kidney Disease - 07/07/2014 - AMENDMENT
Chief Investigator:	Dr Carolyn Clark, Dr David Vesey, A/Prof Glenda Gobe
Supervisor:	A/Prof Glenda Gobe, A/Prof Steven McTaggart
Co-Investigator(s):	David Small, Prof David Fairlie, Dr Michael Ng
School(s):	School of Medicine
Approval Number:	2013001265
Granting Agency/Degree:	Johnson/Gobe Laboratory
Duration:	1st April 2022

Comments/Conditions:

- Protocol Version 2 dated 26/06/2014
- PI&CF Version 3.0 dated 26/06/2014

Note: if this approval is for amendments to an already approved protocol for which a UQ Clinical Trials Protection/Insurance Form was originally submitted, then the researchers must directly notify the UQ Insurance Office of any changes to that Form and Participant Information Sheets & Consent Forms as a result of the amendments, before action.

Name of responsible Committee:
Medical Research Ethics Committee
This project complies with the provisions contained in the *National Statement on Ethical Conduct in Human Research* and complies with the regulations governing experimentation on humans.

Name of Ethics Committee representative:
Professor Bill Vicenzino
Chairperson
Medical Research Ethics Committee

Signature _____ Date 22.7.2014

APPENDIX 3

Queensland Renal Tumours Clinical Record

Date:- _____/_____/_____ **Clinical code:-** C __ __ __ __ __

Patient Name:- _____

Hospital:- _____ **Unit Record No:-** _____

Date of Birth:- _____/_____/_____ **Age at diagnoisis:-** _____

Gender:- ☐ Male. ☐ Female.

Ethnicity:- ☐ Anglo Saxon ☐ Aboriginal / Torres Straits Islander ☐ Arabic
☐ Chinese ☐ Indian ☐ Latin American ☐ Mediterranean ☐ other:- _____

Treating Surgeon:- _____

Oncologist (or other clinician managing systemic therapy):- _____

Person completing this form:- _____

I am ☐ patient's clinician ☐ survey data manager ☐ other:-_____

Initial Section

Date of detection/diagnosis:-

_____/_____/_____ ☐ not known

Primary method of diagnosis:-

☐ US ☐ CT
☐ MRI ☐ biopsy
☐ others:- _____

Result of scan or biopsy:-

_____
_____
_____

Reason for scan/diagnosis:-

☐ Incidental

☐ Surveillance for small renal mass/cyst

☐ Local symptoms

- ☐ haematuria ☐ flank pain
- ☐ palpable abdominal mass
- ☐ Constitutional (loss of weight, loss of appetite etc)
- ☐ Paraneoplastic (fever, hypertension, anaemia, abnormal liver function etc)

☐ Metastatic

- ☐ bone ☐ brain
- ☐ liver ☐ lung

☐ other:- _____

Characteristics of tumour:-

Size:- _____cm. Side:- _____

☐ endophytic(>50%) ☐ exophytic(>50%)

☐ anterior ☐ posterior

☐ upper pole ☐ lower pole ☐ interpolar

☐ single kidney ☐ congenital kidney:-
☐ horseshoe ☐ ectopic kidney

☐ others:- _____

☐ singular ☐ multifocal unilateral
☐ bilateral ☐ multifocal bilateral
☐ Bosniak cyst III / IV ☐ multiple cysts

Invasion:-

☐ renal vein ☐ IVC

☐ Lymph Node

What was the patient's clinical T-Stage (TNM 7th Edition, 2009) **based on imaging?**

☐ 1 **TX** Primary tumour cannot be assessed
☐ 2 **T0** No evidence of primary tumour
☐ 3 **T1** Tumour ≤ 7 cm in greatest dimension
- ☐ 1 **T1a** Tumour ≤ 4 cm in greatest dimension, limited to the kidney
- ☐ 2 **T1b** Tumour > 4 cm and ≤ 7 cm in greatest dimension

☐ 4 **T2** Tumour > 7 cm in greatest dimension, limited to the kidney
- ☐ 1 **T2a** Tumour > 7 cm and ≤ 10 cm in greatest dimension
- ☐ 2 **T2b** Tumour > 10 cm and limited to the kidney

☐ 5 **T3** Tumour extends into major veins or perinephric tissues but not into the ipsilateral adrenal gland and not beyond Gerota fascia
- ☐ 1 **T3a** Tumour grossly extends into the renal vein or its segmental (muscle containing) branches, or tumour invades perirenal and/or renal sinus fat (peri pelvic) fat but not beyond Gerota fascia
- ☐ 2 **T3b** Tumour grossly extends into vena cava below the diaphragm
- ☐ 3 **T3c** Tumour grossly extends into vena cava above diaphragm or invades the wall of the vena cava

☐ 6 **T4** Tumour invades beyond Gerota fascia (including contiguous extension into the ipsilateral adrenal gland)

What was the patient's clinical N-Stage (TNM 7th Edition, 2009) **based on imaging or other clinical evaluation?**

N-stage

☐ 1 **NX** Regional lymph nodes cannot be assessed
☐ 2 **N0** No regional lymph node metastasis
☐ 3 **N1** Metastasis in a regional lymph node(s)

What was the patient's clinical M-Stage (TNM 7th Edition, 2009) **based on imaging or other clinical evaluation?**

M-stage

☐ 1 **MX** Distant metastasis cannot be assessed
☐ 2 **M0** No distant metastasis
☐ 3 **M1** Distant metastasis. Please specify site(s) .....

(clinical) T _ _ N _ M _

Blood tests at diagnosis:-

Haemoglobin :-	☐ Low	☐ Normal	☐ High	specify result:-_____
White cell count	☐ Low	☐ Normal	☐ High	specify result:-_____
Platelets:-	☐ Low	☐ Normal	☐ High	specify result:-_____
Urea:-	☐ Low	☐ Normal	☐ High	specify result:-_____
Creatinine:-	☐ Low	☐ Normal	☐ High	specify result:-_____
GFR:-	☐ Low	☐ Normal	☐ High	specify result:-_____
Albumin:-	☐ Low	☐ Normal	☐ High	specify result:-_____
Calcium:-	☐ Low	☐ Normal	☐ High	specify result:-_____
LDH:-	☐ Low	☐ Normal	☐ High	specify result:-_____
ESR:-	☐ Low	☐ Normal	☐ High	specify result:-_____
CRP:-	☐ Low	☐ Normal	☐ High	specify result:-_____

Urine at diagnosis:-

☐ infection (UTI) ☐ proteinuria ☐ crystals, casts ☐ abnormal cytology

(Yes / No / Unknown)

Height:- ___._____ m **Weight:-** _____kg **BMI:-** _____

Smoking status:-

☐ current smoker ☐ ex-smoker ☐ non smoker **specify amount:-** _____**pack years**

Alcohol intake:- ☐ yes ☐ no specify:- _____

Regular use of NSAIDS /acetaminophen:- ☐ yes ☐ no

Any traditional medications use:- ☐ Yes ☐ No specify:- _____

Relevant comorbidities:- ☐ Yes ☐ No

Comorbidity	Mild	Moderate	Severe
Cardiovascular			
Myocardial Infarct	☐ MI by ECG unknown age	☐ MI > 6 months ago	☐ MI ≤ 6 months
Angina / Ischaemic Heart Disease	☐ ECG or stress test evidence or catheterization evidence of coronary disease without symptoms ☐ Angina pectoris not requiring hospitalization ☐ CABG or PTCA (>6 months) ☐ Coronary stent (>6 months)	☐ Chronic exertional angina ☐ Recent (≤ 6 months) Coronary Artery Bypass Graft (CABG) or Percutaneous Transluminal Coronary Angioplasty (PTCA) ☐ Recent (≤ 6 months) coronary stent	☐ Unstable angina
Congestive Heart Failure (CHF)	☐ CHF with dyspnoea which has responded to treatment	☐ Hospitalized for CHF >6 months prior ☐ CHF with dyspnoea which limits activities	☐ Hospitalized for CHF within past 6 months
Arrhythmias	☐ Sick Sinus Syndrome	☐ Ventricular arrhythmia > 6 months ☐ Chronic atrial fibrillation or flutter ☐ Pacemaker	☐ Ventricular arrhythmia ≤ 6 months
Hypertension	☐ Hypertension, controlled	☐ Secondary cardiovascular symptoms: vertigo, epistaxis, headaches	☐ Severe malignant papilloedema or other eye changes ☐ Encephalopathy
Venous Disease	☐ Old DVT no longer treated with Warfarin or Heparin	☐ DVT controlled with Warfarin or heparin ☐ Old PE > 6 months	☐ Recent PE (≤ 6 months) ☐ Use of venous filter for PE's
Peripheral Arterial Disease	☐ Intermittent claudication ☐ Untreated thoracic or abdominal aneurysm (< 6 cm) ☐ s/p abdominal or thoracic aortic aneurysm repair	☐ Bypass or **amputation for gangrene or arterial insufficiency > 6 months ago ***exclude amputations less than one foot* ☐ Chronic insufficiency	☐ Bypass or amputation for gangrene or arterial insufficiency < 6 months ago ☐ Untreated thoracic or abdominal aneurysm (≥6 cm)
Respiratory			
	☐ Restrictive Lung Disease or COPD (chronic bronchitis, emphysema, or asthma) with dyspnoea which has responded to treatment ☐ Sleep apnoea	☐ Restrictive Lung Disease or COPD (chronic bronchitis, emphysema, or asthma) with dyspnoea which limits activities	☐ Marked pulmonary insufficiency ☐ Restrictive Lung Disease or COPD with dyspnoea at rest despite treatment ☐ Chronic supplemental oxygen ☐ CO_2 retention high pCO_2 ☐ Baseline pO_2 low
Gastrointestinal			
Hepatic	☐ Chronic hepatitis or cirrhosis without portal hypertension ☐ Acute hepatitis without cirrhosis ☐ Chronic liver disease manifested on biopsy or persistently elevated bilirubin	☐ Chronic hepatitis, cirrhosis, portal hypertension with moderate symptoms "compensated hepatic failure"	☐ Portal hypertension and/or oesophageal bleeding ≤ 6 mos. (Encephalopathy, Ascites, Jaundice with elevated Total Bilirubin)
Stomach / Intestine	☐ Diagnosis of ulcers treated with meds ☐ Chronic malabsorption syndrome ☐ Inflammatory bowel disease (IBD) on meds or h/o with complications and/or surgery	☐ Ulcers requiring surgery or transfusion > 6 months ago	☐ Recent ulcers (≤ 6 months ago) requiring blood transfusion
Pancreas	☐ Chronic pancreatitis w/o complications	☐ Uncomplicated acute pancreatitis ☐ Chronic pancreatitis with minor complications (malabsorption, impaired glucose tolerance, or GI bleeding)	☐ Acute or chronic pancreatitis with major complications (phlegmon, abscess, or pseudocyst)
Renal			
End stage renal disease	☐ Chronic renal insufficiency not yet requiring dialysis	☐ Chronic renal insufficiency with chronic dialysis	☐ Multi-organ failure, shock, or sepsis requiring acute emergency dialysis

Endocrine	**(Include comorbid conditions with the (*) in both the Endocrine system and other organ systems if applicable)**		
Diabetes mellitus	☐ Type 2 controlled by oral agents only	☐ Insulin-dependent (Type 1 or Type 2) without complications ☐ Type 2 poorly controlled with oral agents	☐ Hospitalization ≤ 6 months for DKA ☐ Diabetes causing end-organ failure ☐ Retinopathy ☐ Neuropathy ☐ Nephropathy* ☐ Coronary disease* ☐ Peripheral arterial disease*
Neurological			
Stroke	☐ Stroke with no residual ☐ Past or recent TIA	☐ Old stroke with neurological residual	☐ Acute stroke with significant neurological deficit
Dementia	☐ Mild dementia (can take care of self)	☐ Moderate dementia (not completely self-sufficient, needs supervising)	☐ Severe dementia requiring full support for activities of daily living
Paralysis	☐ Paraplegia or hemiplegia, ambulatory and providing most of self care	☐ Paraplegia or hemiplegia requiring wheelchair, able to do some self care	☐ Paraplegia or hemiplegia requiring full support for activities of daily living
Neuromuscular	☐ MS, Parkinson's, Myasthenia Gravis, or other chronic neuromuscular disorder, but ambulatory and providing most of self care	☐ MS, Parkinson's, Myasthenia Gravis, or other chronic neuromuscular disorder, but able to do some self care	☐ MS, Parkinson's, Myasthenia Gravis, or other chronic neuromuscular disorder and requiring full support for activities of daily living
Psychiatric			
	☐ Depression or bipolar disorder controlled with medication	☐ Depression or bipolar disorder uncontrolled ☐ Schizophrenia controlled w/ meds	☐ Recent suicidal attempt ☐ Active schizophrenia
Rheumatological	**(Incl. Rheumatoid Arthritis, Systemic Lupus, Mixed Connective Tissue Disorder, Polymyositis, Rheumatic Polymyositis)**		
	☐ Connective Tissue Disorder on NSAIDS or no treatment	☐ Connective Tissue Disorder on steroids or immunosuppressant medications	☐ Connective Tissue Disorder with secondary end-organ failure (renal, cardiac, CNS)
Immunological	**(AIDS should not be considered a comorbidity for Kaposi's Sarcoma (KS) or Non-Hodgkin's Lymphoma)**		
AIDS	☐ Asymptomatic HIV+ patient. ☐ HIV+ w/o h/o AIDS defining illness $CD4^+ > 200/\mu L$	☐ HIV+ with h/o defining illness $CD4^+ < 200/\mu L$	☐ Fulminant AIDS w/KS, MAI, PCP (AIDS defining illness)
Immunocompromised		☐ Transplant patient ☐ Immunological deficiency	
Malignancy	**(Excluding Cutaneous Basal Cell Ca., Cutaneous SCCA, Carcinoma in-situ, and Intraepithelial Neoplasm)**		
Solid tumour including melanoma	☐ Any controlled solid tumour without documented metastases, but initially diagnosed and treated > 5 years ago	☐ Any controlled solid tumour without documented metastases, but initially diagnosed and treated within the last 5 years	☐ Uncontrolled cancer ☐ Newly diagnosed but not yet treated ☐ Metastatic solid tumour
Leukaemia & Myeloma	☐ H/o leukaemia or myeloma with last Rx > 1 yr prior	☐ 1st remission or new dx <1yr ☐ Chronic suppressive therapy	☐ Relapse ☐ Disease out of control
Lymphoma	☐ H/o lymphoma w/ last Rx >1 yr prior	☐ 1st remission or new dx <1yr ☐ Chronic suppressive therapy	☐ Relapse
Other			
Alcohol	☐ H/o alcohol abuse but not presently drinking ☐ ≥ 8 standard drinks per day	☐ Active alcohol abuse with social, behavioural, or medical complications	☐ Delirium tremens (at time of diagnosis)
Obesity		☐ BMI ≥ 38 (Calculate BMI from height and weight)	

Reference: Jay F. Piccirillo et al, Prognostic Importance of Comorbidity in a Hospital-Based Cancer Registry *JAMA*. 2004;291(20):2441-2447

What was the patient's performance status at diagnosis or initial treatment?

☐ 1 Karnofsky status recorded in medical record. Please supply score;

- ☐ 1 100% Normal; no complaints; no evidence of disease
- ☐ 2 90% Able to carry on normal activity; minor signs or symptoms of disease
- ☐ 3 80% Normal activity with effort; some signs or symptoms of disease
- ☐ 4 70% Cares for self; unable to carry on normal activity, go to school or do active work
- ☐ 5 60% Requires occasional assistance but is able to care for most of his needs
- ☐ 6 50% Requires considerable assistance and frequent medical care
- ☐ 7 40% Disabled; requires special care and assistance
- ☐ 8 30% Severely disabled; hospitalization is indicated though death not imminent.
- ☐ 9 20% Very sick; hospitalization necessary; active supportive treatment necessary
- ☐ 10 10% Moribund; fatal processes progressing rapidly.

☐ 2 ECOG status recorded in medical record. Please supply score;

- ☐ 1 Fully active (ECOG 0)
- ☐ 2 Ambulant and capable of work (ECOG 1)
- ☐ 3 In bed or chair less than 50% of the day, unable to work (ECOG 2)
- ☐ 4 In bed or chair more than 50% of the day (ECOG 3)
- ☐ 5 Bed bound (ECOG 4)

☐ 3 Neither Karnofsky nor ECOG performance status specifically recorded in history but case notes, referral letters etc indicate that patient was

- ☐ 1 Ambulant
- ☐ 2 Not ambulant

☐ 4 American Society of Anesthesiologists Score (ASA) recorded in the medical record

- ☐ 1 A normal healthy patient
- ☐ 2 A patient with mild systemic disease
- ☐ 3 A patient with severe systemic disease, that limits function, but is not incapacitating
- ☐ 4 A patient with severe systemic disease that is a constant threat to life
- ☐ 5 A moribund patient who is not expected to survive without the operation
- ☐ 6 A declared brain-dead patient whose organs are being removed for donor purposes

☐ 5 No indication of patient's performance status in history

Family history:-

☐ renal cancers ☐ other genitourinary tumours ☐ other tumours

Social history :-

Education level (highest):- ☐ primary ☐ secondary ☐ tertiary ☐ not known

☐ other _____

Occupation:- _____ ☐ retired ☐ none

Ipsilateral kidney - previous treatment to the renal tumour.

☐ none ☐ partial nephrectomy ☐ RFA ☐ cryoablation

☐ microwave ablation ☐ laser ablation ☐ HIFU ☐ embolization

☐ systemic therapy ☐ radiotherapy ☐ symptomatic /palliative

☐ metastatectomy (previous/planned) ☐ others, specify_____

Contralateral kidney - Previous treatment to the renal tumour.

☐ none ☐ partial nephrectomy ☐ RFA ☐ cryoablation

☐ microwave ablation ☐ laser ablation ☐ HIFU ☐ embolization

☐ systemic therapy ☐ radiotherapy ☐ symptomatic /palliative

☐ metastatectomy (previous/planned) ☐ others, specify_____

Neoadjuvant therapy:-

☐ no ☐ yes, specify:- ☐ chemotherapy:- _____

☐ immunotherapy:- _____

☐ tyrosine kinase inhibitors:- _____

☐ mTOR inhibitors:- _____

☐ monoclonal antibody:- _____

Neoadjuvant therapy:- date start ____/____/____ date ended ____/____/____

Radiotherapy treatment (Initial):-

☐ no ☐ yes ☐ brain metastases

☐ bone metastases

☐ Others specify:- _____

Radiotherapy:- date started :-____/____/____ date ended:- ____/____/____

Surgical Section

Surgery performed in

☐ private hospital ☐ public hospital

Date of surgery/treatment:- _____

Type of surgery :-

☐ radical nephrectomy ☐ partial nephrectomy

☐ open ☐ laparoscopic ☐ robotic assisted

☐ lymphadenectomy ☐ adrenalectomy ☐ metastatectomy concurrent

☐ IVC thrombectomy ☐ vena caval replacement

☐ cardiopulmonary bypass

Were there any post-operative complications?

☐ 1 No post-operative complications

☐ 2 Yes, please specify which of the following occurred. (*Tick all that apply)*

- ☐ 1 Deep vein thrombosis or pulmonary embolism
- ☐ 2 Wound infection
- ☐ 3 Post-operative pneumonia
- ☐ 4 Haemorrhage requiring blood transfusion
- ☐ 5 Peri-operative death
- ☐ 6 Other, please specify .....

Did the post-operative complications require either of the following?
(tick all that apply)

- ☐ 7 Unplanned return to theatre (for re-operation)
- ☐ 8 Unplanned admission to ICU

Pathology of renal tumour :-

☐ clear cell RCC ☐ papillary RCC ☐ chromophobe RCC ☐ oncocytoma

☐ carcinoma of collecting ducts ☐ others , specify _____

What was the patient's pathological TNM-Stage? (based on TNM 7th Edition)

T-stage

- ☐ 1 **TX** Primary tumour cannot be assessed
- ☐ 2 **T0** No evidence of primary tumour
- ☐ 3 **T1** Tumour ≤ 7 cm in greatest dimension
  - ☐ 1 **T1a** Tumour ≤ 4 cm in greatest dimension, limited to the kidney
  - ☐ 2 **T1b** Tumour > 4 cm and ≤ 7 cm in greatest dimension
- ☐ 4 **T2** Tumour > 7 cm in greatest dimension, limited to the kidney
  - ☐ 1 **T2a** Tumour > 7 cm and ≤ 10 cm in greatest dimension
  - ☐ 2 **T2b** Tumour > 10 cm and limited to the kidney
- ☐ 5 **T3** Tumour extends into major veins or perinephric tissues but not into the ipsilateral adrenal gland and not beyond Gerota fascia
  - ☐ 1 **T3a** Tumour grossly extends into the renal vein or its segmental (muscle containing) branches, or tumour invades perirenal and/or renal sinus fat (peri pelvic) fat but not beyond Gerota fascia
  - ☐ 2 **T3b** Tumour grossly extends into vena cava below the diaphragm
  - ☐ 3 **T3c** Tumour grossly extends into vena cava above diaphragm or invades the wall of the vena cava
- ☐ 6 **T4** Tumour invades beyond Gerota fascia (including contiguous extension into the ipsilateral adrenal gland)

N-stage

- ☐ 1 **NX** Regional lymph nodes cannot be assessed
- ☐ 2 **N0** No regional lymph node metastasis
- ☐ 3 **N1** Metastasis in a regional lymph node(s)

M-stage

- ☐ 1 **MX** Distant metastasis cannot be assessed
- ☐ 2 **M0** No distant metastasis
- ☐ 2 **M1** Distant metastasis. Please specify site(s) .....

(pathological) T _ _ N _ M _

To which specialists was the patient referred for treatment of renal cancer?
(Tick all)

- ☐ 1 Urologist **or** ☐ 2 General surgeon
- ☐ 3 Medical oncologist **or** ☐ 4 General Physician
- ☐ 5 Radiation Oncologist
- ☐ 6 Other, please specify .....
- ☐ 7 Not known

Follow Up Section

Entry into any clinical trials:-

☐ no ☐ yes name of trial:-_____

Since primary treatment any evidence of recurrence:-

☐ no

☐ yes ☐ local recurrence date first detected:- ____/_____/_____

☐ distant metastases date first detected:- ____/_____/_____

☐ not known

Adjuvant therapy:-

☐ no ☐ yes specify:-

☐ chemotherapy:- _____

☐ immunotherapy:- _____

☐ tyrosine kinase inhibitors:- _____

☐ mTOR inhibitors:- _____

☐ monoclonal antibody:- _____

Adjuvant therapy:- Date start _____ date ended _____

Latest Renal function :-

Creatinine at discharge _____

Creatinine on follow up :- _____ (date:- _____)

What is the patient's last known status?

Alive

☐ 1 With no evidence of disease

☐ 2 With recurrent or progressive disease

☐ 3 Disease status unknown

Deceased

☐ 4 From RCC

☐ 5 From other cause, specify .....

☐ 6 Cause of death unknown

Please specify the date of last contact with the patient or date of death

___ / ___ / ____ or ☐ Not known

Please make any comments that you feel may be relevant in the space below:-

Urology Department

Princess Alexandra Hospital
Ph 3176 6946

Director:
Dr Simon Wood

Clinical Coordinator:
Dr Irina Oleinikova
Ph: 07 3176 2217

Radiation Oncology

Dr Margot Lehman
Ph: 07 3176 2111 page 1217

National Chair
Australian Prostate
Cancer BioResource

Prof Judith Clements
Ph: 07 3443 7241

Australian Prostate
Cancer BioResource

Queensland Coordinator:
Allison Eckert
Ph: 07 3176 1891
Fax: 07 3138 6034

National Project Manager:
Dr Trina Yeadon
Ph: 07 3176 1892

Australian Prostate
Cancer Research Centre-
Queensland

Executive Director:
Prof Colleen Nelson
Ph: 07 3176 7443

Clinical Coordinator:
Dr Maggie Fung
Ph: 07 3176 7449

Princess Alexandra
Hospital (MaCH R) Tumour
Bank

Manager:
Ph: 07 3176 2375

HUMAN RESEARCH
ETHICS APPROVAL

Princess Alexandra Hospital
Ph: 07 3176 5856

PARTICIPANT CONSENT FORM

I, the undersigned ..... herby consent to donate tissue/blood samples for Urological Disease and Urological Cancer Research Now and in the Future

- I acknowledge that the nature, purpose and contemplated affects of this project so far as it affects me, have been fully explained to me by the clinical research coordinator and my consent is given voluntarily. I have also read and understand the Participant Information Sheet.
- I am informed that the tissue/blood samples I have donated may be used immediately or may be stored, in a coded state, for a period of time and will be released for future research.
- I understand that local, national and international research collaborations using my blood/tissue will only take place where the researchers abide by equal or more stringent regulations of privacy and ethics as those in Australia, as assessed by a Human Research Ethics Committee.
- I understand that the purpose of my tissue donation is to improve the quality of medical care, it has also been explained that my involvement may not be of any benefit to me.
- I am informed that no information regarding my medical history will be divulged and the results of any tests will not be published or released to a third party so as to reveal my identity.
- I understand that my involvement in the study will not affect my relationship with my medical advisers in their management of my health. I also understand that I am free to withdraw from the study at any stage without my future treatment being affected.
- I understand that if at any time I decide that I no longer wish to participate in the study, the samples will be discarded upon my written request to the Urology Clinical Coordinator. This will not affect my future medical treatment
- I give permission for my tissue to be used in any way that the Urology Research Group deems most beneficial and If my samples lead to the development of a commercial product in the future I assign and waive all claims to patents, commercial exploitation, property or any material or products which may form part of or arise from this study.
- I give permission for the Urology Research Group to access my medical records, for the purpose of these projects.
- I understand that this project will comply with the National Health and Medical Research Council's National Statement on Ethical Conduct in Research involving Humans and in accordance with the relevant State and Federal Privacy Legislation.

Signature of Participant		Date
Name of Witness	Signature of Witness	Date

Urological Disease and Urological Cancer Research Now and in the Future

Consent to be contacted in the future

Principal Investigators: Professor Judith Clements
Project Managers: Ms Allison Eckert (07) 3176 1891 or
Dr Maggie Fung (07) 3176 7449

I, ... the undersigned

am willing to be contacted in the future to consider participating in further research projects ☐Yes ☐No

wish to be contacted if findings are made that have implications for me or my family ☐Yes ☐No

give permission for these findings to be revealed to members of my family if they request the information ☐Yes ☐No

If family is to be notified please contact the following person/s if I am not contactable for any reason:

Name of 1st nominee if I cannot be contacted .....

Relationship to me: .....

Address.....

.....

Phone .....

.....

Name of 2nd nominee if I cannot be contacted .....

Relationship to me: .....

Address.....

.....

Phone .....

.....

_____ _____

Signature of Participant Date

_____ _____ _____

Name of Witness Signature of Witness Date

Urological Disease and Urological Cancer Research Now and in the Future

Consent to receive project specific information on current and future research conducted using my donated tissue samples

Principal Investigators: Professor Judith Clements
Project Managers: Ms Allison Eckert (07) 3176 1891 or
Dr Maggie Fung (07) 3176 7449

I, ... the undersigned

wish to receive information regarding any current research that my will use my donated tissue samples. ☐Yes ☐No

wish to receive information from the Australian Prostate Cancer BioResource regarding any future research that will use my donated tissue samples. ☐Yes ☐No

Signature of Participant _____ Date _____

Name of Witness _____ Signature of Witness _____ Date _____

Urological Disease and Urological Cancer Research Now and in the Future

Withdrawal of consent for the use of donated tissue samples
and associated clinical information.

Principal Investigators: Professor Judith Clements
Project Managers: Ms Allison Eckert (07) 3176 1891 or
Dr Maggie Fung (07) 3176 7449

I ..... hereby wish to WITHDRAW my consent for the use of my donated tissue samples and associated clinical information to be used in Urological disease and Urological cancer research. I understand that such withdrawal WILL NOT jeopardize any treatment or my relationship with Princess Alexandra Hospital and Medical Practitioner

Name: _____
(Please Print Clearly)

Signature: _____ Date:_____

Please return this form to the Urology Research Group Project manager.
A copy of this form will be returned to you.

Urological Disease and Urological Cancer Research Now and in the Future

Principal Investigators: Professor Judith Clements
Project Managers: Ms Allison Eckert (07) 3176 1891 or
Dr Maggie Fung (07) 3176 7449

As you have asked to withdraw from the study we will not contact you again about the Tissue Bank.

As you have asked to withdraw from this project, the Urology Research Group has destroyed the samples and /or data that you had previously donated.

Project Manager: _____
(Please Print Clearly)

Signature: _____ Date:_____

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Princess Alexandra
Hospital (MaCH R) Tumour
Bank

Manager:
Ph: 07 3176 2375

HUMAN RESEARCH
ETHICS APPROVAL

Princess Alexandra Hospital
Ph: 07 3176 5856

Urological Disease and Urological Cancer Research Now and in the Future

Research into men and women with diseases or problems associated with kidneys, genitals, adrenal glands, bladder or the prostate gland

Principal Investigators: Professor Judith Clements
Project Managers: Ms Allison Eckert (07) 3176 1891 or
Dr Maggie Fung (07) 3176 7449

We would like to invite you to be involved with the Urology Department at the Princess Alexandra Hospital by donating tissue samples. Please read through all the documentation supplied before making your decision.

Purpose of Tissue Collection.

Scientific and medical research and genetic research using human tissue, blood, urine and bodily fluid samples have played a significant role in advancing the knowledge and understanding of the causes and the treatment or management of a wide range of diseases. From this research we are able to advise patients with particular diseases which treatment or combination of treatments is best for them. The success of this research relies heavily upon donations of tissue and blood samples for current research, for storage in a tissue bank for future research and access to specimens archived in diagnostic pathology laboratories.

Types of Research Projects

There are several groups of doctors and scientists using tissue samples researching methods to improve the diagnosis, management and outcomes for people with urological disease and urological cancers. The small amount of tissue/blood/body fluids or tissue samples archived in pathology laboratories that we are asking you to donate can be used in a variety of ways in the:-

- Development of new techniques to diagnose urological disease and urological cancer
- Development of new treatments and drugs to treat prostate disease and prostate cancer
- Determining the effects of new treatments and drugs on the growth of the cancer cells. This may also involve studies on laboratory animals.
- Identification of viruses that may be implicated in the development of cancer
- Establishment of Primary cell lines
    - Cancer cells are cultured (grown) in the laboratory. Once the cells are successfully growing, the genes can be studied. This may involve adding or removing genes to the cells to determine if this produces changes to how the cells grow or behave
- Production of Tissue Micro-arrays (TMAs) from specimens archived within diagnostic pathology laboratories. TMAs are a new investigative tool that will ultimately help doctors select proper treatments and provide accurate prognosis for cancer patients

- Adult Stem Cell Research. Stem cells are the foundation cells for every cell in the body. They are essentially "blank" cells that do not yet have a specific function. Under the right conditions or given the right signals, stem cells give rise to many different cell types. Studying these cells will offer insights in the regulation of injury and repair processes and may lead to the development of alternative therapeutic tools.

Further detailed information regarding current individual research projects and future projects using your donated tissue samples will be provided by the Urology Research Group unless you specifically indicate that you do not want to receive this information. Further detailed information regarding future prostate disease or prostate cancer research projects will be provided by the APC BioResource unless you specifically indicate that you do not want to receive this information.

What is involved in Donating Tissue and Information?

Tissue Donation

The tissue removed during a diagnostic procedure or surgical procedure (operation) on any part of the body will be sent to the pathology department for routine tests. The results of these tests will be given to your doctor and will be used to plan your post-operative care.

It is usual that not all of the tissue removed at the time of your procedures is required for your diagnosis. We would like to collect and store small portions of fresh tissue, already removed during your diagnostic or surgical procedure, for the purpose of future biochemical and genetic research. This tissue is collected in such a way that it will not interfere with your surgery, subsequent treatment or the pathology departments examination of the specimen. We would also like to access any "left over tissue" that is not required for examination by the pathology department and the tissue that has been processed and may be stored in the pathology department following routine testing.

According to current legislation your tissue samples that are sent to pathology for routine analysis are retained for a period of up to 30 years. We would also like your permission to access these specimens.

Additional Donation of Blood and Urine.

We would also ask your consent to collect a small amount of blood (30mls or approximately 2 table spoons) and a urine sample from you, either at a routine hospital clinic visit, at the time of your surgery or during your hospital admission. These will be processed and stored for future biomedical and genetic research.

It is possible that your blood sample will be used in developing tests to determine genes that may be involved in whether there is a response to particular types of new or developing therapies. This research may also look at genes that will predict whether or not you will get a particular urological disease, urological cancer or urological metastasis.

You can donate a blood/other biological samples to the Urology Research Group even if you are not donating tissue. If you are having surgery or another diagnostic procedure, it can be arranged for the blood samples to be collected at that time.

If you are suitable and agree to participate in a longitudinal study, we will ask you to visit the clinic 10 times at 6-week intervals (i.e. week 0, 6, 12, 18, 24, 30, 36, 42, 48 and 54). Please see the attached table on page 5 for details.

Information collection from your health records

Information may be collected from you directly in the form of a questionnaire or from your medical records. The Urology Research Group will request your permission to collect information from your medical records about your past and present medical history, such as the names of any medication that you are currently taking, and for what medical condition, the results of any investigations, pathology reports from your operation and hospital admission and other ongoing information about your progress. All information will be collected and stored for an indefinite period of time in accordance with the relevant State and Federal Privacy legislation.

Does the decision to Participate affect my care in any way?

Deciding to donate Tissue samples is voluntary and that refusal to participate will involve no penalty or loss of benefits to which you are otherwise entitled, and you may discontinue participation at any time without comment or penalty. If you do not wish your donated tissue samples to be used in certain types of research all you have to do is let us know and precautions will be taken so that your donated sample is not used.

Benefits of Participating

It is not possible to predict whether any personal benefits to you will result from participation in these research projects. Research using your tissue may have the potential to provide invaluable information about the progression of diseases and may provide information that will assist in studies of therapeutic treatments and the people it afflicts, improving the quality of medical care in the future.

Possible Risks Associated with the Donation of Tissue Samples

Tissue Collection

This tissue is collected in such a way that it will not interfere with your surgery, subsequent treatment or the pathology departments examination of the specimen. However, if the pathologist feels that there is potentially important information related to your diagnosis or treatment within the samples we have stored then we will return the tissue for further examination by the pathologist. In most situations this will not be the case and we will be able to use this tissue for research.

No tissue stored in the tissue bank or APC BioResource will be released prior to the pathologists examination report being finalised.

Blood Collection

If blood is collected you might feel a slight pinch in your arm when the blood is taken and you could develop a small bruise.

What is a Tissue Bank or BioResource?

Tissue that may not be required for current research may be banked in a Tissue Bank or BioResource. A Tissue bank or BioResource is a large not-for-profit repository established for the collection of tissue and data samples from multiple sources for the purposes of use in future for biomedical, genetic analysis and genetic testing research projects. The term "tissue" includes the substance, structure and texture of which the human body or any part or organ of it is composed. The Term "Tissue" includes tumour samples, blood, blood components and other bodily fluid products (ejaculate, sweat, ascetic fluid, urine etc). It also includes tissue derivatives such as DNA, RNA and proteins obtained from human beings. Genetic Analysis research refers to the evaluation of the DNA, RNA and products of cells, where genetic testing research refers to the study of disease-predisposing genetic or hereditary factors which might place people at a greater risk of developing specific diseases or cancers. Tissue samples that are banked will be banked either in the Princess Alexandra Hospital (MaCH R)Tumour Bank or in the Tumour Biobank located in the Centre for Kidney Disease Research within the Translational Research Institute (TRI) or if prostate tissue is collected this may be banked as part of the Australian Prostate Cancer BioResource (APC BioResource).

Future Implications of donating Tissue Samples

Every stored tissue is a potential source of genetic information (DNA). It is possible that future research using your sample may result in new genetic information about your risk of getting cancer. If after extensive testing and validation, it is determined that the research findings may have significant implications for your family, and you have indicated you wish to receive further information, then this will be arranged with the help of appropriate counselling.

It is your choice whether or not you wish to know any important results and we will ask you if you want us to tell you if we do find any information that has significant implications for you or your family. We will also ask you if you want the information to be given to a member of your family if we cannot contact you. We will not give any information about you to members of your family without your permission.

How is My Privacy Protected?

To maintain your privacy each participant is given a unique identification (ID) number or code that is used to track all tissue samples and data. All information that could possibly identify you, such as your name and date of birth, is removed and replaced with this code. However, this code can be used to re-identify you for the purpose of linking important medical information to the stored tissue samples. Only authorised staff within the Urology Research Group will be able to link this code with your information. These authorised staff are, the project manager at the PAH, the manager and Queensland coordinator of the APC BioResource. If your tissue sample is stored for the purpose of future research, researchers accessing these samples are only supplied with coded samples and data. This ensures that nothing that can identify you or your family will ever be sent to other researchers, or appear on any public or published reports.

What are the Financial Implications of Donating Tissue?
You will not receive any financial reward for donating tissue samples and information. In order to achieve our aims, we will seek to use the most advanced technology available and on some occasions this will only be possible through collaboration with other institutions or commercial companies. If new discoveries of potential diagnostic or therapeutic importance are made we will protect this "intellectual property" through the filing of appropriate patents. The development of diagnostic agents and new medicines for cancer patients is likely to be very expensive and may require us to license our "intellectual property" to commercial companies. Such companies would be asked to undertake costly and complicated analyses and, in return, may require commercial rights to benefits arising from any discoveries. In this context, it should be noted that it is the whole collection of hundreds of samples that is of value and that each individual sample has in reality no value of its own. To allow such collaborations to proceed, you are asked to waive any future claim to financial benefit through participation in this research.

Who will use my tissue?
Your tissue sample may be used by local, interstate and international medical researchers for biochemical and genetic studies of diseases. Prior to the use of tissue samples and associated information in current projects and the release of tissue samples and associated information for future research release, these studies must have been approved by a Human Research Ethics Committee as required by the principles set out in the National Health and Medical Research Council of Australia (NHMRC) National Statement on Ethical Conduct in Research involving Humans, and the reporting Scientific Committee.

Will I find out the results of the research using my donation?
The results of any research done with your tissue are not likely to be available in the immediate future. This is because research can take a long time and must use tissue samples from many people before results are known. The researchers will not be able to give you the individual results from your samples except in exceptional circumstances.

What if I change my mind?
At any stage following tissue sample donation you have the right to withdraw any banked tissue samples. If you wish to have your tissue or blood or other samples withdrawn from this tissue bank or to stop access to your health information, please notify the Project Manager. A letter confirming removal of your tissue and or health information will be sent to you.

Who can I contact if I have more questions?
For further information: please contact Prof. Judith Clements (07 3443 7241) or the Project Managers on phone 07 3176 1891 or 07 3176 7449. You may also contact any member of the Urology Research Group.

Ethical Considerations
All work undertaken on your donated tissue samples will comply with the NHMRC National Statement on Ethical Conduct in Research involving Humans (http://www.nhmrc.gov.au/publications/synopses/_files/e72.pdf) and will have approval from the Princess Alexandra Hospital Human Research Ethics Committee. If you would like to discuss your rights as a participant or to discuss the conduct of the study please contact the Ethics Manager, Princess Alexandra Hospital on 07 3176 5856. All matters will be dealt with confidentially.

Attachment

Time and events schedule

	Study Visit									
	1	2	3	4	5	6	7	8	9	10
Week	0	6	12	18	24	30	36	42	48	54
Informed consent	X									
Inclusion criteria	X									
Blood collection	X	X	X	X	X	X	X	X	X	X

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Consent Form

Urological Diseases and Urological Cancer Research Now and in the Future

1. I, the undersigned ..... hereby consent to my involvement in the above study.

2. Include here the details of the procedure proposed including the anticipated length of time it will take, the frequency with which the procedure will be performed, and an indication of any discomfort, which may be expected.

3. I acknowledge that the nature, purpose and contemplated effects of the study so far as it affects me have been fully explained to me by the research worker and my consent is given voluntarily. I have also read and understand the Patient Information Sheet.

4. Although I understand that the purpose of this research project is to improve the quality of medical care, it has also been explained that my involvement may not be of any benefit to me.

5. I have been given the opportunity to have a member of my family or a friend present while the study was explained to me.

6. I am informed that no information regarding my medical history will be divulged and the results of any tests involving me will not be published so as to reveal my identity.

7. I understand that my involvement in the study will not affect my relationship with my medical advisers in their management of my health. I also understand that I am free to withdraw from the study at any stage without my future treatment being affected.

8.* "I understand that where biological material is collected, it may be stored and used for future research purposes either with my further consent or (in circumstances where my further consent cannot be obtained or is impractical to obtain) with the further specific approval of a hospital ethics committee set up in accordance with the NHMRC guidelines".

9.* I give permission for the release of information regarding progress in this study to the study centre, on the understanding that while the study centre will keep confidential results under my name, no published study will identify me in any way.

10.* I authorise the Greenslopes Private Hospital to allow access to relevant medical records to the investigators from Urology Department, Princess Alexandra Hospital.and Centre for Kidney Disease Research, School of Medicine, University of Queensland.....

11. I have been told that this study has been approved by the Ethics Committee at Greenslopes Private Hospital.

Signed ..... **Date .....**

Greenslopes Private Hospital Consent Form - Version 1 - Formulated - 28.09.98

* If applicable (eg No.9 is usually applicable to clinical trials.)
* No. 10 applies when investigators from other institutions are involved.

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PATIENT INFORMATION SHEET

Urological Disease and Urological Cancer Research Now and in the Future

Principal Investigators:	Dr Keng Lim Ng	Ph:- (07) 34437937
Research members:	Dr Simon Wood	Ph:- (07) 3176 6946
	Assoc Prof Glenda Gobe	Ph: (07) 31765655
	Dr Cristudas Morais	Ph: (07) 34438012
	Mr David Small	Ph: (07) 34437938

We would like to invite you to be involved with the Urology Department of Greenslopes Private Hospital by donating kidney and other urological tissue samples. Please read through all the documentation supplied before making your decision.

Purpose of Tissue Collection.

Scientific, medical and genetic research using human tissue, blood, urine and bodily fluid samples has played a significant role in advancing the knowledge and understanding of the causes and management of urological diseases. From this research, we are able to advise patients with particular urological diseases including cancers which treatment or combination of treatments is best for them. The success of this research relies heavily upon donations of kidney tissue, blood and urine samples for current research and for storage in a tissue bank for future research.

Types of Research Projects

There are several groups of doctors and scientists involved in research using donated tissue samples from patients. The small amount of kidney tissue, blood and urine stored in the tissue bank that we are asking you to donate can be used in a variety of ways in the:-

- Development of new techniques to diagnose urological diseases and urological cancers
- Identification of novel markers that can aid in diagnosis of kidney cancers
- Development of new treatments and drugs to treat urological diseases and cancers
- Determining the effects of new treatments and drugs on the growth of the cancer cells. This may also involve studies on laboratory animals.
- Identification of viruses that may be implicated in the development of cancer
- Establishment of Primary cell lines:- Cancer cells are cultured (grown) in the laboratory. Once the cells are successfully growing, the genes can be studied. This may involve adding or removing genes to the cells to determine if this produces changes to how the cells grow or behave
- Production of Tissue Micro-arrays (TMAs) from specimens archived within diagnostic pathology laboratories. TMAs are a new investigative tool that will allow examination of multiple tissue samples under the microscope.

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Further detailed information regarding current individual research projects and future projects using your donated tissue samples will be provided by the researchers mentioned above unless you specifically indicate that you do not want to receive this information.

What is involved in Donating Tissue and Information?

Tissue Donation

The kidney tissue removed during a diagnostic procedure or surgical procedure (operation) will be sent to the pathology department for routine tests. The results of these tests will be given to your doctor and will be used to plan your post-operative care.

It is usual that not all of the kidney tissue removed at the time of your procedure is required for your diagnosis. We would like to collect and store small portions of fresh kidney tissue, already removed during your diagnostic or surgical procedure, for the purpose of research. This kidney tissue is collected in such a way that it will not interfere with your surgery, subsequent treatment or the pathologists' examination of the specimen. We would also like to access any "left over tissue" that is not required for examination by the pathology department which is stored in the pathology department following routine testing.

According to current legislation your tissue samples that are sent to pathology for routine analysis are retained for a period of up to 30 years. We would also like your permission to access these specimens.

Additional Donation of Blood and Urine.

We would also ask your consent to collect a small amount of blood (30mls or approximately 2 table spoons) and a urine sample from you, either at a routine hospital clinic visit, at the time of your surgery or during your hospital admission. These will be processed and stored in tissue bank for future biomedical and genetic research.

It is possible that your blood and urine samples will be used in developing tests to determine genes or markers that may be involved in relation to particular types of new or developing therapies. This research may also look at genes that can predict the likelihood of your kidney tumour being benign or malignant.

Information collection from your health records

Information may be collected from you directly in the form of a questionnaire or from your medical records. We request your permission to collect information from your medical records about your past and present medical history, such as the names of any medication that you are currently taking, and for what medical condition, the results of any investigations, pathology reports from your operation and hospital admission and other ongoing information about your progress. All information will be collected, coded and stored for an indefinite period of time in accordance with the relevant State and Federal Privacy legislation.

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Does the decision to Participate affect my care in any way?
Deciding to donate tissue samples is voluntary and that refusal to participate will involve no penalty or loss of benefits to which you are otherwise entitled, and you may discontinue participation at any time without comment or penalty. If you do not wish your donated tissue samples to be used in certain types of research all you have to do is let us know and precautions will be taken so that your donated sample is not used.

Benefits of Participating
It is not possible to predict whether any personal benefits to you will result from participation in these research projects. Research using your tissue may have the potential to provide invaluable information about the progression of kidney diseases and may provide information that will assist in studies of therapeutic treatments and the people it afflicts, improving the quality of medical care in the future.

Possible Risks Associated with the Donation of Tissue Samples
Tissue Collection
This tissue is collected in such a way that it will not interfere with your surgery, subsequent treatment or the pathology department's examination of the specimen. However, if the pathologist feels that there is potentially important information related to your diagnosis or treatment within the samples we have stored, then we will return the tissue for further examination by the pathologist. In most situations this will not be the case and we will be able to use this tissue for research.
Blood Collection
If blood is collected you might feel a slight pinch in your arm when the blood is taken and you could develop a small bruise.
Urine collection
If urine is collected, there are no possible risks associated with donation of this sample.
By donation of the above samples, it will not jeopardise the treatment in anyway and if there were adverse events, then the providing team of doctors will provide the appropriate assistance.

What is a Tissue Bank or BioBank?
Tissue that may not be required for current research may be banked in a Tissue Bank or BioBank. A BioBank is a large not-for-profit repository established for the collection of tissue samples from multiple sources for the purposes of use in future for biomedical, genetic analysis and genetic testing research projects.
The term "tissue" includes the substance, structure and texture of which the human body or any part or organ of it is composed. The term "tissue" includes kidney tumour or healthy kidney samples, blood, and urine. It also includes tissue derivatives such as DNA, RNA and proteins obtained from human beings. Tissue samples that are banked will be banked in the Princess Alexandra Hospital and BioBank at Centre for Kidney Disease Research at Translational Research Institute.

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Future Implications of donating Tissue Samples

Every stored tissue is a potential source of genetic information (DNA). It is possible that future research using your sample may result in new genetic information about your risk of getting cancer or disease. If after extensive testing and validation, it is determined that the research findings may have significant implications for your family, and you have indicated you wish to receive further information, then this will be arranged with the help of appropriate counselling. It is your choice whether or not you wish to know any important results and we will ask you if you want us to tell you if we do find any information that has significant implications for you or your family. We will also ask you if you want the information to be given to a member of your family if we cannot contact you. We will not give any information about you to members of your family without your permission.

How is My Privacy Protected?

To maintain your privacy each participant is given a unique code that is used to track all tissue samples and data. All information that could possibly identify you, such as your name and date of birth, is removed and replaced with this code. However, this code can be potentially identifiable and traced back to you for the purpose of linking important medical information that can be of a benefit to you. Only authorised staff involved in this research will be able to de-identify the code and link this code with your information. These authorised staff are the members of the research team above. If your tissue sample is stored for the purpose of future research, researchers accessing these samples are only supplied with coded samples and data. This ensures that nothing that can identify you or your family will ever be sent to other researchers, or appear on any public or published reports.

What are the Financial Implications of Donating Tissue?

You will not receive any financial reward for donating tissue samples and information. In order to achieve our aims, we will seek to use the most advanced technology available and on some occasions this will only be possible through collaboration with other institutions or commercial companies. If new discoveries of potential diagnostic or therapeutic importance are made, we will protect this “intellectual property” through the filing of appropriate patents. The development of diagnostic agents and new medicines for cancer patients is likely to be very expensive and may require us to license our “intellectual property” to commercial companies. Such companies would be asked to undertake costly and complicated analyses and, in return, may require commercial rights to benefits arising from any discoveries. In this context, it should be noted that it is the whole collection of hundreds of samples that is of value and that each individual sample has in reality no value of its own. To allow such collaborations to proceed, you are asked to waive any future claim to financial benefit through participation in this research.

Who will use my tissue?

Your tissue sample may be used by local, interstate and international medical researchers for biochemical and genetic studies of kidney diseases. Prior to the use of tissue samples and associated information in current projects and the release of

tissue samples and associated information for future research release, these studies must have been approved by a Human Research Ethics Committee as required by the principles set out in the National Health and Medical Research Council of Australia (NHMRC) National Statement on Ethical Conduct in Research involving Humans, and the reporting Scientific Committee.

Will I find out the results of the research using my donation?
The results of any research done with your tissue are not likely to be available in the immediate future. This is because research can take a long time and must use tissue samples from many people before results are known. The researchers will not be able to give you the individual results from your samples except in exceptional circumstances.

Information to other doctors or investigators
That the subjects should advise the investigator of any other studies in which they are participating in.

What if I change my mind?
At any stage following tissue sample donation you have the right to withdraw any banked tissue samples. If you wish to have your tissue or blood or other samples withdrawn from this tissue bank or to stop access to your health information, please notify any member of the research team. A letter confirming removal of your tissue and or health information will be sent to you.

Who can I contact if I have more questions?
For further information: please contact Dr Keng Lim Ng (07 34437937) or you may also contact any research members mentioned above.

Ethical Considerations
All work undertaken on your donated tissue samples will comply with the NHMRC National Statement on Ethical Conduct in Research involving Humans and will have approval from the Greenslopes Hospital Research and Ethics Committee.

Thank you for your time and contribution.

APPENDIX 4

Authorized Ethics Committee
Aquesta Specialized Uropathology
21 Lissner Street, Toowong
Queensland 4066

AQUESTA®
SPECIALISED UROPATHOLOGY
Into Focus

NATA
MUTUAL RECOGNITION ACCREDITATION

RCPA
The Royal College of Pathologists of Australasia

Ethics Application Form

Version 2013 V.3.0

To be filled out by authorized personnel only

Date Approved: 1st April 2014

Protocol number: 14/02

To be filled out by the applicant

For detailed information on how to fill out this application, please request your copy of local guidelines.

NOTE! Only one project per application

Short description of project

Investigation of biomarkers in renal neoplasms

Authorized Ethics Committee
Aquesta Specialized Uropathology
21 Lissner Street, Toowong
Queensland 4066

Responsible pathologist

Name: Hemamali Samaratunga

Address: Aquesta Specialized Uropathology
21 Lissner Street, Toowong
Queensland 4066

E-mail: hema@aquesta.com.au

Telephone:

Associated laboratories (if applicable):

Name: Keng Lim Ng

Title: Dr
Address: Urology Department, Princess Alexandra Hospital
Wollongabba 4102 QLD
E-mail: kenglimng@yahoo.com
Telephone: 0418621234

Detailed description of project:

Abstract Title:- Investigation of Biomarkers to aid in the differentiation of renal tumours, especially renal Oncocytoma and Chromophobe renal cell carcinoma.

Hypothesis:- There are distinct differences in molecular signatures between renal oncocytoma and chromophobe RCC that can be exploited to distinguish benign from malignant phenotypes.

Aim:- To investigate novel and established molecular biomarkers through analysis of blood, urine and tissue samples from patients with oncocytoma and chromophobe RCC.

Method:- One method will be to develop and investigate archived tissue blocks of these disease entities via immunohistochemical stains. To assess if there are any difference in the expression intensities of immunohistochemical stains between oncocytoma and Chromophobe RCC.

Authorized Ethics Committee
Aquesta Specialized Uropathology
21 Lissner Street, Toowong
Queensland 4066

Description of tissue and data collection:
Archived tissue blocks (oncocytoma & chromophobe RCC) of patients with renal cell carcinoma stored at Aquesta Pathology.

Patient selection:
Archived tissue blocks of patients with renal cell carcinoma (oncocytoma & chromophobe RCC) from Aquesta Pathology

Ethical considerations:
This study involves the use of existing collections of material that contain only non-identifiable data. There is no risk to patients

Funding:
No funding is needed to carry out this experiment.

..... Block capitals KENG LIM NG
I hereby declare that all information stated above is true and that this project will be carried out according to Australian law

Approved by Aquesta Pathology Ethics Committee:

.....
Signature

Name J. PERRY-KEENE

.....
Signature

Name KIARA KLOPFER

K Samaraliinga
.....
Signature

Name

1/4/2014 Brisbane
.....
Date and place

UNIVERSITI MALAYA
PUSAT PERUBATAN UM

JAWATANKUASA ETIKA PERUBATAN
PUSAT PERUBATAN UNIVERSITI MALAYA
ALAMAT: LEMBAH PANTAI, 59100 KUALA LUMPUR, MALAYSIA
TELEFON: 03-79493209 FAKSIMILI: 03-79494638

No. Rujukan: PPUM/MDU/300/04/03

22 April 2011

Dr. Retnagowri Rajandram
Jabatan Surgeri
Pusat Perubatan Universiti Malaya

Puan,

SURAT PEMAKLUMAN KEPUTUSAN PERMOHONAN MENJALANKAN PROJEK PENYELIDIKAN
Caspase-8 and TRAF1 as biomarkers of progression of renal cell carcinomas
Protocol No : -
MEC Ref. No : 848.17

Dengan hormatnya saya merujuk kepada perkara di atas.

Bersama-sama ini dilampirkan surat pemakluman keputusan Jawatankuasa Etika Perubatan yang bermesyuarat pada 20 April 2011 untuk makluman dan tindakan puan selanjutnya.

2. Sila maklumkan kepada Jawatankuasa Etika Perubatan mengenai butiran kajian samada telah tamat atau diteruskan mengikut jangka masa kajian tersebut.

Sekian, terima kasih.

"BERKHIDMAT UNTUK NEGARA"

Saya yang menurut perintah,

Norashikin Mahmood
Setiausaha
Jawatankuasa Etika Perubatan
Pusat Perubatan Universiti Malaya

s.k Ketua
Jabatan Surgeri

UNIVERSITY OF MALAYA
KUALA LUMPUR
UM MEDICAL CENTRE

MEDICAL ETHICS COMMITTEE
UNIVERSITY MALAYA MEDICAL CENTRE
ADDRESS: LEMBAH PANTAI, 59100 KUALA LUMPUR, MALAYSIA
TELEPHONE: 03-79493209 FAXIMILE: 03-79494638

NAME OF ETHICS COMMITTEE/IRB: Medical Ethics Committee, University Malaya Medical Centre ADDRESS: LEMBAH PANTAI 59100 KUALA LUMPUR	ETHICS COMMITTEE/IRB REFERENCE NUMBER: 848.17
PROTOCOL NO: TITLE: Caspase-8 and TRAF1 as biomarkers of progression of renal cell carcinomas	
PRINCIPAL INVESTIGATOR: Dr. Retnagowri Rajandram TELEPHONE: KOMTEL:	SPONSOR: FRGS

The following item [✓] have been received and reviewed in connection with the above study to be conducted by the above investigator.

[✓] Borang Permohonan Penyelidikan	Ver date: 29 Mar 11
[ ] Study Protocol	Ver date:
[ ] Investigator Brochure	Ver date:
[✓] Patient Information Sheet	Ver date:
[✓] Consent Form	
[ ] Questionnaire	
[✓] Investigator(s) CV's (Dr. Retnagowri Rajandram)	

and have been [✓]

[✓] Approved
[ ] Conditionally approved (identify item and specify modification below or in accompanying letter)
[ ] Rejected (identify item and specify reasons below or in accompanying letter)

Comments:

Investigator are required to:

1) *follow instructions, guidelines and requirements of the Medical Ethics Committee.*
2) *report any protocol deviations/violations to Medical Ethics Committee.*
3) *provide annual and closure report to the Medical Ethics Committee.*
4) *comply with International Conference on Harmonization – Guidelines for Good Clinical Practice (ICH-GCP) and Declaration of Helsinki.*
5) *note that Medical Ethics Committee may audit the approved study.*

Date of approval: 20th APRIL 2011

c.c Head
Department of Surgery

Deputy Dean (Research)
Faculty of Medicine

Secretary
Medical Ethics Committee
University Malaya Medical Centre

PROF. LOOI LAI MENG
Chairman
Medical Ethics Committee

APPENDIX 5

Protocol # 8 : Cytokeratin-7 (03/09/2015)

Procedure: RUO DISCOVERY Universal (v0.00.0201)

Discovery ULTRA Staining Module

Translational Research Institute, 37 Kent Street Woolloongabba QLD 4102

Step No	Procedure Step
1	Enable Mixers
2	Warmup Slide to 37 Deg C
3	[Delay refers to a time delayed start: Select time until run start]
4	[RECOMMENDED: Set temperature to 60°C and time to 8 minutes]
5	Disable Mixers
6	Warmup Slide to [60 Deg C], and Incubate for [8 Minutes] (Baking)
7	Disable Slide Heater
8	Enable Mixers
9	Disable Mixers
10	Warmup Slide to 58 Deg C, and Incubate for 4 Minutes
11	Apply CC Coverslip Long
12	Incubate for 8 Minutes
13	Apply CC Coverslip Long
14	Incubate for 8 Minutes
15	Apply CC Coverslip Long
16	Incubate for 8 Minutes
17	Apply EZPrep Volume Adjust
18	Enable Mixers
19	[RECOMMENDED: Set temperature to 69°C and time to 8 minutes foreach cycle]
20	[Depar Cycle 1]
21	Warmup Slide to [69 Deg C], and Incubate for [8 Minutes] (Cycle 1)
22	Rinse Slide With EZ Prep
23	Apply Coverslip
24	Incubate for 4 Minutes
25	Apply EZPrep Volume Adjust
26	[Depar Cycle 2]
27	Incubate for [8 Minutes] (Cycle 2)
28	Rinse Slide With EZ Prep
29	Apply Coverslip
30	Incubate for 4 Minutes
31	Apply EZPrep Volume Adjust
32	[Depar Cycle 3]
33	Incubate for [8 Minutes] (Cycle 3)
34	Rinse Slide With EZ Prep
35	Apply Depar Volume Adjust
36	Apply Coverslip
37	Warmup Slide to 37 Deg C
38	Disable Slide Heater
39	Pause Point (Landing Zone)
40	Warmup Slide to 37 Deg C
41	Rinse Slide With EZ Prep
42	Apply Long Cell Conditioner #1
43	Apply CC Coverslip Long
44	Warmup Slide to [95 Deg C], and Incubate for 4 Minutes (Cell Conditioner #1)
45	Incubate for 4 Minutes

CC1 buffer pH6

* one drop is one reagent dispense

Protocol # 8 : Cytokeratin-7 (03/09/2015)

Procedure: RUO DISCOVERY Universal (v0.00.0201)

Discovery ULTRA Staining Module

Translational Research Institute, 37 Kent Street Woolloongabba QLD 4102

Step No	Procedure Step
46	Incubate for 8 Minutes
47	Apply Cell Conditioner #1
48	Apply CC Medium Coverslip No BB
49	Incubate for 8 Minutes
50	Apply Cell Conditioner #1
51	Apply CC Medium Coverslip No BB
52	Apply Cell Conditioner #1
53	Apply CC Medium Coverslip No BB
54	Apply Cell Conditioner #1
55	Apply CC Medium Coverslip No BB
56	Disable Slide Heater
57	Apply Cell Conditioner #1
58	Apply CC Medium Coverslip No BB
59	Warmup Slide to 37 Deg C
60	Rinse Slide With Reaction Buffer
61	Adjust Slide Volume With Reaction Buffer
62	Apply Coverslip
63	Disable Slide Heater
64	Pause Point (Landing Zone)
65	Warmup Slide to 37 Deg C
66	[Select an Inhibitor NOTE: Inhibitor CM comes packaged with Chromomap DAB]
67	[DISCOVERY Inhibitor is a stand alone product for use with all other HRP substrates]
68	[Inhibitor CM will be applied]
69	Rinse Slide With Reaction Buffer
70	Adjust Slide Volume With Reaction Buffer
71	Apply Coverslip
72	Rinse Slide With Reaction Buffer
73	Adjust Slide Volume With Reaction Buffer
74	Apply Coverslip
75	Apply One Drop of Inhibitor CM, and Incubate for [8 Minutes]
76	Disable Slide Heater
77	Pause Point (Landing Zone)
78	Warmup Slide to 37 Deg C
79	Rinse Slide With Reaction Buffer
80	Adjust Slide Volume With Reaction Buffer
81	Apply Coverslip
82	Disable Slide Heater
83	Disable Mixers
84	Wait For Button (Antibody)
85	Enable Mixers
86	Warmup Slide to 37 Deg C
87	Incubate for 4 Minutes
88	Rinse Slide With Reaction Buffer
89	Adjust Slide Volume With Reaction Buffer
90	Apply Coverslip

Protocol # 8 : Cytokeratin-7 (03/09/2015)

Procedure: RUO DISCOVERY Universal (v0.00.0201)

Discovery ULTRA Staining Module

Translational Research Institute, 37 Kent Street Woolloongabba QLD 4102

Step No	Procedure Step
91	Incubate for 4 Minutes
92	Warmup Slide to [37 Deg C] from Very Low Temperatures (Primary Antibody
93	Hand Apply (Primary Antibody), and Incubate for [60 Minutes]
94	Rinse Slide With Reaction Buffer
95	Adjust Slide Volume With Reaction Buffer
96	Apply Coverslip
97	Disable Slide Heater
98	Pause Point (Landing Zone)
99	Warmup Slide to 37 Deg C
100	[Requires DETECTION dispensers]
101	[These selections may be used for haptenated linking antibodies]
102	Rinse Slide With Reaction Buffer
103	Adjust Slide Volume With Reaction Buffer
104	Apply Coverslip
105	Rinse Slide With Reaction Buffer
106	Adjust Slide Volume With Reaction Buffer
107	Apply Coverslip
108	Warmup Slide to [37 Deg C] from Very Low Temperatures (2nd Antibody
109	Apply One Drop of [Anti-Mouse HQ] (Detection #1), and Incubate for [0 Hr 16 Min]
110	Rinse Slide With Reaction Buffer
111	Adjust Slide Volume With Reaction Buffer
112	Apply Coverslip
113	Disable Slide Heater
114	Pause Point (Landing Zone)
115	Warmup Slide to 37 Deg C
116	Rinse Slide With Reaction Buffer
117	Adjust Slide Volume With Reaction Buffer
118	Apply Coverslip
119	Rinse Slide With Reaction Buffer
120	Adjust Slide Volume With Reaction Buffer
121	Apply Coverslip
122	[Select Multimer]
123	Apply One Drop of [Anti-HQ HRP] (Conjugate #1), and Incubate for [16 Minutes]
124	Rinse Slide With Reaction Buffer
125	Adjust Slide Volume With Reaction Buffer
126	Apply Coverslip
127	Rinse Slide With Reaction Buffer
128	Adjust Slide Volume With Reaction Buffer
129	Apply Coverslip
130	Rinse Slide With Reaction Buffer
131	Adjust Slide Volume With Reaction Buffer
132	Apply Coverslip
133	Apply One Drop of H2O2 CM, and Incubate for 4 Minutes
134	Apply One Drop of DAB CM, and Incubate for 8 Minutes
135	Rinse Slide With Reaction Buffer

1:75 SantaCruz mouse anti CK7

* one drop is one reagent dispense

Protocol # 8 : Cytokeratin-7 (03/09/2015)

Procedure: RUO DISCOVERY Universal (v0.00.0201)

Discovery ULTRA Staining Module

Translational Research Institute, 37 Kent Street Woolloongabba QLD 4102

Step No	Procedure Step
136	Adjust Slide Volume With Reaction Buffer
137	Apply One Drop of Copper CM, Apply Coverslip, and Incubate for 4 Minutes
138	Rinse Slide With Reaction Buffer
139	Adjust Slide Volume With Reaction Buffer
140	Apply Coverslip
141	Rinse Slide With Reaction Buffer
142	Adjust Slide Volume With Reaction Buffer
143	Apply Coverslip
144	Apply One Drop of [HEMATOXYLIN II] (Counterstain), and Incubate for [8 Minutes]
145	Rinse Slide With Reaction Buffer
146	Adjust Slide Volume With Reaction Buffer
147	Apply Coverslip
148	Rinse Slide With Reaction Buffer
149	Adjust Slide Volume With Reaction Buffer
150	Apply Coverslip
151	Apply One Drop of [BLUING REAGENT] (Post Counterstain), and Incubate for [4 Minutes]
152	Rinse Slide With Reaction Buffer
153	Adjust Slide Volume With Reaction Buffer
154	Apply Coverslip
155	Rinse Slide With Reaction Buffer
156	Rinse Slide With Reaction Buffer

APPENDIX 6

Please refer to the publication: Ng KL, Morais C, Bernard A, Saunders N, Samaratunga H, Gobe G, Wood S. 2016. A systematic review and meta-analysis of immunohistochemical biomarkers that differentiate chromophobe renal cell carcinoma from renal oncocytoma. Journal of Clinical Pathology 0 :1-11 at hyperlink :- DOI:10.1136/jclinpath-2015-203585