The role of stem cells and WNT signalling pathway in renal cell carcinoma

by

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DECLARATION

I, Dr Siphelele Clifford Madlala, hereby declare that the work on which this dissertation is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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DEDICATION

This work is dedicated with gratitude to my wife and to my mentors.

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ABSTRACT

Introduction: Renal cell carcinoma (RCC) accounts for 87% of all kidney cancers. Despite advances in diagnostic techniques and management, renal cell carcinoma remains a lethal tumour accounting for substantial mortality and morbidity. The poor prognosis arises from metastasis, chemoradiation resistance and disease relapse. Cancer stem cells, a subpopulation of tumour cells with capacity to self-renew and reconstitute tumour heterogeneity have been implicated as the root cause of poor prognosis. Therefore, a better understanding of biomarkers of cancer stem cells will be useful for risk stratification, prognostication and may lead to novel targeted therapies that will ultimately alter the management of many patients.

Aims and objectives: To review the morphological subtypes of renal cell carcinomas diagnosed in the Division of Anatomical Pathology, National Health Laboratory Service, Groote Schuur Hospital over a 10 year period. To identify cancer stem cells in various histopathological subtypes of renal cell carcinoma using immunohistochemical markers (CD133 and CD105). To review the WNT signalling pathway in renal cell carcinomas using selected protein expression by immunohistochemistry (β-Catenin).

Materials and methods: Ten-year retrospective study in which sixty-four cases of renal cell carcinoma were retrieved and reviewed. Four immunohistochemical stains (β-catenin, HIF-1α, CD133 and CD105) were performed and scored in tumour tissue. Data were analysed to determine if there was any correlation between expression of the biomarkers and the histopathological subtypes of renal cell carcinoma.

Results: The mean age of the patients was 56-years (range, 35 to 81 years). Females constituted just over half (52%, n = 33) of the study patients. All 64 cases were confirmed as renal cell carcinomas, with 29 (45%) clear cell renal cell carcinomas, 14 (22%) papillary renal cell carcinomas, 9 (14%) chromophobe renal cell carcinomas, 9 (14%) multicystic renal cell carcinomas and 3 (5%) sarcomatoid renal cell carcinomas. Ten (16%) cases showed abnormal β-Catenin cytoplasmic localisation. The majority of cases (n=6, 60%) showing abnormal β-Catenin localisation were clear cell renal cell carcinomas. However, there was no significant correlation between abnormal and normal β-Catenin localisation and RCC histopathological subtype (p = 0.766). CD133 immunohistochemical studies showed low expression in 52 (81 %) cases and high expression in 12 (19 %) cases. There was no correlation between low and high CD133 expression and histopathological RCC subtype (P = 0.800). CD105 immunostaining showed tumour cell immunopositivity in one case of clear cell renal cell carcinoma whilst the rest of the cases were negative. The low, moderate and high microvascular density categories had 24, 10 and 32 cases respectively. There was no significant correlation between low, moderate and high microvascular densities and the histopathological RCC subtype (P = 0.320). HIF-1α immunohistochemical studies showed low expression in 39 (61 %) cases and high expression in 25 (39 %) cases. There was no significant correlation between levels of HIF-1α expression and the histopathological RCC subtype (P = 0.972).

Conclusion: Within the power limitations of this small study, β-catenin abnormal expression, microvascular densities and levels cytoplasmic CD133 and HIF-1α were not associated with any histopathological subtype of renal cell carcinoma.

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LIST OF ABBREVIATIONS

ccRCC……..	Clear cell RCC
CDC……	Collecting duct carcinoma
CKD……..	Chronic kidney disease
chRCC……..	Chromophobe RCC
CSC……..	Cancer stem cell
CXCR-4…….	CXC chemokine receptor-4
CSS……	Cancer-specific survival
DAB……	3,3'-Diaminobenzidine
ECM…….	Extracellular matrix
EDTA……..	Ethylenediaminetetraacetic acid
EMT…….	Epithelial-to-mesenchymal transition
EPO……..	Erythropoietin
EPOR…….	EPO receptor
ESC…….	Embryonic Stem Cell
FFPE…….	Formalin-fixed paraffin-embedded
HIF……	Hypoxia-inducible factor
HRP……..	Horseradish peroxidase
ISUP…….	International Society of Urological Pathology
mTOR……..	Mammalian target of rapamycin
MVD……	Microvascular density
PI3K……..	Phosphoinositide 3-kinase

PDGF…… Platelet-derived growth factor

PN..... Partial nephrectomy

pRCC…… Papillary RCC

pVHL…… von Hippel-Lindau protein

RCC…….. Renal cell carcinoma

RN…….. Radical nephrectomy

SDH…….. Succinate dehydrogenase

sRCC..... Sarcomatoid RCC

TCGA…… The Cancer Genome Atlas

TEDTA…….. Tris Ethylenediaminetetraacetic acid

TGF…… Transforming growth factor

VEGF…….. Vascular endothelial growth factor

VHL…….. von Hippel Lindau

WHO…….. World Health Organization

CHAPTER 1

INTRODUCTION

Worldwide, renal cancer represents 2.4% of all adult malignancies, with about 403 000 new cases having been reported in 2018 [1]. The World Health Organization (WHO) recognises a wide variety of histopathological subtypes of renal cell carcinoma [2]. The main histopathological subtypes are clear cell (ccRCC), papillary (pRCC) and chromophobe (chRCC). Clear cell renal carcinoma accounts for 70% of all renal cell carcinomas (RCCs) and contributes a significant proportion of the mortality and morbidity attributed to RCC. The ccRCC has a high metastatic index and low survival rates of 10-20% for metastasised RCC [2]. The current treatment modalities for localised RCC include partial or total nephrectomy which are associated with 30% chance of developing metastatic disease and a 40% chance of local recurrence [3,4]. The resulting, metastatic disease is usually chemoradiation resistant and this results in renal malignancy being the most lethal type of genitourinary malignancy [5].

Advanced disease is managed using a multi-therapy approach which includes total nephrectomy and systemic targeted-drug therapies [6,7]. Targeted drug therapies which are commonly used include mammalian target of rapamycin (mTOR) inhibitors and vascular endothelial growth factor receptor (VEGFR) inhibitors [6,7]. VEGFR inhibitors target to reduce neovascularisation (angiogenesis) while mTOR inhibitors reduce cell proliferation and thus inhibiting tumour progression [6,7]. However, patients may present with primary

or develop acquired resistance, which leads to failure of these targeted drug therapies and subsequent tumour progression [8].

Some of the common molecular alterations observed in RCC are loss of function mutation of the von Hippel Lindau (*VHL*) gene and deletions of the short arm of chromosome 3 [9,10]. Absence of active VHL protein (pVHL) is responsible for the loss of VHL-associated proteolysis of HIF-1α and HIF-2α [11.12]. This subsequently leads to accumulation of hypoxia inducible factor (HIF) which results in alterations that promote progression to RCC [11,12]. The adaptive response to hypoxia involves the transcription factor, hypoxia-inducible factor -1 (HIF-1) which consists of two subunits (HIF-1α and HIF-1β) [11,12]. HIF-1α is thought to be linked to the pathogenesis of RCC. Cases of ccRCC have been observed to express high levels of HIF-1α which is also considered a favourable independent prognostic factor [13].

Recent investigations have confirmed the presence of a subpopulation of tumour cells referred to as cancer stem cells (CSC) which exhibit the capacity to self-renew and reconstitute tumour heterogeneity [6]. The CSCs have been identified as the key drivers of tumour progression, metastasis and chemoradiation resistance [14].

Researchers have identified developmental signalling pathways including Wnt signalling pathway as being critical for the survival of CSCs [15]. The Wnt/β-catenin signalling pathway plays a crucial role in regulating differentiation of stem cell populations, cell proliferation

and induction of apoptosis [16]. Activation of the Wnt signalling can co-operate with RAS and hedgehog signalling pathways in solid tumours like renal and colon carcinomas which makes it an attractive target for anti-tumour therapeutics [17].

There exists great interest in the elucidation of mechanisms that operate in CSCs. Embryonic stem cell (ESC) markers such as NANOG, OCT4, KLF4, c-MYC and progenitor cell marker CD133, CD105 and CD44 can be used to identify CSCs [14,18]. Cancer stem cells have been identified in a number of solid tumours including RCC [17,19]. Some of the progenitor cell markers which have been reported as potential markers of CSCs in RCC include CD44, CD105 and CD133 [5,20].

CSCs are often linked to therapy resistance, poor prognosis and might therefore represent a prime target for novel treatment strategies [20]. Currently available targeted treatment options do not target CSCs, emphasising the need to identify and characterise them. This in turn may potentially lead to the development of targeted therapy against this subpopulation of tumour cells.

In this study we characterised CSCs in RCC using immunohistochemistry (IHC) staining for CD105, CD133 and β-catenin. We also characterised expression of HIF-1α.

CHAPTER 2

LITERATURE REVIEW

2.1. Epidemiology

Renal cancer represents 2.4% of all adult malignancies worldwide, with incidence and mortality rates which have been on the increase [1,2]. South Africa's National Cancer Registry reported that in 2014, kidney cancer contributed approximately 0.64% and 1.11% of all cancers in females and males respectively [21]. Renal cell carcinoma is more common in males with a male-to-female ratio of 2:1 [2]. The carcinoma is rare in children and the incidence increases with age until peaking at ages 60 to 70 years [2]. There were an estimated 143 000 deaths worldwide from renal malignancy in 2012 (91 000 in men, 52 000 in women) [1].

Several aetiological agents and risk factors have been identified for renal cell carcinoma, and these include smoking, obesity, hypertension, chronic renal disease and occupational exposure to specific carcinogens, such as trichloroethylene [1,2].

Widespread use of radiological imaging modalities in clinical practice has inadvertently led to an increase in incidentally reported renal cancers. These kidney incidentalomas are usually small in size and have better prognosis [22].

2.2. Histopathological diagnosis

The current WHO classification of renal tumours describes a broad spectrum of histopathological entities [2]. Renal cancer is a heterogeneous disease. RCC is the most frequent (87%) malignancy affecting the adult kidney [23]. The most common histopathological subtype of RCC is ccRCC (70%). Other morphological subtypes of RCC include pRCC (10–15%), chRCC (5%), and collecting duct carcinoma (CDC) (< 2%) [1,2].

The 2016 WHO Classification of RCC also lists other subtypes including those that were previously considered as potential emerging entities [2]. The new entities include hereditary leiomyomatosis-associated RCC (HLRCC), renal medullary carcinoma, MiT family translocation RCC, succinate dehydrogenase-deficient RCC (SDH deficient RCC), mucinous tubular and spindle cell carcinoma, tubulocystic RCC, acquired cystic disease-associated RCC, clear cell papillary RCC and RCC, unclassified [2].

2.2.1. Clear cell renal cell carcinoma

Macroscopically, ccRCC is a well circumscribed, pseudo-encapsulated tumour with golden-yellow appearance on cut surface, often with haemorrhage and necrosis. Microscopically the neoplasm shows predominantly nested or acinar growth pattern. A common diagnostic clue is the presence of a regular network of "chicken wire" capillaries. The cytoplasm of the cells is filled with glycogen and lipids [2].

2.2.2. Papillary renal cell carcinoma

Papillary RCC (pRCC) is the second most commonly encountered histopathological subtype of RCC [2]. Papillary RCC has traditionally been subdivided into Type 1 and 2 which have distinct clinical and molecular features [24].

Grossly, pRCC is well circumscribed with a pseudocapsule and a yellow to brown appearance on cut surface depending on the extent of intratumoral haemorrhage. Microscopically, the tumour shows papillae with delicate fibrovascular cores which contain foamy macrophages and psammoma bodies. Type 1 carcinomas display papillae lined monolayer of nuclei and scanty pale cytoplasm. Type 2 carcinomas show high grade nuclear pseudostratification with cells containing abundant eosinophilic cytoplasm [2].

The tumour is commonly organ confined (pT1-2 N0M0) and has a better prognosis than ccRCC [25].

2.2.3. Chromophobe renal canal cell carcinoma

Macroscopically, chRCC appears as a well circumscribed tumour with a pale tan cut section. Morphologically, the tumour shows cells arranged in solid sheets with intervening hyalinised vascular septa. The neoplastic cells are large with prominent 'plant cell-like' cell membranes and reticular cytoplasm. The nuclei often have a distinctive irregular wrinkled (raisinoid) appearance, with coarse chromatin and perinuclear haloes [2].

Fuhrman or WHO/ISUP grading system is not applicable to chRCC as the cells have innate nuclear atypia. The tumour has a relatively good prognosis with a high ten-year cancer-specific survival (CSS) [25].

2.3. Molecular basis of clear cell renal cell carcinoma

The molecular pathogenesis of most ccRCC involves biallelic loss of function of the *Von Hippel-Lindau (VHL)* tumour suppressor gene [27]. VHL-associated proteolysis of HIF-1α and HIF-2α is done by E3 ubiquitin ligase complex during normoxia [28].

HIF-1α and HIF-2α are both normally involved in activating target genes for transcription in response to reduced oxygen tension. Under normal conditions (functional *VHL*), reduced oxygen tension can inhibit HIF hydroxylase enzymes and lead to HIF-1α protein accumulation, which may translocate to the cell nucleus, dimerise with HIF-1β, and activate the transcription of some HIF target genes (**Figure 1**) [13] .

In ccRCC, constitutive activity of HIF-1α and HIF-2α independent of the oxygen levels is observed due to loss of VHL-associated proteolysis of HIF-1α and HIF-2α [27]. Absence of active pVHL is responsible for the loss of VHL-associated proteolysis of HIF-1α and HIF-2α (**Figure 1**) [27,29].

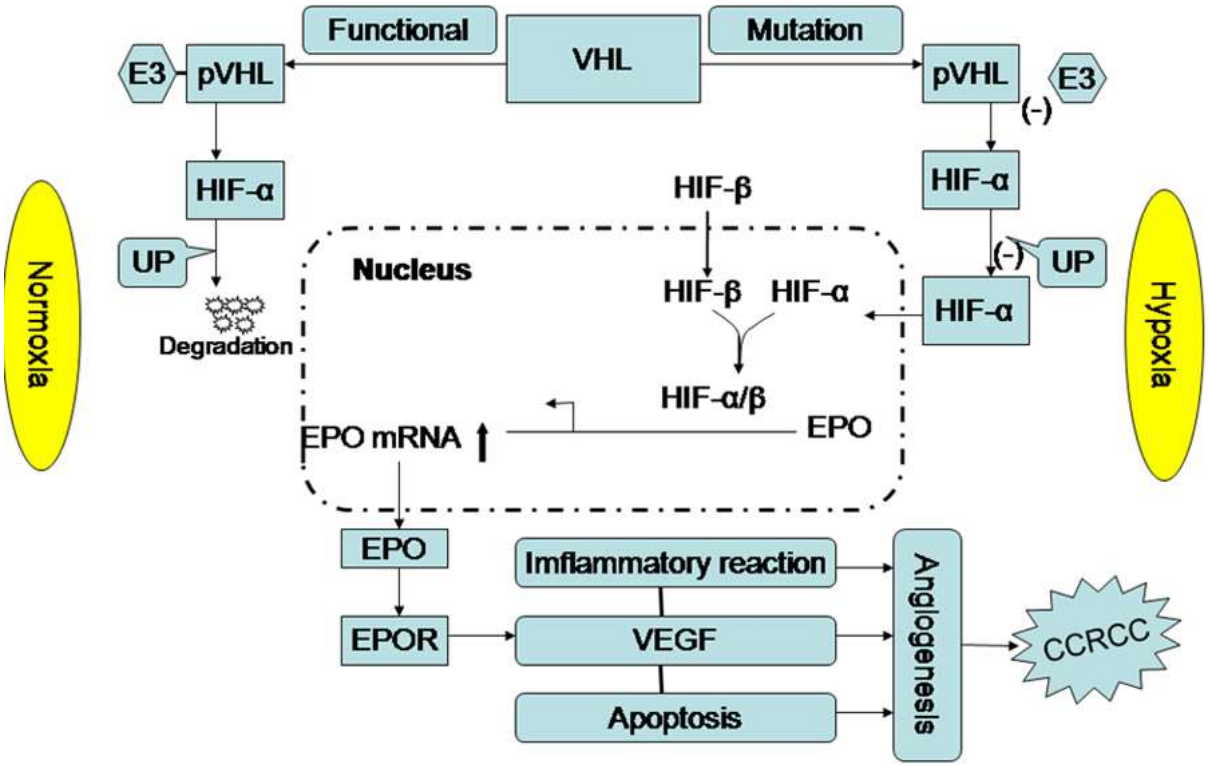

Figure 1: Role of the VHL-HIF pathway in ccRCC (Reprinted from Bai et al. [29])

In ccRCC, HIF-1α has been identified as a potent tumour suppressor whilst HIF-2α has been identified as the key oncoprotein crucial for tumour progression [13]. Loss of regulated HIF degradation which emanates from loss of the *VHL* tumour suppressor gene, leads to increase in HIF-2α target gene transcription [30,31]. Erythropoietin (EPO) is one class of HIF-target genes. Vascular endothelial growth factor (VEGF) and platelet-derived growth factor β (PDGF-β) which are directly involved in angiogenesis are activated by HIF-2α [30,31].

The high vascularity evident in ccRCC is a direct effect of increased expression of VEGF, and this has led to the development of novel therapies aimed at inhibiting angiogenesis. Bevacizumab, an immunotherapy drug that targets and inactivates *VEGF*, has been developed to treat advanced ccRCC [32].

The Cancer Genome Atlas (TCGA), describes recurrent mutations in the PI3K/AKT/mTOR pathway, mutations in *SETD2* and mutations involving the *SWI/SNF* chromatin remodelling complex as additional molecular alterations which may be found in ccRCC [23]. The mTOR complex activation results in translation of *HIF-1α*, whilst inhibition of mTOR complex inhibition results in decreased *HIF-1α* translation [33]. Targeted therapy against the mammalian target of rapamycin (mTOR) such as temsirolimus has been developed based on this knowledge [34].

In addition to the mTOR, VHL and VEGF signalling, additional signalling pathways which have been implicated in RCC pathogenesis, include Wnt/β- catenin [35].

2.4. Wnt signalling pathway

The Wnt family is comprised of 19 diverse secreted lipid-modified glycoproteins involved in signalling [36]. The signalling glycoproteins are involved in regulation of cell proliferation, differentiation, survival, migration and stem cell self-renewal [36].

The two Wnt signalling pathways are the canonical and non-canonical Wnt signalling pathways [36,37]. The canonical pathway involves β-catenin whilst the non-canonical pathway functions independent of β-catenin (**Figure 2**) [36,37].

When Wnt signalling is in its resting state, cytosolic β-catenin forms a destruction complex composed of adenomatous polyposis coli (APC), axin, glycogen synthase kinase-3 β (GSK-3 β) and casein kinase (CK) which results in the phosphorylation of β-catenin. The

ubiquitination of this phosphorylated β-catenin by cellular β-transducin repeat-containing proteins (β-TrCP) subsequently leads to its degradation by a proteasome [37].

Binding of Wnt-ligands to Frizzled (Fzd) receptors and the co-receptor low density lipoprotein receptor-related protein 5 (LRP5) or LRP6 in the canonical Wnt-pathway results in the phosphorylation of the cytoplasmic adaptor protein dishevelled (Dvl), leading to inhibition of glycogen synthase kinase (GSK-3 β) activity and thereby promoting a dephosphorylation and stabilisation of β-catenin which accumulates within the nucleus. Its interaction with members of the lymphoid enhancer-binding factor (LEF) and T-cell factor (TCF) family promotes the transcription of critical Wnt-target genes governing cell proliferation such as *c-MYC* and *cyclin D1* [38,39].

Therefore, influencing the Wnt signalling pathway could lead to novel targeted strategies in cancer therapy.

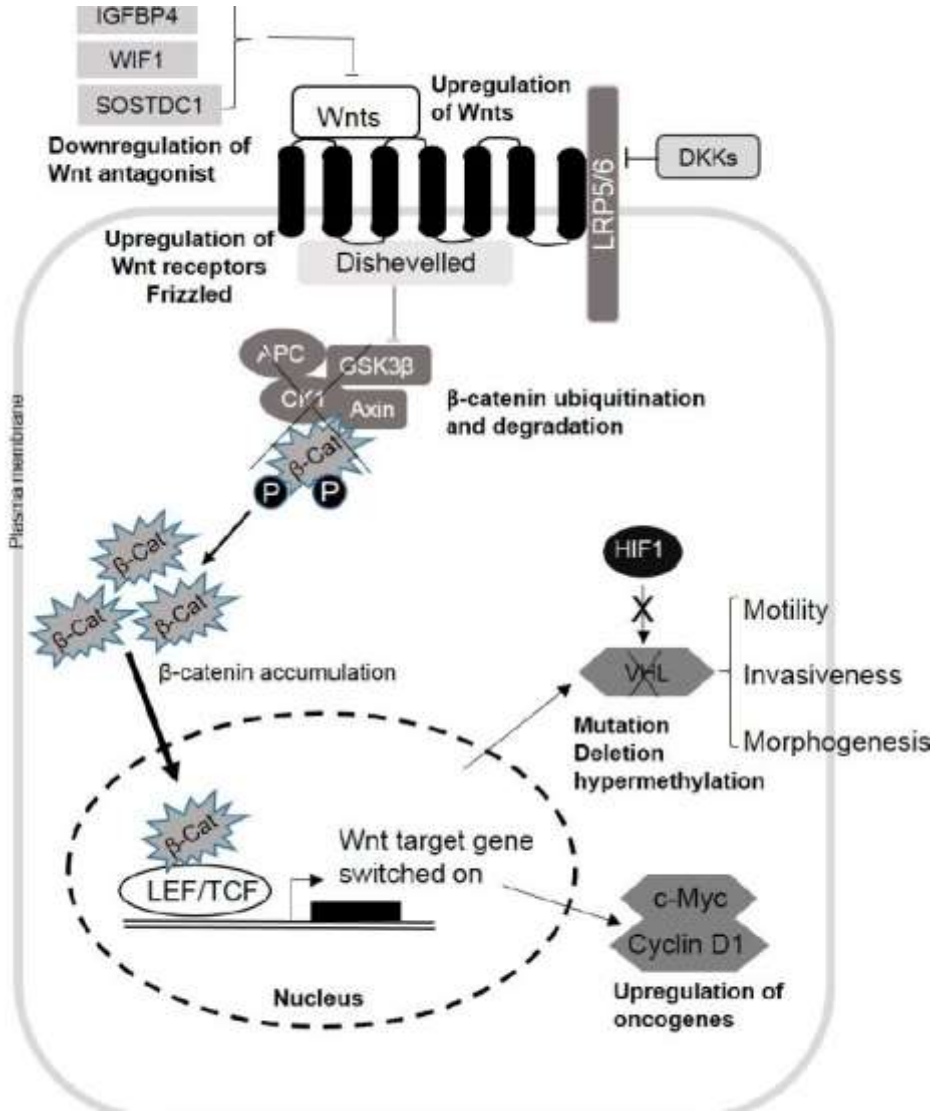

Figure 2: Wnt signalling pathway in RCC (Reprinted from Xu et al. [36])

2.4.1. Wnt Signaling in Renal Cell Carcinoma

Altered expression of different Wnt proteins, Wnt receptors (Fzds) and Wnt antagonists identified in ccRCC highlight the important role of Wnt signalling in RCC. Wnt1 expression, which is a canonical Wnt, is associated with large tumour diameter and invasiveness [40].

Wnt7A expression, which is a non-canonical Wnt is downregulated in the majority of ccRCCs [41]. Wnt receptors Fzd5 and Fzd8 are increased in RCC with an associated downstream increase in Cyclin D1 [42].

Cytoplasmic overexpression of β-catenin is related to high rates of morbidity and mortality in patients with RCC [38,40]. Patients with cytoplasmic β-catenin localisation are associated with large tumour size, more advanced stage and lymphovascular invasion [40]. Currently, no mutations have been detected in the β-catenin gene, *CTNNB1* [43,44].

Wnt/β-catenin signalling pathway has been identified as one of the pathways involved in regulation of cancer stem cells (CSCs) [45,46]. As a result it has been postulated that inhibition of Wnt/β-catenin signalling will inhibit RCC progression [45]. Thus, the Wnt signalling pathway has emerged as one of the potential targets for novel therapy for RCC.

The Wnt/β-catenin pathway is involved in regulating cell proliferation, differentiation and apoptosis induction. In ccRCC, an enhanced Wnt signalling pathway has been suggested as an aetiological factor involved in carcinogenesis and tumour progression through exaggerated cell proliferation and differentiation [15,45,47]. Expression of tumour suppressor proteins and oncoproteins in RCC is altered as a downstream effect of the activation of the Wnt/β signalling pathway [36]. Some of the downstream Wnt-regulated genes upregulated in ccRCC are oncogene, *c-MYC* and cell cycle regulator, cyclin D1 (*CCND1*) [36].

Three Wnt inhibitors (piroctoneolamine, ciclopiroxolamine and ethacrynic acid), which are selective inhibitors of Wnt signalling pathways have been found to be potent inducers of apoptosis in RCC [16,48].

Limited data is available on the possible role of non-canonical Wnt signalling [36]. Overexpression of RTK-like orphan receptor 2 (Ror2), a Wnt ligand receptor whose expression is normally confined to embryogenesis, has been found in ccRCC [36].

2.5. Cancer stem cells

Cancer stem cells are a small subpopulation of tumour cells capable of self-renewing and have the potential to form new cancer colonies [49]. Wnt signalling has been found to be crucial for the maintenance of the CSC population [45,46]. Research has shown that the capacity of a tumour to grow and propagate resides in this small population of cancer stem cells (CSCs) [50]. This population of CSC has also been found to be chemoradiation resistant and therefore can persist after treatment, promoting tumour recurrence [51,52].

Due to the ability of CSCs to recapitulate the tumour, strategies of selectively targeting the Wnt signalling pathway and CSCs could prove to be effective therapeutic options.

2.5.1. Cancer stem cell biomarkers

Many studies employing different approaches have been conducted to identify CSCs. Consequently, several biomarkers have been identified and these include ALDH1, CXCR-4, OCT4, CD133, CD105 and CD44 (**Figure 3**) [20, 53-55].

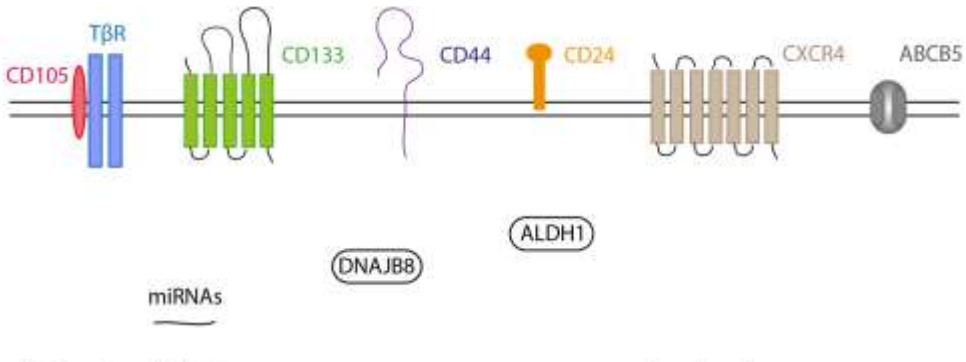

Figure 3: Cancer stem cells surface markers (Adapted from Corro et al. [20])

2.5.1.1. CD133

The CD133 (Prominin-1) is a pentaspan membrane glycoprotein arranged on the cell membrane in a way which results in the N-terminal domain on the extracellular compartment and the C-terminal domain on the cytoplasmic aspect [56,57]. CD133 is encoded for by the *PROM1* gene located on chromosome 4p15 [20]. CD133 is thought to be involved in membrane organisation due to the preferential localisation of the glycoprotein on apical plasma membranes protrusions and microvilli [57].

CD133 act as an upstream activator of the PI3K/AKT signalling pathway through interaction with p85 [20,58]. Tumour cells attain self-renewal capacity, metastasis and chemoradiation resistance capacity through utilising the PI3K/AKT signalling pathway [57].

High CD133 expression is correlated with hypoxia, mTOR inhibition and TGF-β1 in solid carcinomas including lung carcinoma and hepatocellular carcinoma [57]. Reduced oxygen tension increases HIF-1α expression which is known to inhibit mTOR activity [57].

CD133 has been identified as a biological marker of cancer stem cells in solid malignancies including kidney, central nervous system and prostate [59]. CD133 expression has been identified in a small proportion (<1% of tumour cells) of renal carcinoma cells [55].

CD133-positive cells in normal kidneys have been found to demonstrate stem cell characteristics which include multipotency and self-renewal [53]. CD133 expression has also been documented as a poor prognostic indicator in solid tumours [55,60].

The CD133 positive tumour cells have also been found to be chemoradiation resistant [51,52].

Using CD133 to identify the small population of CSC will not only enable identification of tumours with potential to recur but also suggest a potential target for novel therapy [51,52].

2.5.1.2. CD105

CD105 (endoglin) is a transmembrane glycoprotein predominantly overexpressed in proliferating endothelial cells [20]. The protein is encoded by the endoglin (*ENG*) gene located on chromosome 9q34. [20].

CD105 is one of the accessory proteins of the TGF-β complex. Activation of the TGF-β complex results in the binding of endoglin which also subsequently results in the activation of SMAD proteins. The ultimate result of these serial activations is regulation of various cellular processes such as cell proliferation, migration, differentiation, and angiogenesis [19].

CD105 is primarily expressed in vascular endothelial cells and has a role in angiogenesis [20]. CD105 is highly expressed in tumour neovasculature and is weakly expressed or negative in neoplastic cells and resident blood vessels [61]. Endoglin is currently the most reliable marker of abnormal endothelial cell proliferation in tumours [61]. Neovascularisation is an essential requirement for growth and metastatic potential of solid tumours and treatment strategies aimed at inhibiting angiogenesis may play crucial role in management of the tumours [61].

In ccRCC, less than 10% of the tumour cells have been reported to show CD105 upregulation and this has been reported as a potential biomarker for CSC in ccRCC [58]. The expression of CD105 in these neoplastic cells is however usually weak [61]. CD105 is a CSC marker which thus implies its potential use to identify tumour cells with capacity to self-renew and

contribute to chemoradiation resistance and tumour recurrence [62]. Endoglin expression has been found to be associated with aggressive tumour behaviour and poor outcome in ccRCC patients [63]. This association has not been confirmed in other RCC subtypes [63].

2.5.1.3. CD44

CD44 is a single span transmembrane glycoprotein with more than 20 isoforms due to RNA alternative splicing of variable exons [20,64,65]. The glycoprotein is encoded by the *CD44* gene located on human chromosome 11p13 [64]. CD44 is involved in multiple biological processes which include cell-cell interaction, cellular proliferation, migration and angiogenesis [65].

The primary ligand in the extracellular matrix (ECM) components which interacts with CD44 is glycosaminoglycan, hyaluronic acid [66]. The interaction of hyaluronic acid with CD44 leads to activation of multiple pathways which include activation of receptor tyrosine kinases (RTK), TGF-β, MAPK, PI3K/AKT which in turn promote cell proliferation and invasion [20,65,66]. CD44 is involved in the regulation of CSCs through its interaction with the Wnt/β-Catenin signalling and protein kinase C (PKC) [68].

CD44 has been found to be correlated to Fuhrman grade, primary tumour stage, and poor prognosis in patients with RCC [20]. High CD44 expression is associated with poor five-year overall survival [68,69]. CD44 expression as a result may function as a CSC biomarker, prognostic and predictive biological marker [69].

2.5.1.4. CD24

CD24 is a heavily glycosylated small cell surface protein molecule composed of 27 - 30 amino acids, and is encoded by the *CD24* gene located on chromosome 6q21 [20,70]. Expression of CD24 is observed in a wide variety of cell types, including progenitor, stem cells and haematopoietic cells, [70]. A subset of tumour cells expressing stem cell transcription factors and showing self-renewal properties have been observed to express CD24 [70,71].

CD24 has been suggested as an enhancer of metastatic potential of tumour cells and is associated with poor prognosis in haematological and solid tumours [70]. Limited studies have been done on CD24 and no substantial observation has been made on whether it can be used as a biological marker of CSC [20].

2.5.1.5. CXCR-4

The CXC-chemokine receptor-4 (CXCR-4), a G-protein-coupled receptor (GPCR), is an integral part of the cell membrane and contains 7 membrane-spanning helices [20]. The receptor is encoded by *CXCR-4* gene which is located on human chromosome 2q22 [20].

CXCR-4 acts by combining with stromal cell derived factor 1 (SDF-1) which results in the activation of genes responsible for cellular proliferation, metastasis and neovascularisation [19,72].

High expression of CXCR-4 has been shown to be correlated with poor overall survival in patients with advanced and metastatic RCC [73,74].

2.5.1.6. ALDH-1

Aldehyde dehydrogenase-1 (ALDH-1) is a cytosolic enzymatic protein encoded by the *ALDH-1* gene located on chromosome 9q21 and is involved in the formation of carboxylic acids from aldehydes [20].

Amongst its other functions, ALDH-1 plays a critical role in cell differentiation and proliferation [75]. Cytosolic expression ALDH-1 has been identified as a CSCs biological marker in RCC and other solid tumours where it is also considered a poor prognostic indicator [76].

2.6. Prognostic factors

The main prognostic factors for RCC: tumour stage, histopathological subtype, histopathological features and symptoms score [2].

2.6.1. Stage classification

Higher tumour stage is associated with a poorer prognosis [2]. Parameters included in assessment of stage are tumour size, extent of regional spread (venous invasion, renal capsular invasion, adrenal involvement) and lymph node and distant metastasis [77].

2.6.2. Histopathological subtype

The 5-year cancer-specific-survival (CSS) rates for patients with ccRCC are 91%, 74%, 67% and 32% for stages I, II, III and IV respectively [78]. Generally, ccRCC has a poorer prognosis when compared to pRCC and chRCC, even after stratifying for tumour stage and nuclear grade [79]. Patients with early pRCC have significantly less risk of cancer-specific death, when compared to ccRCC [80]. However, advanced or metastatic pRCC and chRCC have poor five-year overall survival (OS) comparable to that of ccRCC [81]. Papillary RCC type 1 has a higher incidence and better prognosis when compared to pRCC type 2 [2]. Chromophobe RCC has a 5-year survival of 78 - 100% [2].

Sarcomatoid RCC has a five-year survival of 15 - 22% whilst a histopathological diagnosis of collecting duct carcinoma is an adverse prognostic factor [2].

2.6.3. Histopathological factors

Histopathological factors which have an impact on the prognosis include tumour nuclear grade, lymphovascular invasion, sarcomatoid differentiation and invasion of the urinary collecting system [82]. Sarcomatoid RCC is classified as a high grade tumour and is very aggressive and associated with poor prognosis [2].

The new WHO/ISUP nuclear grading system has replaced the Fuhrman grading and has been validated as a prognostic indicator for ccRCC and pRCC [2]. In ccRCC and pRCC, prognosis worsens with increasing histopathological grade [2]. The grading system is not used for chRCC which has innate nuclear atypia [2].

2.6.4. Symptom score

The factors which are assessed include performance status (PS), local symptoms, cachexia, anaemia, platelet count, neutrophil/lymphocyte ratio, C-reactive protein (CRP) and albumin [83,84]. High levels of C-reactive protein (CRP), thrombocytosis and hypoalbuminaemia in patients with RCC predict a poor prognosis. Cachexia-like symptoms (loss of weight, anorexia and hypoalbuminemia) independently predict a poor patient outcome in patients with early RCC [84].

2.7. Disease management

Nephrectomy remains the preferred treatment option for localised renal carcinoma. When technically feasible, partial nephrectomy for small renal cancers, is the favoured operative technique [85,86]. Radical nephrectomy (RN) and partial nephrectomy (PN) have comparable oncologic outcomes, but RN is, in addition, associated with an increased risk of chronic kidney disease (CKD) [87].

Currently, an effective postsurgical adjuvant therapy for patients with locally advanced disease has not been established [88]. Most metastatic disease remains highly chemoradiation resistant and only a small fraction have benefited from immunotherapy with cytokines, such as interferon-α and interleukin-2 (IL-2) [88].

Tumour metastasis and relapse are the major contributors to poor survival observed in ccRCC patients. Cancer stem cells are thought to be directly responsible for tumour progression, metastasis and chemoradiation resistance in solid tumours like ccRCC [14,20]. Therefore, a better understanding of biomarkers of cancer stem cells will be useful for risk stratification, prognostication and may lead to novel targeted therapies that will ultimately alter the management of many patients.

2.8. Aim and objectives

To review the morphological subtypes of renal cell carcinomas diagnosed in the Division of Anatomical Pathology, National Health Laboratory Service, Groote Schuur Hospital over a 10 year period.

To identify cancer stem cells in various histopathological subtypes of renal cell carcinoma using immunohistochemical markers (CD133 and CD105).

To review the WNT signalling pathway in renal cell carcinomas using β-Catenin expression by immunohistochemistry.

CHAPTER 3

MATERIALS AND METHODS

3.1. Study design

The study design was that of a laboratory-based descriptive retrospective study which involved purposive sampling and evaluation of all biopsies diagnosed and coded as 'renal cell carcinoma'. A search of the database in the Division of Anatomical Pathology, National Health Laboratory Service (NHLS), Groote Schuur Hospital, Cape Town, South Africa for all nephrectomy cases with a diagnosis of renal cell carcinoma was done. Demographic information was retrieved from the DISA database (age and sex).

3.2. Sample selection

In order to estimate the sample size, the marker β-catenin which has the lowest prevalence in RCC amongst the markers being investigated was used. The estimate that 22.7% (p) of all RCC cases have abnormal β-catenin localisation was used [89]. Calculating the minimum sample size for a large population also involves critical value for confidence level at 95% from standard statistical tables (1.96) and an acceptable margin of error (ε) taken here as 0.07 (7%):

$$n = \frac{1.96^2 \, \mathrm{P}(1 - \mathrm{P})}{\varepsilon^2}$$

However, the maximum available sample size for the ten year study period is expected to be only 830 because the total population of Western Cape province is estimated to be 6 844 272 [90]. The adjusted cases per 100 000 per year is 1.55 for males and 0.87 for females [21]. This equates to about 83 patients/year, or 830 for 10 years. From the sample of 830, the minimum sample size was 159 as the equation was adjusted for limited sample available and assumed a normal distribution [91]. Adding 10% for incomplete and missing datasets, n = 175. However, all available data from the registers was used for analysis in this retrospective cohort study.

All slides of nephrectomy cases diagnosed as RCC from 1st January 2004 to 31st December 2013 were retrieved from the archives. A total of 73 cases were obtained and reviewed. On histopathological review, nine cases had predominantly necrotic tumour with minimal viable tumour. These were excluded. The study cohort consisted of 64 cases of renal cell carcinoma. All data on demographics, histomorphology and immunohistochemical staining were entered into a Microsoft Excel 2016® spread sheet (**Appendix 2**). To maintain confidentiality, data was de-identified at source and cases were allocated study numbers.

3.3. Immunohistochemistry method

Blocks showing representation of viable tumour were selected. One section for haematoxylin and eosin (H&E) stain and 4 sections for immunohistochemistry were cut on each case. Each case was stained with four antibodies using the manual immunohistochemistry staining method (**Table 1**).

Table 1: Primary antibody information

PRIMARY ANTIBODY	CLONE/ SPECIES	SUPPLIER	ANTIGEN RETRIEVAL	DILUTION	INCUBATION TIME	POSITIVE CONTROL
Beta-Catenin	17C2/ Mono	Novacastra	TEDTA	1:50	I Hr	Normal breast
CD105	SN6h/ Mono	Dako	Protease enyme digestion	1:50	Overnight	Kidney
CD133	NB120-16518/ Poly	Novus	TEDTA	1:250	1 Hr	Placenta
HIF-1α	28b/ Mono	Santa Cruz	Citric acid	1:100	Overnight	Urothelial carcinoma

Key: Mono – Monoclonal

Poly – Polyclonal

Supplier information:

Dako – Denmark

Novacastra – United Kingdom

Novus – United Kingdom

Santa Cruz – Europe

3.3.1. Staining procedure

- Three micron paraffin wax embedded tissue sections were cut, picked up onto Histobond slides (Marienfeld-Germany) and heat fixed on a hotplate for 10-15 minutes.
- Sections were dewaxed through xylene, cleared in ethanol and rehydrated in water.

- Endogenous peroxidase activity was blocked by treating the slides with a 3% hydrogen peroxide (H_2O_2) solution for 10 minutes.
- Slides were washed well in water.
- Antigen retrieval was performed by pressure-cooking slides in Tris EDTA (TEDTA) or Citric acid for 1 minute 30 seconds at full pressure, or Enzyme digestion with protease at 37°C for 15 minutes (Refer to **Table 1**).
- This was followed by washing in tap water.
- Thereafter, slides were rinsed with phosphate buffered saline solution (PBS pH 7.6), (Oxoid-Hampshire, England).
- Non-specific binding was blocked by treating slides with a 5% Goat Serum Solution (DAKO - Denmark).
- Serum was then drained off and sections were incubated with primary antibody at room temperature at specified times and dilutions (Refer to **Table 1**).
- The slides were then washed well with PBS Buffer.
- After this step, only the HIF-1α antibody required additional treatment with the DAKO Envision Flex+ Linker for 15 minutes to enhance the staining of the primary antibody.
- This was followed by incubation with the respective (Monoclonal/Polyclonal) DAKO Envision labeled Polymer, HRP (DAKO- USA) (**Table 2**) for 30 minutes at room temperature.
- Sections were washed well with PBS buffer.
- Positivity was developed by applying the chromogenic substrate 3.3-diaminobenzidine (DAB), (DAKO- USA) for 5-10 minutes.

- Slides were washed in running tap water and counterstained with Mayer's haematoxylin for approximately 3 minutes.
- After a good washing in running tap water, sections were blued in ammoniated water.
- Finally, the slides were dehydrated through alcohols, cleared with xylene and mounted with Entellan (MERCK- Germany).

Controls:

A negative reagent control in which the primary antibody was replaced with PBS was used together with a positive control as per manufacturer's recommendation for each immunohistochemistry run (**Table 1**).

Table 2: Kits used in the study

Kits	**Supplier**
EnVision+ System-HRP Labelled Polymer Anti-mouse	Dako - CA, USA
EnVision+ System-HRP Labelled Polymer Anti-Rabbit	Dako - CA, USA
Liquid DAB + Substrate Chromogen system	Dako - CA, USA
Envision Flex+ kit - (Mouse-Linker)	Dako - CA, USA

3.3.2. Scoring of immunohistochemical stains

Intensity of immunohistochemical staining for β-catenin were scored according to method used by Kim *et al.* [89]. Membranous β-catenin staining was classified as normal and abnormal β-catenin localisation was defined as either cytoplasmic or nuclear localisation.

Immunohistochemical staining for CD133 and HIF-1α were scored according to Zeng *et al.* [92] as follows:

- Strongly positive (3+): Cytoplasmic staining in >75% of cells.
- Positive (2+): Cytoplasmic staining in 50 - 75% of cells.
- Weakly positive (1+): Cytoplasmic staining in 5 - 50% of cells.
- Negative (0): < 5% of the tumour cells are stained.

Expressions were considered high expression when scores were 2 or more and low expression when scores were 1 or less.

The tumour cell staining with CD105 was recorded as either negative or positive.

Microvascular density (MVD) was used to assess expression of CD105 by using the number of positive vessels (endothelial cells). MVD was enumerated using some modifications of the criteria previously described in literature [93,94]. Three hotspots (most vascular areas) were identified at low power magnification (X100), and blood vessels counted in each case at X200 magnification. The average number of microvessels in the selected three hotspots

was considered as the mean vascular density. A microvessel was counted for any single cell or spot that stained with CD105.

Tumours were categorised into 3 groups according to the number of microvessels which showed immunopositivity for CD105 marker: low (1+; 3 - 24 vessels), moderate (2+; 25 - 80 vessels) and high (3+; >80 vessels).

3.4. Inclusion / Exclusion Criteria

The inclusion criteria were those cases that had histopathological features compatible with all subtypes of renal cell carcinoma. Cases with predominantly necrotic tissue and no residual tissue for further immunohistochemical staining were excluded from the study.

3.5. Study location

This study was conducted in the laboratory of the Division of Anatomical Pathology, Faculty of Health Sciences, University of Cape Town and National Health Laboratory Service (NHLS), Groote Schuur Hospital, Cape Town, South Africa.

3.6. Ethics approval

The Human Research Ethics Committee (HREC) of the Faculty of Health Sciences, University of Cape Town approved the study (HREC reference number: 832/2015). Furthermore, permission was also granted by the National Health Laboratory Service (NHLS) to retrieve and use archived material and data.

Funding for this study was obtained from the National Health Laboratory Service (NHLS) Research Trust.

3.7. Statistical analysis

All data on demographics, histomorphology and immunohistochemical staining were entered into a Microsoft Excel 2016® spread sheet. Statistical analysis was done using Stata 16.0 statistical software (Stata Corp LP, College Station, TX, USA). The data was descriptive and consisted of frequencies that were expressed in percentages and graphs. Descriptive statistics included calculations of means and standard deviations.

Analysis of variance (ANOVA) was performed on variables to determine if there was a significant correlation between levels of expression (CD133, CD105 and HIF-1α) and the histopathological RCC subtype.

Fisher's exact test was adopted to determine if there was statistically significant correlation between abnormal and normal β-Catenin localisation and RCC subtype.

All the results were considered statistically significant if the p-value was less than 0.05.

CHAPTER 4

RESULTS

4.1. Study population

A total of seventy-three cases with a diagnosis of renal cell carcinoma from 1st January 2004 and 31st December 2013 were retrieved from the archives of the Division of Anatomical Pathology, Groote Schuur Hospital. Nine cases which demonstrated predominantly necrotic tissue with insufficient viable tissue for further immunohistochemical studies were excluded, from the study cohort. Sixty-four cases made up the final study cohort.

4.2. Demographics

4.2.1. Age

Patient age at diagnosis of renal cell carcinoma ranged from 35 to 81 years with a mean age of 56 years (SD = 10.9 years); the age of one patient was unknown (**Figure 4**).

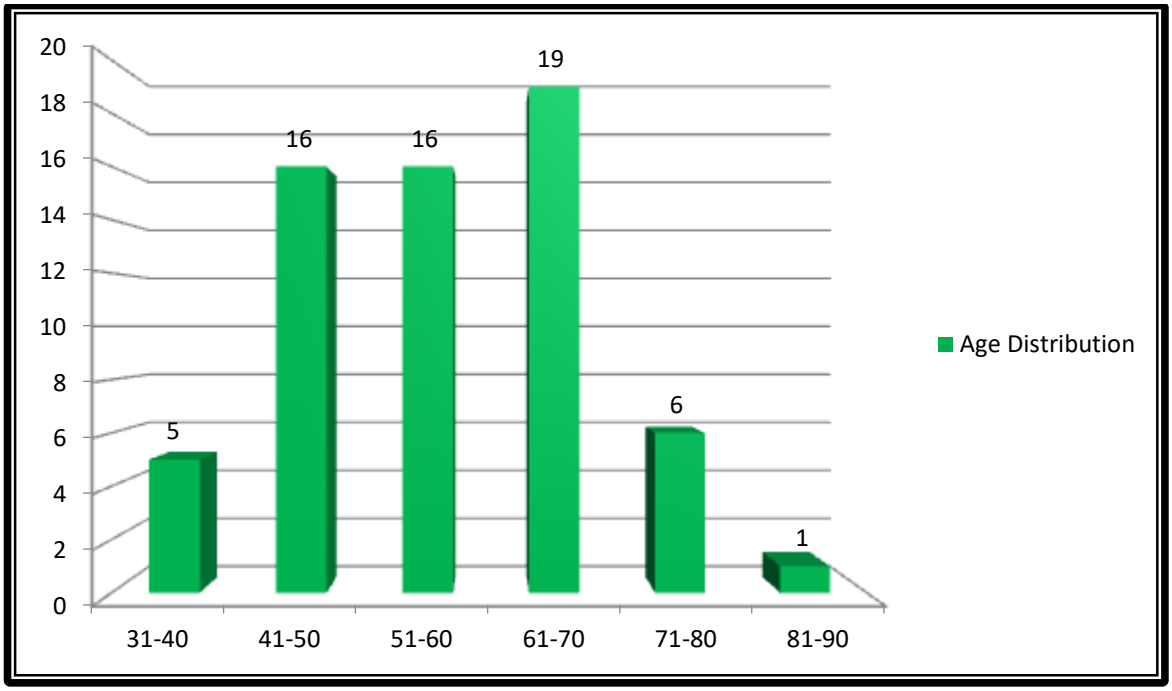

Figure 4: Age distribution in patients with RCC

4.2.2. Sex

Females constituted just over half (52%, n = 33) of the patients in the study cohort.

4.3. Histopathological Diagnosis

Haematoxylin and eosin (H&E) staining confirmed the presence of RCC in all the 64 cases used for DAB IHC staining (**Figure 5 - 8**). The most common tumour subtype was ccRCC in 45% (n = 29) of the patients, followed by pRCC (22%, n = 14), chRCC (14%, n=9), multicystic RCC (14%, n=9) and sRCC (5%, n=3) (**Figure 9**). The pRCC cases consisted of type 1 (13%, n = 8) and type 2 (9%, n = 6).

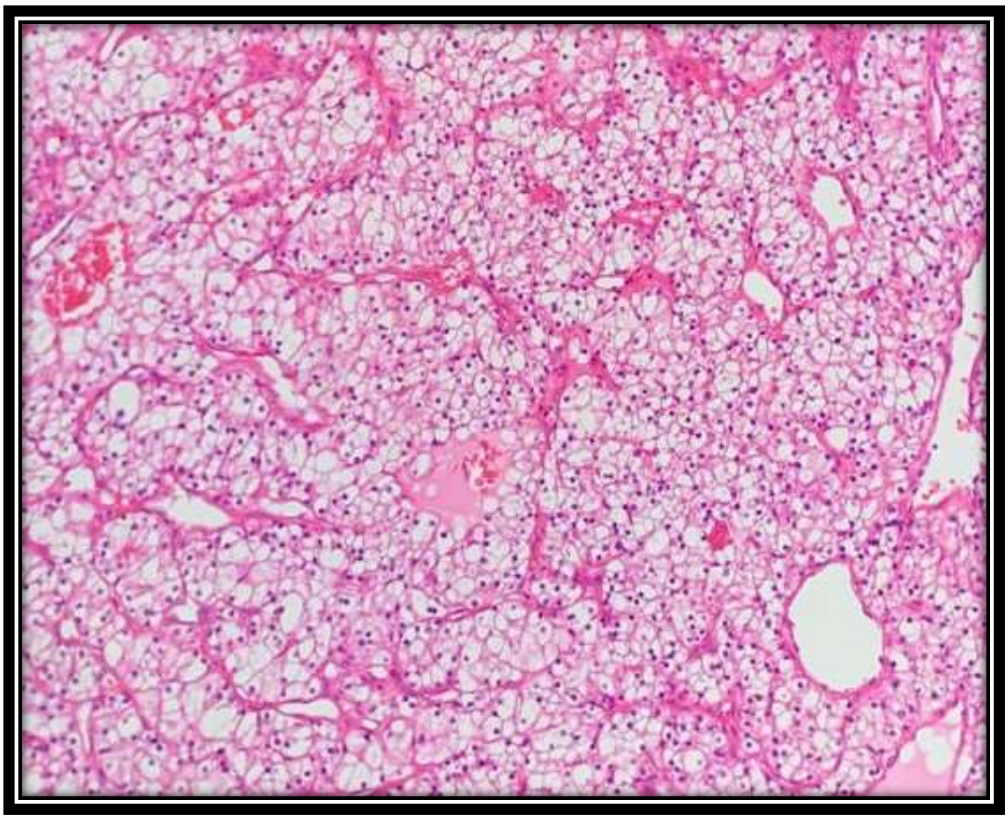

Figure 5: Clear cell RCC (H&E, magnification X400)

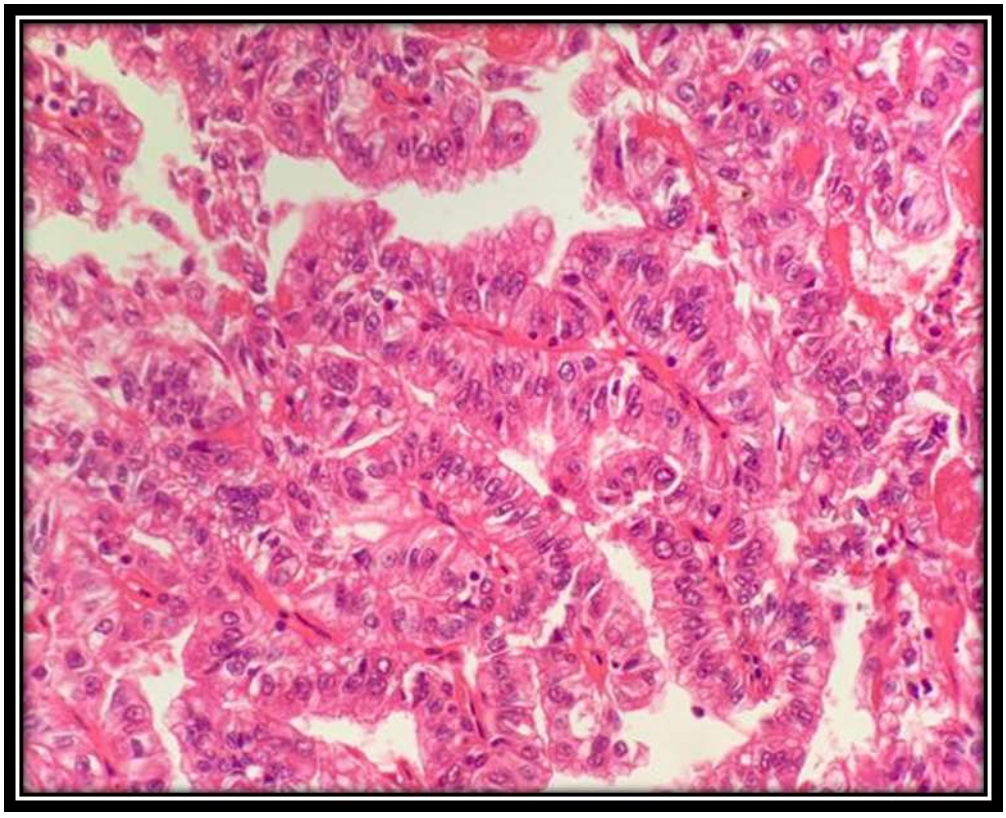

Figure 6: Papillary RCC (H&E, magnification X400)

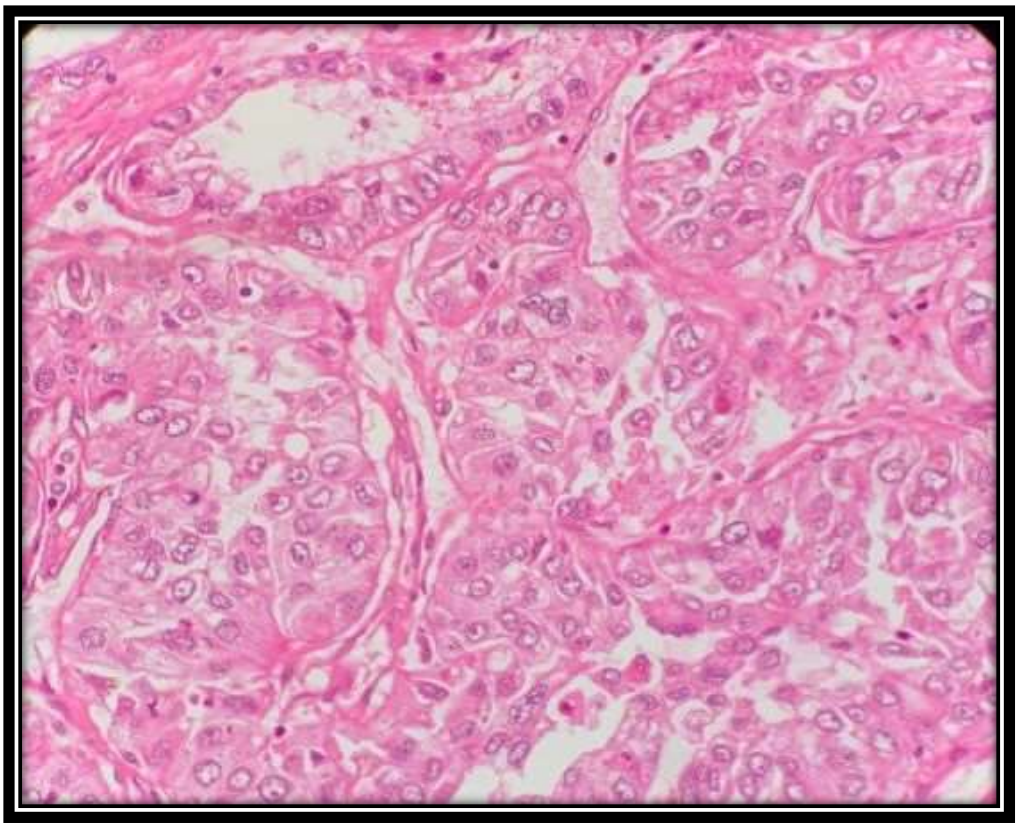

Figure 7: Chromophobe RCC (H&E, magnification X400)

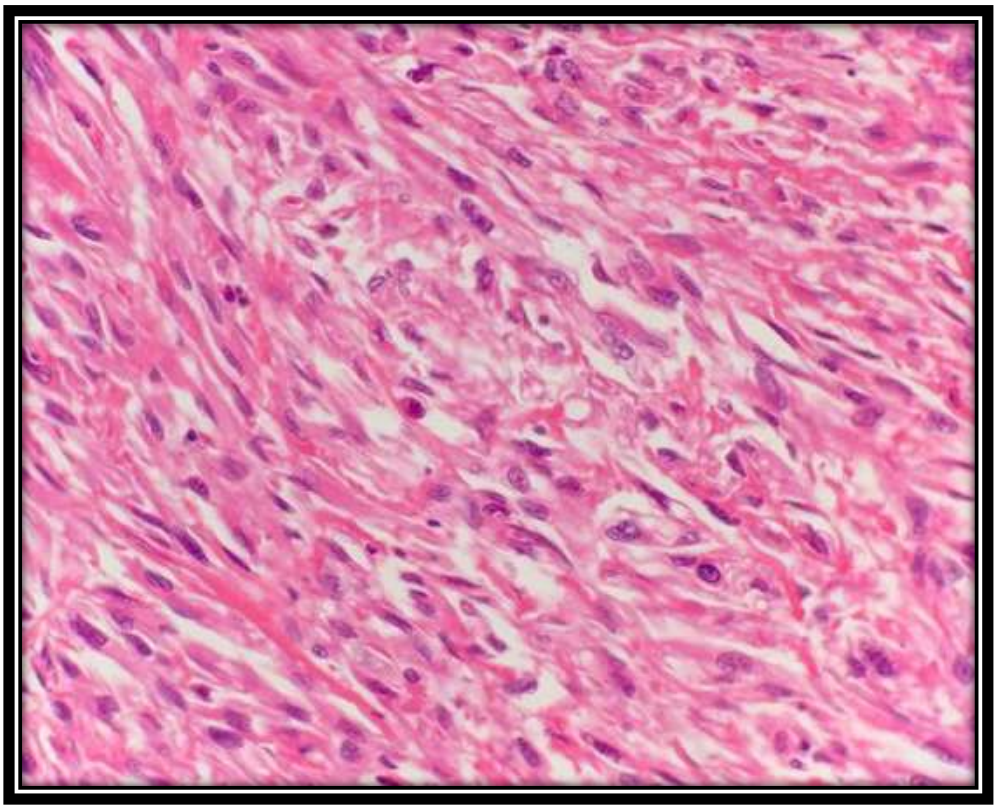

Figure 8: Sarcomatoid RCC (H&E, magnification X400)

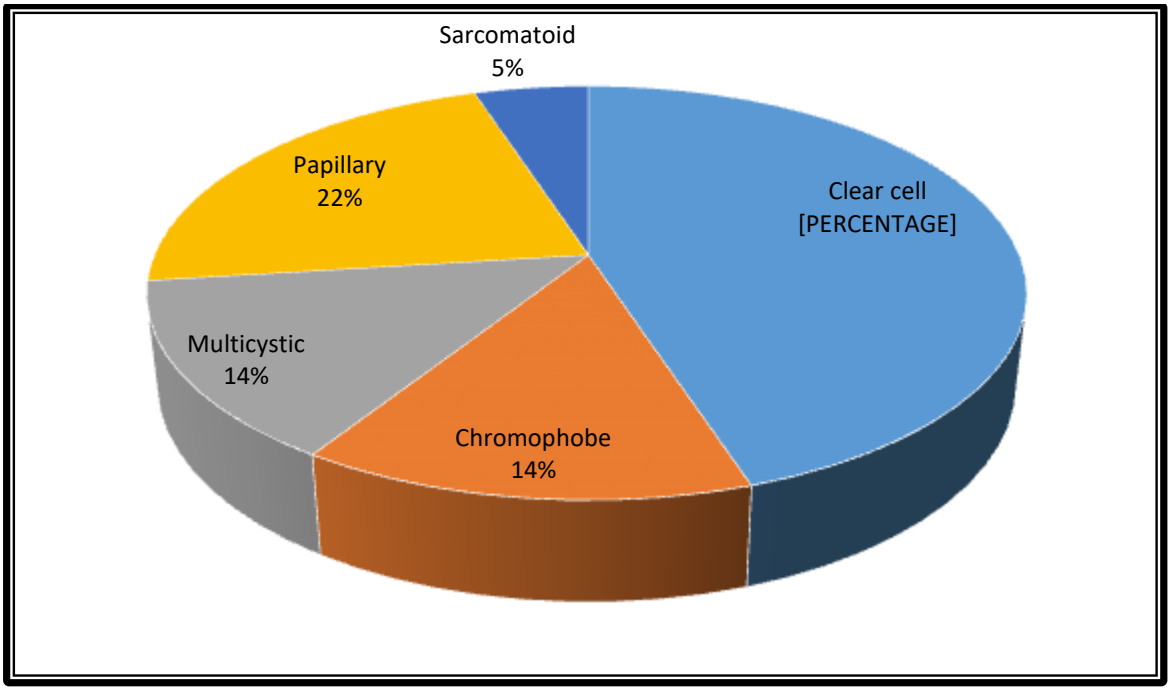

Figure 9. Tumour subtypes

4.4. Immunohistochemical stain analysis

4.4.1. β-catenin

Abnormal β-Catenin cytoplasmic localisation was observed in 16% (n=10) of the cases (**Figure 10**). None of the cases in the study cohort demonstrated nuclear localisation of β-Catenin. Abnormal β-Catenin localisation was predominantly observed in ccRCC (60%, n=6) (**Figure 11**). However, there was no statistically significant correlation between abnormal and normal β-Catenin localisation and RCC subtype (Fisher's Exact test: p = 0.766).

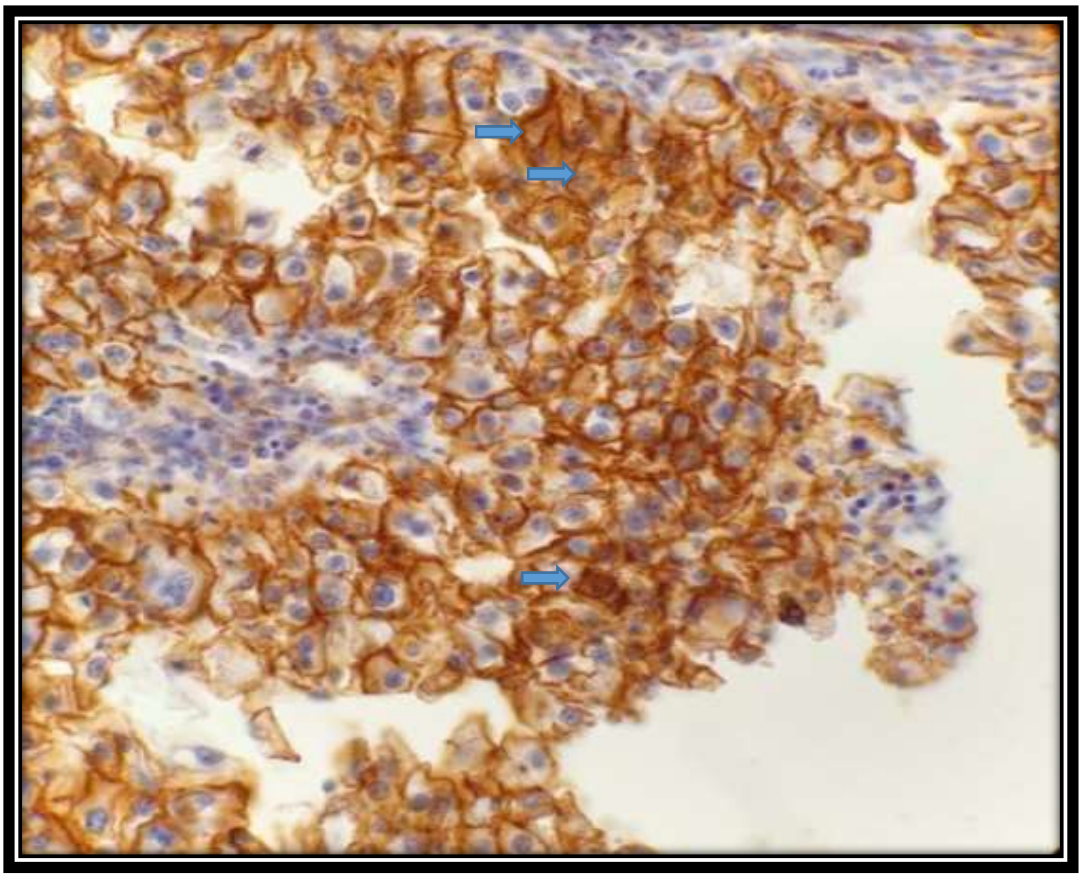

Figure 10: Predominant membrane staining with focal cytoplasmic (arrow) β-Catenin localisation in pRCC (β-Catenin, magnification X400)

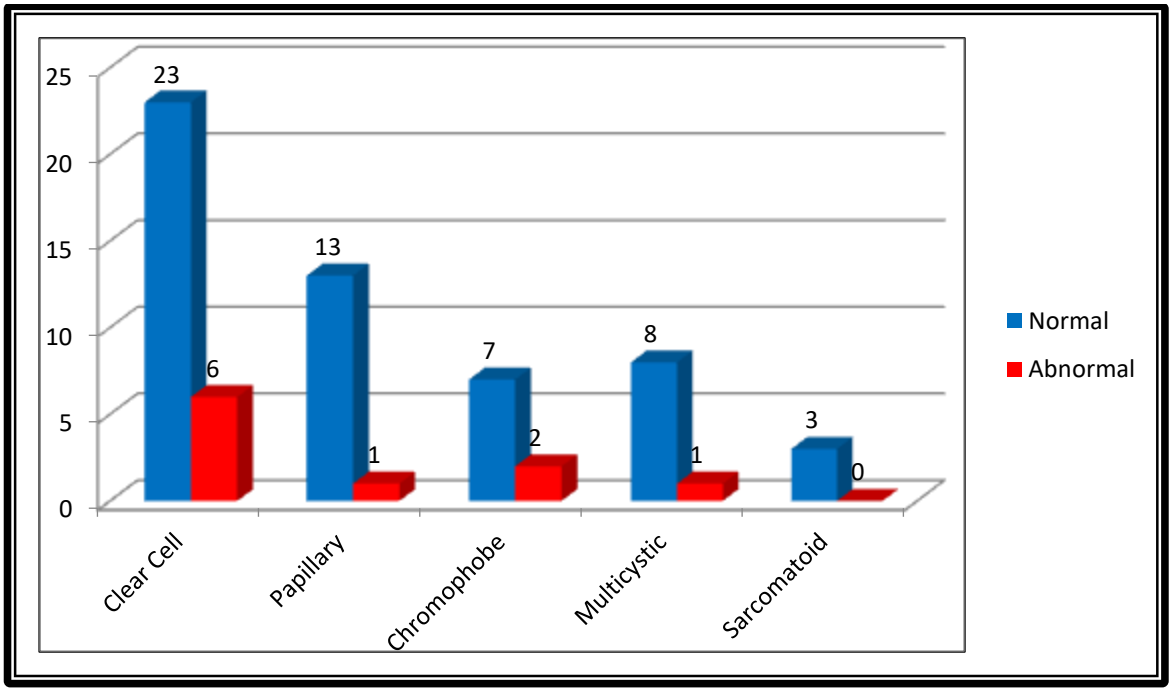

Figure 11: β-Catenin staining according to histopathological subtype

4.4.2. CD133

Immunopositivity to CD133 showed cytoplasmic staining in the positive tumour cells (**Figure 12**). CD133 immunohistochemical studies showed low expression in 52 (81 %) cases and high expression in 12 (19 %) cases (**Figure 13**). There was no correlation between level of CD133 expression and RCC subtype (One way ANOVA: p = 0.800).

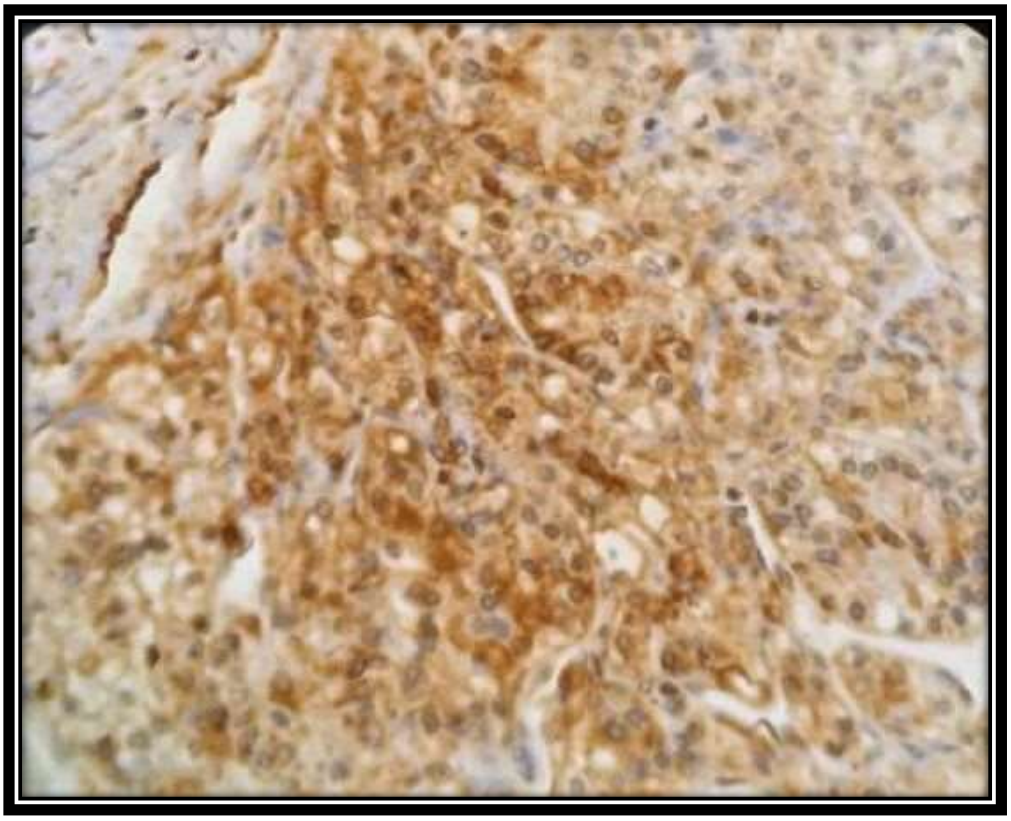

Figure 12: Cytoplasmic CD133 positive staining in pRCC (CD133, magnification X400)

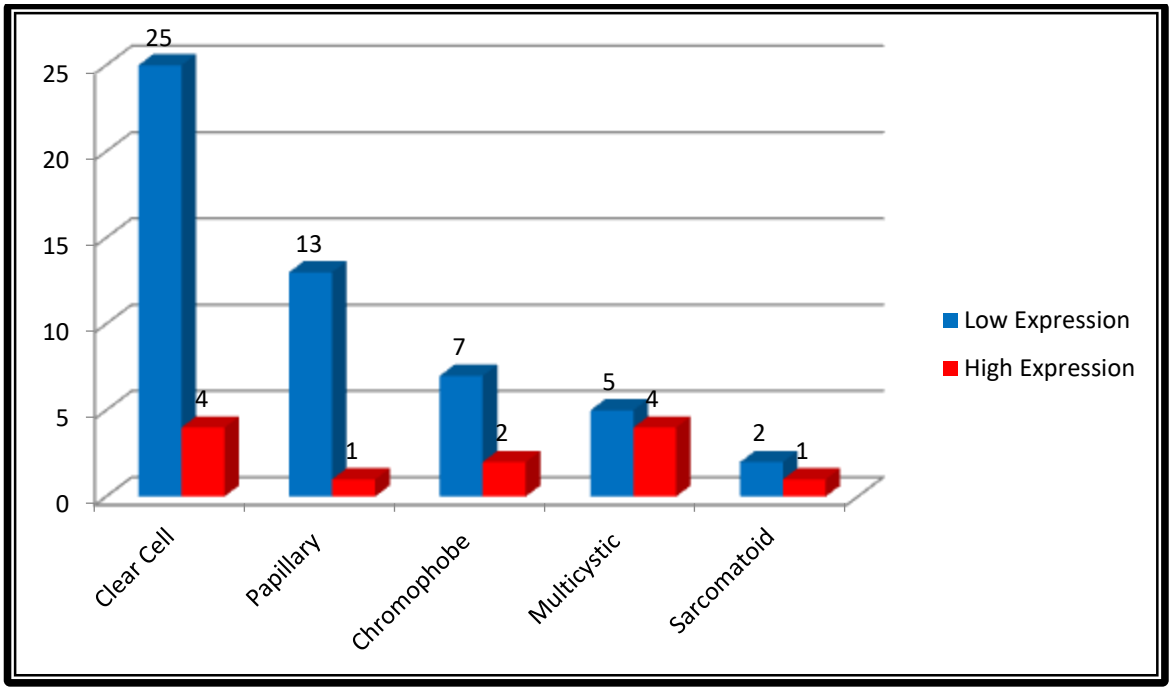

Figure 13: Low and high CD133 staining according to histopathological subtype

4.4.3. CD105

CD105 immunopositivity in tumour cells was demonstrated in one case of ccRCC whilst the rest of the cases were negative. Microvascular density (MVD) using CD105 was used to assess neovascularisation in RCC subtypes (**Figure 14**).

The low, moderate and high microvascular density categories had 24, 10 and 32 cases each, respectively (**Figure 15**).

There was no significant correlation between level of CD105 expression (microvascular density) and the histopathological RCC subtype (One way ANOVA: p = 0.320).

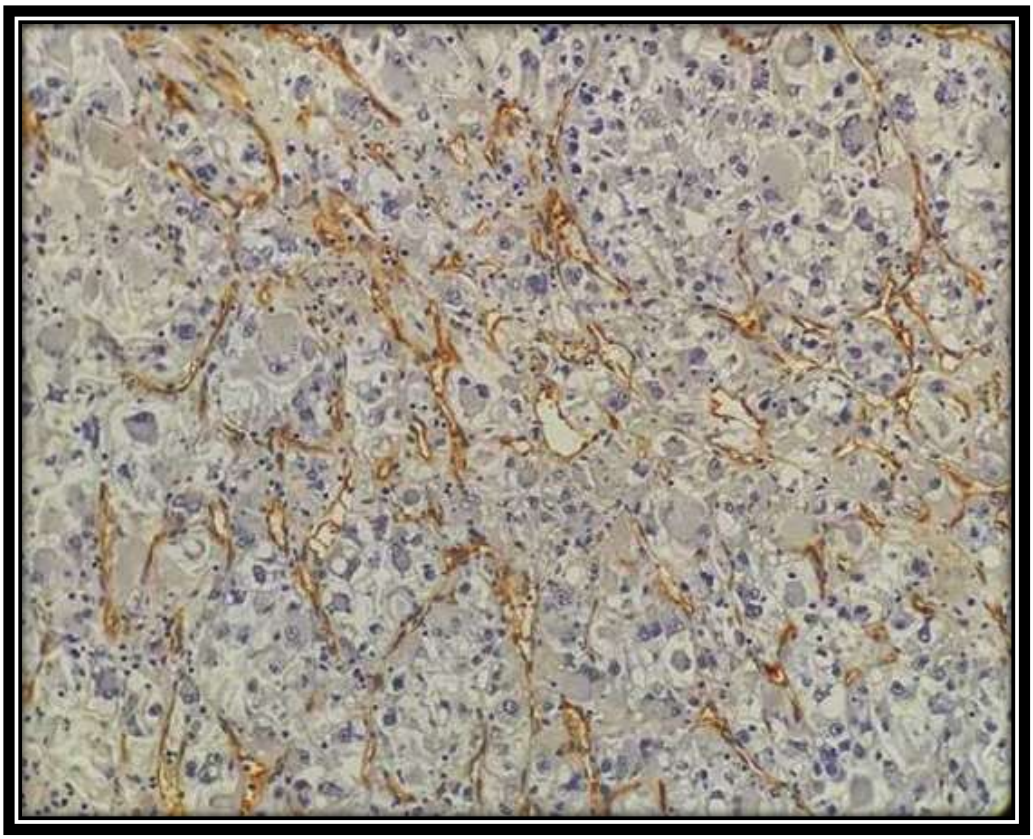

Figure 14: CD105 staining outlining tumour capillaries but negative in ccRCC tumour cells (CD105, magnification X200)

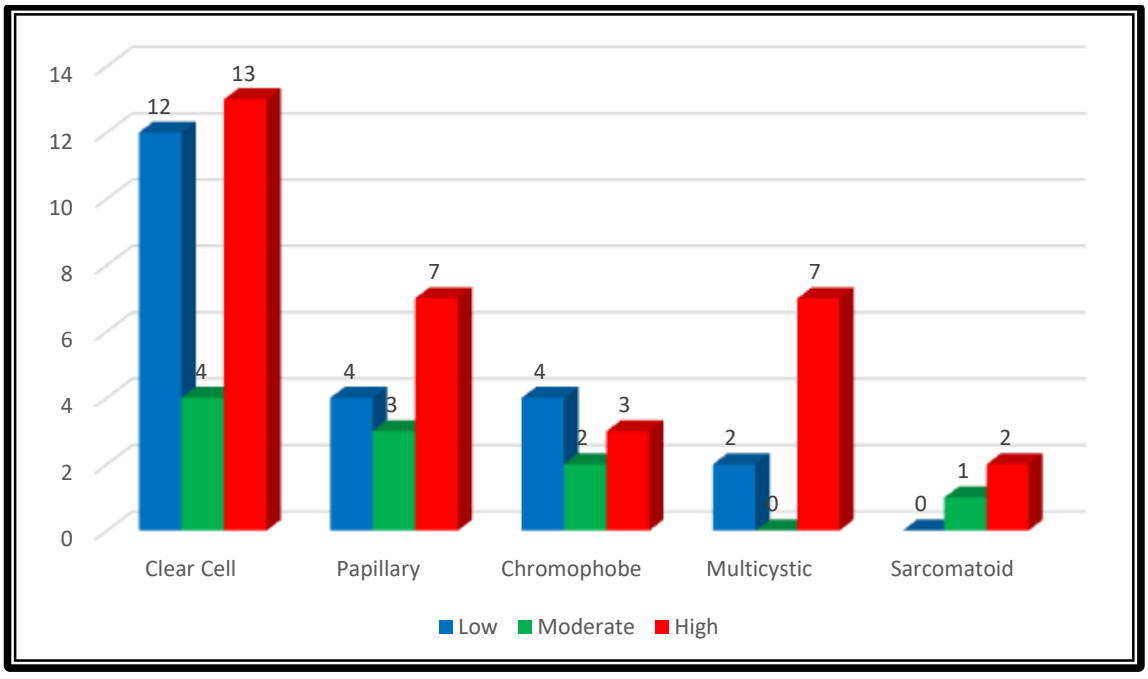

Figure 15: Endothelial CD105 expression (microvascular density) in different RCC subtypes

4.4.4. HIF-1α

HIF-1α showed cytoplasmic staining in positive tumour cells (**Figure 16**). Nuclear staining was not identified. Among the 64 RCCs in the study cohort, 25 (39 %) showed high expression of HIF-1α and 39 (61 %) displayed low expression (**Figure 17**). There was no significant correlation between levels of HIF-1α expression and the histopathological RCC subtype (One way ANOVA: p = 0.972).

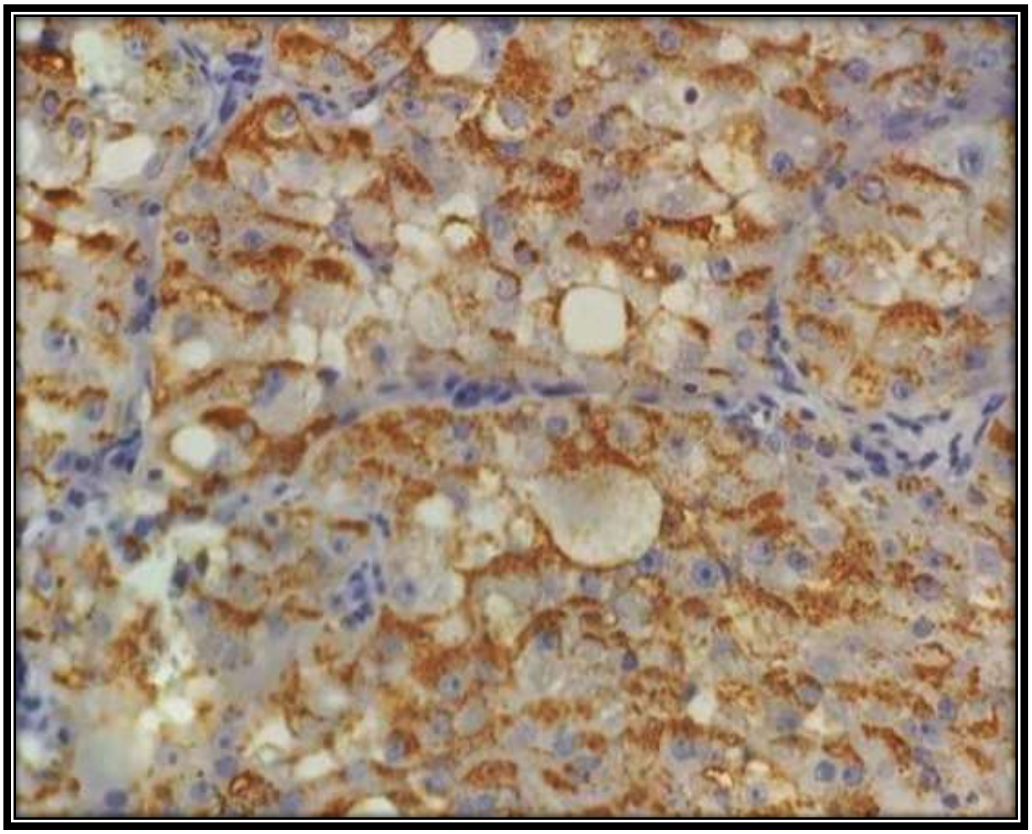

Figure 16: HIF-1α positive cytoplasmic staining in ccRCC (HIF-1α, magnification X400)

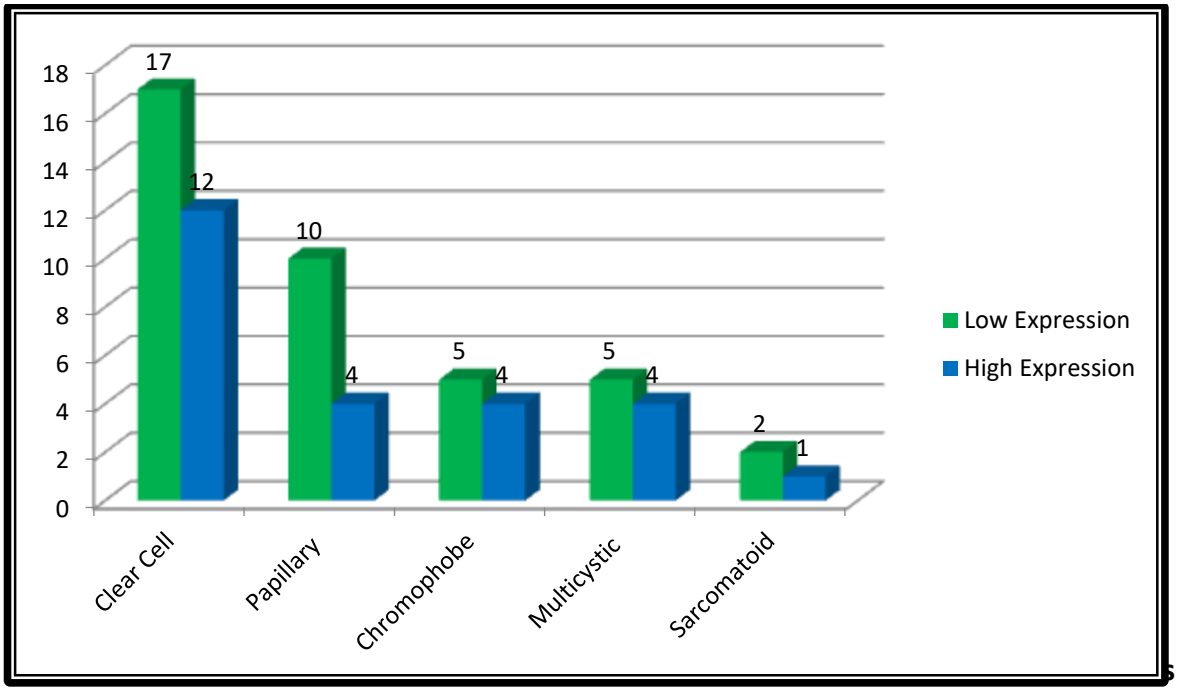

Figure 17: Low and high HIF-1α staining according to histopathological subtype

CHAPTER 5

DISCUSSION

5.1. Demographics

The mean age of the patients in the study cohort was 56-years (range 35 -81-years). This is similar to the findings described in literature [2].

There were slightly more females (52%) than males (48%) in the study cohort. This finding differs markedly from the 2:1 male- to-female ratio described in various reports [1,2]. The possible reasons for this variation include the small sample size and the exclusion of some cases from the study.

5.2. Histopathological subtypes

A predominance of ccRCC (45%, n=29) followed by pRCC (22%, n= 14), chRCC (14%, n = 9) and sRCC (5%, n = 3) was observed in this study cohort. These figures are in keeping with findings described within broader literature [1,2]. Multicystic renal cell carcinoma constitutes less than 1% of all renal cell carcinomas [2]. Nine (14%) cases in the current study cohort were of multicystic RCC subtype. The possible reasons for the observed discrepancy could be due to small sample size, exclusion of some cases and possible histologic undercall of clear cell renal cell carcinoma with cystic/regressive changes as multicystic renal cell carcinoma.

5.3. β-Catenin

Abnormal WNT/β-catenin signalling plays a pivotal role in tumour progression and maintenance of CSCs in several types of solid tumours including RCC [15,44-46]. In the canonical Wnt signalling pathway, activation of the Frizzled (Fzd) receptors results in cytoplasmic accumulation of β-catenin. Subsequent nuclear translocation of β-catenin, through the activation of lymphoid enhancer-binding factor (LEF) and T-cell factor (TCF) family signalling pathway, results in higher cyclin D1 expression in RCC [38,39].

In the current study we found that only 16% of RCC showed abnormal cytoplasmic accumulations of β-catenin pointing to stabilisation of the protein. None of the cases in the study cohort demonstrated nuclear localisation of β-Catenin. There was no statistically significant association between normal and abnormal β-catenin accumulation and RCC histopathological subtype. This finding is comparable to findings by Kim *et al* [89] who identified abnormal cytoplasmic β-catenin localisation in 22.7% of their cases with no cases showing nuclear staining. However, in contrast to our study, abnormal cytoplasmic β-catenin localisation in the study by Kim *et al* [89] was only observed in ccRCC subtype and was not evident in pRCC and chRCC. Bruder *et al* [95] also observed strong cytoplasmic expression with no nuclear expression of β-catenin expression in ccRCC. Some researchers have reported higher cytoplasmic β-catenin expression (91.7% of cases) in ccRCC [96].

In the current study, the most common tumour subtype with abnormal β-catenin localisation was ccRCC in 60% (n = 6) of the cases, followed by cRCC (20%, n = 2), pRCC (10%, n=1) and multicystic RCC (10%, n=1). However, in contrast to the current study, abnormal

cytoplasmic β-catenin localisation has only observed in ccRCC subtype and not evident in pRCC and chRCC [89,95,96]. The possible reason for this variation includes the use different antibody clones.

Ronkaineinen *et al* [97] have reported on nuclear β-catenin immunopositivity in RCCs. In a study cohort of 152, 44% (n= 65) of the cases expressed nuclear β-catenin immunopositivity. The discrepancy in the study by Ronkaineinen *et al* [97] with the current study and other previous studies may likely be due to the use of different antibody clones.

Cytoplasmic β-catenin has been identified as a possible indicator of poor prognosis in patients with RCC [40]. The cases with abnormal cytoplasmic β-catenin localisation, which is an indicator of its abnormal stabilisation, are characterised by larger tumour size, vascular invasion and late stage disease at presentation [40].

5.4. CD133

Fifty-two cases (81%) of the study cohort showed low expression of CD133 and 12 (19%) showed high expression. In contrast to this study, Da Costa *et al.* [98] in a study involving 142 RCC patients showed low CD133 expression in 54% of the cases and 46% with high CD133 expression. In another study by Kim *et al*. [99] which had 119 RCC cases, high expression of CD133 was seen in 17.8% of cases, a figure which is comparable to that from our study. While there were no significant associations between levels of CD133 expression according to the subtype, tumour stage, tumour size and TNM stage, CD133 expression significantly correlated with Fuhrman nuclear grade [99]. Low grade tumours showed high

levels of CD133 expression whilst high grade tumours displayed low levels of CD133 expression [97]. The levels of CD133 expression observed in this study did not show any statistically significant association with the histopathological RCC subtypes.

During hypoxia, HIF-1α is involved in the regulation of CD133 expression [100]. CD133 being a marker of CSCs, its expression may be associated with metastasis, chemoradiation resistance and tumour recurrence [14].

5.5. CD105

Microvascular density has been reported to be a reliable semi-quantitative method to evaluate angiogenesis and various immunohistochemical markers have been proposed [93,94,101]. CD105 has been observed to more accurately reflect the angiogenic status of tumours when compared to other endothelial markers [100]. CD105 is robustly expressed by tumour microvasculature and is negative or weakly expressed in blood vessel of resident tissue [102,103].

CD105 has been identified as a prognostic marker and a potential target for anti-angiogenesis agents [63]. The prognostic value of CD105 has however only been validated in ccRCC and may not be of any value in the other histopathological subtypes of RCC [63]. In the current study, levels of angiogenesis (microvascular density) were not significantly associated with the histopathological RCC subtype. The results from this study seem to suggest that as an immunohistochemical marker for angiogenesis, CD105 may be used in all subtypes of RCC, which is at variance with the results from previous studies [62,63,104]. The

exact reason for this difference is unclear, though possible reasons could include the non-standardisation of the microvascular density enumeration methods and the small sample size.

Only one case of ccRCC showed tumour cell immunopositivity. CD105 tumoral expression is associated with poor overall survival rates [104].

5.6. HIF-1α

In the current study, levels of HIF-1α expression were not significantly associated with the histopathological subtype of RCC. In a previous study in which analysis was done using Western blot, higher HIF-1α levels were observed in ccRCC compared to other RCC subtypes [105]. The possible reasons for the observed differences include employing different methods to demonstrate and enumerate HIF-1α expression and the small sample size.

In ccRCC, constitutive activity of HIF-1α and HIF-2α independent of the oxygen levels is observed due to loss of VHL-associated proteolysis of HIF-1α and HIF-2α [27]. Absence of active pVHL is responsible for the loss of VHL-associated proteolysis of HIF-1α and HIF-2α [27].

Hypoxia and *VHL* gene mutation are responsible for the high levels of HIF-1α observed in ccRCC [27,28]. Hypoxia is thought to be the main regulator of HIF-1α in RCC subtypes which do not harbour *VHL* gene mutation (pRCC and chRCC) [2]. As a result, the expectation would

be for ccRCC to demonstrate a higher expression of HIF-1α when compared to other histopathological subtypes as it carries the genetic alterations involving chromosome 3.

High expression of HIF-1α is associated with better prognosis and survival in ccRCC but not in pRCC and chRCC [106].

5.7. Limitations of study

This was a single-centre, laboratory based retrospective study with a relatively small number of cases.

A total of seventy-three cases which were diagnosed as renal cell carcinoma during the study period were retrieved. However, only 64 cases were included in the study cohort, as nine of the cases were excluded due to insufficient viable tumour tissue on the paraffin wax blocks. This may have inadvertently altered the epidemiological data collected in this study.

There was limited clinical information and follow-up details. This greatly restricted performance of a detailed clinicopathological correlation analysis.

Due to financial constraints other immunohistochemical markers such as CD44 and SMAD4 could not be assessed.

CHAPTER 6

CONCLUSION AND RECOMENDATIONS

The most common renal cell carcinoma histopathological subtype over the ten year study period was clear cell renal cell carcinoma (45%). Sarcomatoid differentiation was reported in three (5%) cases of RCC during the study period.

Within the power limitations of this small study, abnormal β-catenin localisation was observed in 16% of the cases and was not correlated with any histopathological subtype of renal cell carcinoma.

CD133 was expressed in all RCCs (100%). The level of CD133 expression has no correlation with histopathological RCC subtype. Therefore, we consider the CD133 to be a useful CSC marker in all histopathological RCC subtypes. Further studies combining CD133 and other novel CSCs markers are required to identify CSCs and investigate their potential use in predicting patients' prognosis and survival analysis.

Angiogenesis as assessed using microvascular density was not significantly different between various histopathological subtypes of RCC. This is in contrast to other reports. Further studies to standardise the enumeration of microvascular density using CD105 to enable better comparisons between studies is recommended.

Our results revealed no association between levels of HIF-1α and the histopathological subtypes. Studies integrating clinicopathological factors to assess its potential use in predicting patients' survival outcomes are recommended.

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APPENDIX 1: Ethics Clearance

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

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10 December 2015

HREC REF: 832/2015

Prof D Govender
Public Health & Family Medicine
Falmouth Building

Dear Prof Govender

PROJECT TITLE: THE ROLE OF STEM CELLS AND THE WNT SIGNALLING PATHWAY IN RENAL CELL CARCINOMA - (MMed candidate- Dr S Madlala)

Thank you for your response to the Faculty of Health Sciences Human Research Ethics Committee dated 8 December 2015.

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

Approval is granted for one year until the 30th December 2016.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

Please quote the HREC REF in all your correspondence.

We acknowledge that the student, Dr Siphelele Madlala will also be involved in this study.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Yours sincerely

Signature removed to avoid exposure online

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN RESEARCH ETHICS COMMITTEE
Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938
This serves to confirm that the University of Cape Town Human Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Convention on Harmonisation Good Clinical Practice (ICH GCP), South African Good Clinical Practice Guidelines (DoH 2006), based on the Association of the British Pharmaceutical Industry Guidelines (ABPI),and Declaration of Helsinki (2013) guidelines.

HREC 832/2015

APPENDIX 2: Data collected

Case N	Age	Gende	Diagnosis	β-Cateni	CD133	CD105 Tumou	CD105 Endo	HIF-1α
1	66	F	Clear Cell RCC	Normal	1	1	3	3
2	48	M	Clear Cell RCC	Abnormal	2	0	3	3
3	43	F	Papillary RCC type 2	Normal	1	0	3	0
4	57	M	Papillary RCC type1	Normal	0	0	2	0
5	68	F	Clear Cell RCC	Normal	0	0	3	0
6	62	M	Chrophobe RCC	Normal	0	0	2	3
7	63	M	Clear Cell RCC	Abnormal	0	0	1	2
8	67	F	Multicystic RCC	Normal	2	0	1	3
9	45	F	Papilllary RCC type 1	Normal	0	0	3	0
10	61	F	Papillary RCC type 2	Normal	0	0	1	2
11	63	F	Clear Cell RCC	Normal	1	0	3	0
12	66	M	Multicystic RCC	Abnormal	3	0	3	3
13	73	F	Clear Cell RCC	Abnormal	1	0	1	3
14	71	F	Multicystic RCC	Normal	3	0	3	0
15	58	M	Chrophobe RCC	Normal	1	0	3	0
16	50	M	Chrophobe RCC	Abnormal	0	0	1	2
17	75	F	Sarcomatoid [ccrcc]	Normal	3	0	3	0
18	45	M	Papillary RCC type1	Abnormal	0	0	3	0
19	64	F	Papillary RCC type1	Normal	1	0	3	2
20	56	F	Clear Cell RCC	Normal	2	0	3	0
21	71	M	Clear Cell RCC	Abnormal	0	0	1	0
22	50	M	Chrophobe RCC	Normal	2	0	1	0
23	51	F	Papillary RCC type 2	Normal	1	0	1	1
24	71	M	Chrophobe RCC	Abnormal	0	0	3	0
25	51	F	Papillary type1	Normal	1	0	1	0
26	49	F	Clear Cell RCC	Abnormal	1	0	1	0
27	35	F	Clear Cell RCC	Normal	0	0	3	0
28	68	M	Clear Cell RCC	Normal	2	0	2	3
29	54	M	Chrophobe RCC	Normal	2	0	2	3
30	81	F	Multicystic RCC	Normal	1	0	3	0
31	57	M	Chrophobe RCC	Normal	1	0	3	0
32	48	F	Clear Cell RCC	Normal	0	0	1	0
33	53	F	Papillary RCC type 2	Normal	1	0	3	3
34	54	F	Papillary RCC type 1	Normal	1	0	2	3
35	36	F	Papillary type1	Normal	0	0	2	0
36	47	M	Clear Cell RCC	Normal	0	0	1	0
37	52	M	Chrophobe RCC	Normal	1	0	1	0
38	37	F	Clear Cell RCC	Normal	1	0	3	3
39	54	F	Clear Cell RCC	Normal	1	0	2	3
40	42	F	Clear Cell RCC	Normal	0	0	1	3
41	42	M	Clear Cell RCC	Normal	0	0	1	0
42	?	F	Clear Cell RCC	Normal	1	0	2	1
43	60	M	Multicystic RCC	Normal	2	0	3	0
44	57	M	Papillary RCC type1	Normal	2	0	3	3
45	41	F	Clear Cell RCC	Normal	1	0	1	0
46	44	M	Papillary type 2	Normal	0	0	3	2
47	61	M	Multicystic RCC	Normal	0	0	3	3
48	63	M	Clear Cell RCC	Normal	0	0	2	0
49	56	F	Clear Cell RCC	Normal	2	0	3	0
50	63	M	Multicystic RCC	Normal	1	0	1	1
51	50	F	Clear Cell RCC	Normal	0	0	3	0
52	56	M	Clear Cell RCC	Normal	0	0	3	0
53	63	M	Sarcomatoid [ccrcc]	Normal	0	0	2	1
54	47	M	Clear Cell RCC	Normal	0	0	1	3
55	74	F	Multicystic RCC	Normal	1	0	3	0
56	41	M	Clear Cell RCC	Normal	1	0	3	0
57	65	M	Clear Cell RCC	Normal	1	0	3	0
58	53	F	Chrophobe RCC	Normal	1	0	1	3
59	62	M	Papillary RCCtype 2	Normal	1	0	1	0
60	40	M	Clear Cell RCC	Normal	1	0	3	3
61	57	M	Clear Cell RCC	Normal	1	0	2	2
62	69	F	Multicystic ccrcc	Normal	1	0	3	3
63	40	M	Clear Cell RCC	Abnormal	1	0	1	2
64	66	M	Sarcomatoid [chromo	Normal	0	0	3	2