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Hypoxia Regulated Pathways in Urological Malignancies

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ABSTRACT
Hypoxia Regulated Pathways in Urological Malignancies
Philip John Stuart Charlesworth

Introduction: Kidney Cancer accounts for approximately 2% of all new cancer diagnoses in the UK each year. Patient survival has improved over the past few decades; however the mechanisms of this are yet to be fully elucidated. Hypoxia inducible factor isoforms, HIF-1 and HIF-2, are constitutively expressed in many clear-cell RCCs due to loss of pVHL tumour suppressor function within the tumour. In vitro, HIF-1 and HIF-2 regulate a differential set of target genes, although their expression in primary ccRCC clinical samples and their effects on patient prognosis has yet to be fully understood.

Methods: In this thesis analysis has been performed on all Nephrectomies performed at Oxford Radcliffe Hospitals for Renal Cell Carcinoma (RCC) from 1983 to 2007. Data extracted from Charlesworth Research Uro-Oncology Database, CRUD©, provided long-term survival data, maximal tumour diameter, Fuhrman grade, T-Staging and patient age. A subset of RCCs from this series (170 consecutive clear cell renal tumours from 1983 to 1999) were analysed within a tissue microarray and expression of HIF-1 and HIF-2, together with seven primary target genes (BNIP3, CAIX, CyclinD1, GLUT1, LDH5, Oct-4 and VEGF) was assessed. Comparison was made with tumour angiogenesis (CD31), tumour stage, Fuhrman grade, maximum tumour diameter and patient survival. Further work in this thesis analysed a series of paired VHL (functional and non-functional) ccRCC cell lines, assessing for hypoxic differential MicroRNA expression.

Results: Analysis of 664 RCCs demonstrated a clear change in kidney cancer specific survival over the past 24 years, with 5-year survival improving from 42% (1983-1986) to 73% (1999-2002). The incidence of RCC has increased 10 fold and has a significant association with 4-year survival. There was no significant change in operative mortality, patient age, Fuhrman grade, Pathological T-Stage or mean tumour size. However, there was a 5-fold increase in tumours <6cm, corresponding to an equal fold decrease in tumours 6-8cm, and no change in tumours >8cm. Tumour size >8cm was a significant prognostic marker. HIF-1 and HIF-2 showed no correlation and individually, neither HIF-1 nor HIF-2 expression had any prognostic utility; however a significant time-dependent deterioration of HIF-1 and HIF-2 antigenicity within paraffin blocks was identified. Angiogenesis (VVI CD31) had a strong negative correlation with Fuhrman grade and maximal tumour diameter and had prognostic significance, with high levels associated with good overall survival. Results from microRNA expression arrays found a specific microRNA (MIR-23a) that was differentially expressed depending upon VHL functionality and hypoxic conditions. Furthermore microRNA-23a was up-regulated in cells that expressed both HIF isoforms, and down-regulated in cells that only expressed HIF-2.

Conclusions: Outcome following Nephrectomy for Renal Cell Carcinoma has dramatically improved over the past 24 years. Increasing incidence and decreasing tumour size at operation combined with the lack of statistical variation in Fuhrman grade, suggests that earlier detection of tumours offers subsequent curative treatment by Nephrectomy. Furthermore, stable incidence rates of tumours >8cm potentially represent alternative tumour biology, which grow rapidly, avoiding early detection and curative treatment. Although neither HIF isoform nor the seven HIF target genes was found to influence disease prognosis, the discovery of HIF antigenicity deterioration with time, is a very important finding and casts into doubt previous literature about HIF-1 immunostaining in human cancers. The prognostic significance of CD31+ angiogenesis appears initially counterintuitive, however, CD31+ endothelial cells may represent functional vessels which protect the tumour from sustained periods of ischaemia, unlike the low VVI group, from which hypoxia death-resistant clones could arise facilitating tumour metastasis. This could be very important when considering the effects of biologically targeted antiangiogenic therapies. Furthermore, the negative correlation with angiogenesis (CD31+) and Fuhrman grade suggests that vessel functionality and tumour aggressiveness may change with tumour size. The finding of a specific microRNA that appears to have VHL and HIF dependent expression extends our understanding of the hypoxic pathway and opens the possibility of further development of novel targeted therapies.
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Adenosine Triphosphate, ATP – universal form of energy used by living cells

Angiopoetin2, Ang2 – pro-angiogenic polypeptide

Basic Fibroblast Growth Factor, bFGF – growth factor present in basement membranes and in the subendothelial extracellular matrix of blood vessels, and when activated can mediate the formation of new blood vessels

BCL2/adenovirus E1B 19kDa interacting protein 3, BNIP3 – human gene involved in cell apoptosis and autophagy

Body Mass Index, BMI – statistical measurement of weight according to height = weight (kg) / height² (m²)

Cancer Research UK, CRUK – National Cancer Charity based in United Kingdom.

Clear Cell Renal Cell Carcinoma, CC-RCC – pathological variant of renal cell carcinoma distinguished by classical clear cytoplasm filled with lipid.

Computed Tomography, CT – Radiological body imaging

Cyan, Cy – colour

Cyclooxygenase, COX – an enzyme that converts arachidonic acid to prostaglandin H₂, the precursor for prostanoids

Carbonic Anhydrase IX, CAIX - is an enzyme that catalyzes the rapid conversion of carbon dioxide and water into carbonic acid, protons and bicarbonate ions.
Deoxyribonucleic Acid, DNA – contains genetic instructions for development and function of all known living organisms

Department of Health, DOH – organization (part of British Government) responsible for the running of health services within the UK

Erythropoietin, EPO - is a glycoprotein hormone produced in the kidney that regulates red blood cell production, and consequently increases systemic oxygen delivery.

Epidermal Growth Factor Receptor, EGFR – transmembrane receptor involved in cellular proliferation and angiogenesis. Primary ligand in Epidermal Growth Factor, EGF, although many other growth factors can bind to it.

Ferrous Ion, Fe2+ - iron ion, an essential trace element in humans

Glucose Transporter, GLUT1 - is a transmembrane protein involved in passive transport of glucose through cellular membranes

Haematoxylin and Eosin, H&E, common histological cellular stain

Insulin-like Growth Factor-2, IGF-2 - The insulin-like growth factors are polypeptides with high sequence similarity to insulin that form part of the IGF axis.

Lactate Dehydrogenase, LDH – catalyses interconversion of pyruvate to lactate
Magnetic Resonance Imaging, MRI – medical imaging instrument which uses magnetic fields as its primary method to create computer images of the body.

Matrix Metalloprotease, MMP – are zinc dependant endopeptidases capable of degrading extracellular matrix proteins and can process a number of bioactive molecules.

Messenger RNA, mRNA – translates DNA into protein

MicroRNA, miR – small non-coding RNA

Monoclonal Antibody Ki-67, Ki-67 – proliferation marker

Phosphatase and Tensin Homologue Gene, PTEN – tumour suppressor gene which plays a key role as the inhibitor to the Akt pro-tumourigenic pathway

Placental Growth Factor, PIGF – a member of the Vascular Endothelial Growth Factor family expressed not only in placental cells but also in non-placental cells such as endothelial cells.

Polymerase Chain Reaction, PCR – amplification of small pieces of DNA for experimental quantification

Protein53, p53 – tumour suppressor gene that blocks progression through the cell cycle in the event of DNA damage

Reactive Oxygen Species, ROS – oxygen free radicals

Renal Cell Carcinoma, RCC – cancer of the renal parenchyma
Ribonucleic Acid, RNA – translates DNA into protein

Ribonucleic Acid interference, RNAi – molecular biological technique to block the translation of certain proteins.


Thrombospondin, TSP – glycoprotein involved in cell adhesion, platelet aggregation, cell proliferation, tumour metastasis, and tissue repair. A major suppressor of angiogenesis.

Tissue Factor, TF - membrane protein, which binds to coagulation factor VII/VIIa and initiates the coagulation cascade.

Transforming Growth Factor alpha, TGF alpha – growth factor that binds the Epidermal Growth Factor Receptor.

Transforming Growth Factor beta, TGF beta – growth factor with several receptors including endoglin.

Tumour Associated Macrophage, TAM – Macrophages that are present within tumor stroma, that have a different phenotype from normal tissue macrophages.

Tumour Necrosis Factor, TNF - a multifunctional cytokine with effects on lipid metabolism, coagulation, insulin resistance and the function of endothelial cells lining blood vessels.
Tumour / Node / Metastasis, TNM – universal medical classification used to stage cancer advancement

Ultrasound Scan, USS – sonographical medical imaging

Vascular Endothelial Growth Factor, VEGF – a key angiogenic growth factor induced by hypoxia
Chapter 1: Introduction
1.1 Kidney Cancer

1.1.1 Epidemiology
Carcinoma of the kidney accounts for approximately 2% of all cancers in the UK, with on average 6700 new cases diagnosed each year (CRUK). The incidence is increasing by approximately 2% each year, due in part to the increased use of imaging modalities (Murai and Oya 2004). Despite this increase in early detection, about one third of patients who present with localised kidney cancer develop metastases and over half will die from the disease. Renal cancer, therefore, remains a significant cause of mortality and morbidity.

1.1.2 Pathology
Cancer of the renal parenchyma is termed Renal Cell Carcinoma, RCC and makes up approximately 85% of all tumours of the kidney. Further subclassification according to histopathological characteristics divides RCCs into Clear Cell (70-80%), Papillary (10-15%), Chromophobe (5%), Collecting Duct (Rare), and Unclassifiable (5%). The remainder of renal tumours consist of Transitional Cell Carcinomas (TCCs, 5-10%), Sarcomas, Wilms’ Tumour, as well as benign conditions such as renal cell adenoma, oncocytopma, and angiomyolipoma.

The large majority of Kidney Cancers are Clear Cell RCCs, defined by their translucent cytoplasm under the light microscope, due to lipid accumulation by the tumour cells. Current techniques of DNA sequencing demonstrate that approximately 80% of these tumours have a mutation, large scale deletion or methylation in the Von-Hippel Lindau (VHL) tumour-suppressing gene, rendering it non-functional (Kaelin 2002). The VHL gene is located on the short arm of chromosome 3 (3p26-25, molecular location...
10,158,318 to 10,168,761) (See Figure 1.3). The significance of this will be discussed later in this chapter.

Figure 1.1 - Fixed Surgical Specimen of RCC

Figure 1.2 - Clear Cell RCC with H & E staining, demonstrating translucent cytoplasms

Figure 1.3 - Position of VHL Tumour-suppressor gene on short-arm of Chromosome 3

1.1.3 Primary Surgical Management

Patients suspected to have a renal tumour will be further investigated using USS, CT or MRI imaging modalities. Approximately 75% of patients have no distant metastases at diagnosis, and can be offered curative treatment. Although novel treatments and being developed, such as Radio-Frequency Ablation and High-Intensity Focused Ultrasound, surgical resection remains
the gold-standard. Resection can be radical, partial or laparoscopic nephrectomy depending upon the size and position of the tumour as well as other variables such as premorbid conditions and patient body habitus. Following nephrectomy the surgical specimen is analysed by a specialised pathologist to determine pathological tissue diagnosis, tumour stage and Fuhrman grade.

1.1.4 Tumour T Stage
Pathological stage using the TNM classification has been modified over the past few decades. The principle of staging by defining a tumour by its size and level of invasion remains the same, but the boundaries of invasion have evolved as we have discovered the significance of each. Before 2002 the four stage system described T1 as less than 7cm; T2 as greater than 7cm but contained within the renal capsule; T3a as invasion into the renal capsule or adrenal gland; T3b invasion into renal vein or IVC below the diaphragm; T3c as invasion into the IVC above the diaphragm; and T4 as invasion beyond Gerota’s fascia. In 2002, the American Association of Pathologists made three changes to the classification. These were: i) sub-division of T1 into T1a <4cm, and T1b 4-7cm; ii) invasion of the renal sinus to be classified with extracapsular invasion (T3a); and iii) invasion of the muscular segmental branches of the renal vein within the renal sinus, to be equivalent to renal vein invasion (T3b) (Cancer 2002). These changes, especially the inclusion of renal sinus invasion, was often not specifically assessed in pathological examination prior to 2002 and has meant that as many as 80% of T2 tumours before the new system, would now be classified as T3 (Bonsib 2005).

1.1.5 Fuhrman Grade
Nuclear grade in RCCs was evaluated in 1982 by S. A. Fuhrman, based on 103 RCC H & E sections assessed under the light microscope. Fuhrman grade has subsequently been adopted as the standard classification of
nuclear grade for RCCs and is defined as a score of 1 to 4 based on increasing nuclear size, irregularity and nucleolar prominence (Fuhrman, Lasky et al. 1982).

1.1.6 Management of Advanced RCC

Approximately one third of patients with RCC will have metastasis at diagnosis, whilst another third will develop metastasis following primary nephrectomy. These patients have a poor prognosis with 2-year survival only 10-20% (Campbell, Flanigan et al. 2003). Historically, biologic therapy with interferon (IFN-alpha) and interleukin-2 (IL-2) has been the mainstay of treatment for patients with metastatic RCC, largely due to the radioreistance and chemoresistance of RCC (Yagoda, Petrylak et al. 1993; Deschavanne and Fertil 1996; Motzer and Russo 2000). Treatment with IFN-alpha and/or high dose IL-2, however only achieves response rates in 10-20% of patients (Fyfe, Fisher et al. 1995; Yang, Sherry et al. 2003; McDermott, Regan et al. 2005).

There is some evidence of spontaneous regression of distant metastatic renal deposits following resection of the primary tumour, however this phenomenon remains rare. However, several other potential arguments for the role of cytoreductive surgery in advanced RCC exist. These include reducing the tumour burden and hence removing a source for growth factors and cytokines and avoiding the possibility of an immune sink. Several studies have evaluated various combinations of immunotherapy with and without adjuvant nephrectomy. The European Organisation for Research and Treatment of Cancer (EORTC) 30947 and Southwest Oncology Group (SWOG) 8949 trials were prospectively randomised clinical trials comparing radical nephrectomy plus INF-a compared with IFN-a alone (Mickisch, Garin et al. 2001; Flanigan, Salmon et al. 2001). Combined analysis of the two trials showed a median survival of 13.6 months for nephrectomy plus IFN-a compared with 7.8 months for IFN-a alone. There
has not yet been a randomised trial to compare the efficacy of IFN-a to IL-2 after cytoreductive nephrectomy. However, Pantuck et al. have performed a retrospective study using the eligibility criteria from the SWOG 8949 trial to identify a comparable population treated with IL-2 following nephrectomy from the UCLA Kidney Cancer Database. They identified 89 patients and compared the survival of these patients with the survival of the 120 patients that received surgery plus IFN-a in the SWOG trial. They found that the median survival in patients treated with nephrectomy plus IL-2 was approximately 30% (4 months) longer than the nephrectomy plus IFN-a arm in the SWOG trial. There are currently no data on nephrectomy following any of the novel tyrosine kinase inhibitors, and this must be further evaluated using randomised trials in metastatic RCC.

Recent discoveries in the genetic basis and activating mechanisms of RCC have led to the development of targeted approaches to therapy. Bevacizumab, a VEGF neutralising antibody, was the first of these novel therapies to demonstrate a benefit in advanced RCC, prolonging time to progression (Yang, Haworth et al. 2003). More recently Sunitinib and Sorafenib, multi-target tyrosine kinase inhibitors, have both been shown to significantly improve progression-free survival (Escudier, Eisen et al. 2007; Motzer, Hutson et al. 2007), whilst other trials are ongoing to evaluate inhibitors to the mTOR pathway (located upstream from the HIF pathway), as well as other potential targets (including HIF) as our understanding of the biology improves.

1.1.7 Mortality

Kidney cancer is the 10th commonest cause of death in men and the 13th commonest in women, with a rate of 3600 deaths per year in the UK. The 1, 5 and 10-year survival rates in the UK are 60%, 42% and 38% respectively (data from 2000/2001) (CRUK; Office for National Statistics, 2007). See Table 1.1.
<table>
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<tr>
<td>1 year survival</td>
<td>50%</td>
<td>60%</td>
</tr>
<tr>
<td>5 year survival</td>
<td>35%</td>
<td>42%</td>
</tr>
<tr>
<td>10 year survival</td>
<td>28%</td>
<td>38%</td>
</tr>
</tbody>
</table>

Table 1.1 – 1, 5 and 10-year survival (kidney cancer specific death) following nephrectomy (Office for National Statistics, 2007).

1.1.8 Trends over time

From 1975 to 2003 incidence rates in men and women have increased by 69% and 100% respectively, in the UK. This may be partly due to the increasing average age of the population, with the majority of the increases seen are in men over 65, and in women over 55 years old (Coleman, Rachet et al. 2004; Office of Public Sector information). Another contributing factor is the increasing use of diagnostic imaging, with a subsequent rise in the incidental finding rate of a renal mass. There has been a 73% rise in the use of USS and CTs in the USA from 1986 to 1994 (Chow, Devesa et al. 1999). However, this has also demonstrated a significant rise in asymptomatic metastatic tumours, so the improved survival of kidney cancer may not be wholly accountable to this (Chow, Devesa et al. 1999). Furthermore there have been stable rates of pelvic cancer diagnoses over this time, where a similar rise would otherwise be expected (Chow, Devesa et al. 1999; Tate, Iddenden et al. 2003).
1.1.9 Risk Factors

Established risk factors for kidney cancer are increasing age, sex (male > female), obesity (approximately 25% of RCC could be attributable to obesity, with a relative two-fold increase in risk for patients with a BMI in the highest category); smoking (two-fold increase in risk for RCC); diet (possibly meat and dairy products increase risk, whilst antioxidants within fruit and vegetables decrease risk); other medical conditions (such as acquired cystic kidney disease, end-stage renal failure, hypertension). Reviewed in (CRUK).

1.1.10 Prognostic Algorithms

Several groups have sought to build prognostic algorithms by combining multiple prognostic factors. These may provide superior predictive information for individual patients and identify those at low risk for whom follow-up, including imaging can be safely reduced and select those at high risk for adjuvant therapies.

The UCLA Integrated Staging System (UISS) uses stage (1997 TNM), Fuhrman Grade, and the Eastern Cooperative Oncology Group (ECOG) performance status to stratify patients into low, intermediate or high risk categories (Zisman, Pantuck et al. 2002).

The Memorial Sloan Kettering Cancer Centre (MSKCC) nomogram includes clinical presentation (incidental or symptomatic) (Sorbellini, Kattan et al. 2005). This was of prognostic significance in cohort surgical studies (Ficarra, Prayer-Galetti et al. 2003; Lee, Katz et al. 2002; Patard, Ridriguez et al. 2002) but not in a population based study Gudbjartsson, Thoroddsen et al. 2005). Other factors in the nomogram are tumour size, stage (2002), Fuhrman grade, the presence of necrosis, and/or vascular
invasion. The nomogram of a 5-year predicted probability of freedom from recurrence is available at nomograms@mskcc.org.

The Mayo Clinic algorithm (Leibovich, Blute et al. 2003) is based on pathological tumour stage (2002), nodal status, tumour size (10cm cut-off), nuclear grade, and histological tumour necrosis. This large study including 1671 patients shows good discrimination for the 5-year risk of recurrence between low, intermediate and high risk groups, and has been independently validated (Ficarra, Martignoni et al. 2006).

The prognostic significance of molecular markers is discussed later in this chapter. Prognostic algorithms including these markers are yet to improve on the MSKCC or Mayo nomograms, however their continued investigation is of great interest as this may elucidate which patients are likely to respond to the developing novel therapies in development.

1.2 Data Collection

There is an increasing need to collect data on cancer patients from surgical departments, oncological departments and for research purposes. Clinical audit is an essential requirement of clinical governance for health care professionals and good data collection is a vital component of this process. Furthermore, National Health Service (NHS) trusts are putting increasing emphasis on clinical audit since it has become a requirement for foundation trust status.

Professional bodies such as the British Association of Urological Surgeons (BAUS) are also increasingly keen to audit all complex cancer surgery for the assessment of these procedures, and to evaluate developing techniques in urology cancer surgery. The expanding knowledge of molecular biology and the subsequent growing number of novel targeted therapeutic agents in
Oncological treatment will require careful patient/tumour selection. The assessment of which tumour molecular profile suits which targeted therapy will largely depend upon large scale profiling on tumours and their response to these new treatments.

1.3 Tissue Collection

The collection of cancer specimens for molecular biological assessment is clearly important to further our knowledge of cancer biology and allow translation into targeted therapies. The introduction of the Human Tissue Act in 2004 has meant that human tissue collection for research purposes is now carefully regulated by the Human Tissue Authority under the guidelines of the Act. It is therefore important that we have robust systems in place to fulfil these requirements.

1.4 Tumour Biology

1.4.1 Hypoxia

Oxygen homeostasis and protection from episodes of low oxygen tension in human tissues is important for cell survival. There are a number of physiological and pathological scenarios that place the cell in hypoxic conditions warranting adaptation to the stressful environment.

In hypoxia, oxidative phosphorylation is deceased with subsequent reduction of ATP production. A responsive increase in glycolysis compensates for this ATP reduction to some degree, but many ATP-dependent processes such as protein translation are decreased in hypoxic cells. Despite this, the cell must adapt to the hypoxic environment via increased oxygen delivery systemically and locally, as well as protect itself from secondary effects of hypoxia, such as decreased pH. Therefore, in a background of decreased total protein translation, specific upregulation of
protective mechanisms safeguard the cell from hypoxic stress. This process is primarily regulated by a transcription factor known as Hypoxia Inducible Factor, HIF.

In renal tumours, neovascularisation often lags behind tumour growth, leaving areas of hypoxia. This decrease in oxygen tension activates HIF, causing transcription of many protumourigenic factors that protect the tumour cells from the stressful environment. Induced genes include those involved in angiogenesis (VEGF, VEGFR-1, TP, Ang2), glucose metabolism (glucose transporters GLUT1, aerobic glycolysis LDH5), apoptosis / autophagy (BNIP3), cellular de-differentiation (Oct-4), proliferation (insulin-like growth factors IGF-2, cell cycle progression cyclin D1) and pH regulation (carbonic anhydrase IX) (Harris 2002).

The loss of pVHL has been demonstrated as a characteristic finding in clear cell carcinoma of the kidney, and in the key genetic variant in the pathogenesis of the VHL syndrome. As discussed shortly, a lack of a functional VHL protein product leads to constitutive activation of the HIF pathway.

1.4.2 Hypoxia Inducible Factor, HIF

1.4.2.1 Background

Hypoxia-Inducible Factor 1, HIF-1 was initially identified as a transcriptional activator of erythropoietin (a glycoprotein regulating red cell production in the kidney), under hypoxic conditions (Semenza and Wang 1992) (Wang and Semenza 1995). Numerous other factors have subsequently been identified to be induced by HIF and many of these genes are also known to be similarly regulated in cancer, suggesting an important regulatory role of HIF in tumours. HIF is highly conserved in eukaryotes from C elegans through to humans, highlighting its fundamental role in normal physiological cell functions.
1.4.2.2 Structure

HIF1 is composed of a 120-kDa HIF1-alpha subunit complexed with a 91- to 94-kDa HIF1-beta subunit. They are both basic-helix-loop helix proteins containing a PAS domain. HIF1-alpha located on chromosome 14q21-q24 (Semenza, Agani et al. 1997) is closely related to Sim, and HIF1-beta is aryl hydrocarbon receptor nuclear translocator ARNT, which heterodimerise with either HIF1-alpha or AHR. HIF1-alpha protein levels increase under hypoxic conditions and subsequently return to normal state under normoxia (Wang, Jiang et al. 1995). HIF alpha contains an N-terminus bHLH domain that mediates binding to consensus DNA sequences (CTACGTGCT) in the promoter region of target genes (Murre, McCaw et al. 1989) (Semenza and Wang 1992) (Semenza, Koury et al. 1991; Wang, Jiang et al. 1995).

![Figure 1.4 – Structure of HIF alpha](image)

1.4.2.3 HIF Degradation Pathway

In normal physiological conditions HIF-alpha undergoes rapid degradation. The proline residues (Pro402 - Pro564 in HIF-1 and Pro 406 – Pro 531 in HIF-2) (Bruick and McKnight 2001) (Epstein, Gleadle et al. 2001) within its oxygen-dependent degradation domain (ODD) are hydroxylated by one of a 3 member family of prolyl hydroxylase domain, containing proteins 1-3 (PHDs 1-3). These enzymes are oxygen, Fe2+, ascorbate and 2 oxoglutarate dependent and their action is enhanced by the presence of OS-9, (a ubiquitously expressed protein of no other known function). This hydroxylation allows interaction with von Hippel Lindau protein (pVHL),
(the recognition component of an E3 ubiquitin ligase) which subsequently promotes a larger complex formation with elongin-B, elongin-C and cullin-2. This is then rapidly destroyed by 26S proteasomes.

**Figure 1.5** – HIF alpha degradation pathway

### 1.4.2.4 HIF Activation in Hypoxia

In times of insufficient oxygen the prolyl hydroxylation of HIF-alpha is reduced, allowing the HIF alpha and beta subunits to combine at nuclear HREs (hypoxic response elements) at a number of target genes (Ivan, Kondo et al. 2001) (Jaakkola, Mole et al. 2001). Subsequent interaction with coactivators CBP and p300 activates transcription and thus gene expression (Ruas, Poellinger et al. 2005).

**Figure 1.6** – HIF activation
In many target genes HIF binding to HREs is sufficient for gene induction, although in others synergistic co-operation with other transcription factors such as Smad3, HNF4, ATF1/CREB1, AP1 and Ets-1 is required (Bracken, Whitelaw et al. 2003). (See recent reviews (Pugh and Ratcliffe 2003) (Harris 2002; Hirota and Semenza 2006)).

1.4.2.5 Alternative Pathways of HIF Activation

An alternative mechanism for HIF-alpha activation in hypoxia is regulated by Factor Inhibiting HIF (FIH). HIF has two transactivation domains, NAD (N-terminal activation domain) and CAD (C-terminal activation domain). The CAD overlaps with the ODD binding pVHL complex and the NAD offers the alternative regulatory mechanism. Oxygen dependent hydroxylation of the asparagine residue (Asn 803 – HIF1, Asn 851 – HIF2) on the CAD blocks interaction with the transcriptional coactivators CBP (CREB binding protein) and p300 previously mentioned. This hydroxylation is enabled through the enzyme FIH that requires oxygen as a co-factor. Therefore in hypoxic conditions, hydroxylation of this asparagine residue is avoided allowing HIF transcriptional activation (recently reviewed in (Liu and Simon 2004)).

Figure 1.7 – Alternative pathway of HIF alpha activation

HIF-1 can also be activated by interaction with reactive oxygen species (ROS) possible via inhibition of PHD hydroxylation (Liu and Simon 2004), the source of which may be from mitochondria as oxygen tension drops.
Increased ROS production is also seen in response to radiation treatment, where there is subsequent increased HIF-1 expression and VEGF production (Moeller, Cao et al. 2004). The source of which is likely to be inflammatory cells such as macrophages.

Low oxygen tension is the primary activator of the HIF pathway, although growth factors (e.g. insulin, EGF, PDGF) and cytokines (e.g. TNF alpha, interleukin-1 beta) have also been shown to initiate the HIF pathway although the amplitude of induction is lower (Zelzer, Levy et al. 1998; Haddad and Land 2001; Stiehl, Jelkmann et al. 2002; Treins, Giorgetti-Peraldi et al. 2002; Zhou, Schmid et al. 2003). HIF hydroxylation and subsequent degradation is also dependent upon sufficient levels of iron, ascorbate and 2-oxoglutarate. Therefore a deficiency of any of these cofactors can activate the HIF system in-spite of sufficient oxygen levels. HIF activity is also enhanced by oncogenes such as Human Epidermal Growth Factor Receptor 2 (HER2), H-ras and v-Src (Jiang, Agani et al. 1997; Chen, Pore et al. 2001; Laughner, Taghavi et al. 2001). Interestingly, every oncogene pathway investigated has been reported to enhance HIF function (Blancher, Moore et al. 2000; Vogelstein and Kinzler 2004). This is likely related to a fundamental link between proliferation, the generation of hypoxia, metabolite consumption and the need to increase blood flow and oxygen delivery locally and systemically (Maxwell, Pugh et al. 2001).

1.4.2.6 Regulation of HIF Synthesis

Regulation of HIF-1 mRNA translation to protein is still necessary, although not mediating rapid responses to hypoxia, it is clearly essential to maintain protein synthesis. It can be an important contribution to the mechanisms by which oncogenes activate HIF. Thus HER2 can activate two intracellular signalling pathways PI3K and MAPK, which in turn lead to activation of the eukaryotic translation initiation factor 4E (eIF-4E) which increases the rate at which HIF-1 mRNA is translated into protein (Semenza 2003). These
pathways are subject to positive feedback loops as HIF-1 can induce expression of many growth factors, and their receptors.

1.4.2.7 HIF isoforms
HIF family comprises HIF-1 alpha (aka Member of PAS1 (MOP1)); HIF-2 alpha (aka endothelial PAS domain protein 1 (EPAS1)); HIF-3 alpha (aka MOP3); HIF-1 alpha like protein (MOP2 / HLF); and HIF related factor (HRF). HIF-1 alpha and HIF-2 alpha both dimerise with HIF-beta and subsequently bind to nuclear Hypoxia Response Elements, HREs to regulate certain genes in a hypoxia-inducible manner (Wiesener, Turley et al. 1998; Ema, Hirota et al. 1999). HIF-1 and HIF-2 contain 48% sequence homology but have significant differences in their C-terminal sequences. This may explain the findings in specific knockdown studies of HIF-1 and HIF-2 in embryonic stem cells, mouse embryos and human cancer cells that there is differential function and target gene expression between the two isoforms (Ema, Taya et al. 1997; Hu, Wang et al. 2003; Pugh and Ratcliffe 2003) (Raval, Lau et al. 2005) (Park, Dadak et al. 2003; Sowter, Raval et al. 2003). This is particularly apparent in the difference between HIF-2 predominance in renal cancer which promotes tumour growth (Kondo, Klco et al. 2002; Maranchie, Vasselli et al. 2002) and breast cancer where growth is inhibited (Blancher, Moore et al. 2000). There is increased selectivity of HIF target genes to HIF-1 or HIF-2 in VHL defective cells (such as the majority of CC-RCC) compared to other cells. In CC-RCC cell lines and a mouse model HIF-2 encourages tumour growth whilst HIF-1 overexpression suppressed this growth, and that the two isoforms have a reciprocal relationship (Raval, Lau et al. 2005).

The distribution of HIF-1 and HIF-2 differs in normal tissue of rodents. HIF-1 widely expressed – predominantly in epithelial cells, while HIF-2 has a more localised expression pattern, predominantly in interstitial fibroblasts and endothelial cells (Rosenberger, Mandriota et al. 2002). HIF2 has higher
expression in highly vascular organs such as the heart, placenta and lungs, bone marrow macrophages, kidney epithelial cells, liver parenchyma, cardiac myocytes and pancreatic parenchymal cells in the adult, and lung and neural crest derivatives in the embryo. (Tian, McKnight et al. 1997; Talks, Turley et al. 2000; Wiesener, Jurgensen et al. 2003) (Ema, Taya et al. 1997). In humans however, there are many cells that express both isoforms of HIF alpha or neither.

An alternative splice variant of the HIF-3 locus, termed inhibitory PAS, negatively regulates HIF induced gene expression, in particularly VEGF. This HIF-3 alpha isoform may have a role in maintaining an avascular environment in certain tissues such as the cornea, and possibly forms a negative feedback loop to regulate HIF hypoxic response (Makino, Kanopka et al. 2002).

1.4.2.8 HIF and Embryonic Vascular Development

HIF-1 is expressed in all tissues during development and is essential for embryonic vasculogenesis, angiogenesis and haematopoiesis in part by promoting the production of VEGF.

Mesenchyme derived endothelial progenitor cells are produced in the yolk sac and embryo in a HIF dependent manner (Ramirez-Bergeron, Runge et al. 2004). They differentiate during development under the regulation of VEGF and bFGF mediated signalling (Kennedy, Firpo et al. 1997; Faloon, Arentson et al. 2000; Conway, Collen et al. 2001) to form the primary capillary plexus of the embryo.

Via studies in HIF1-alpha negative embryonic stem cells, Iyer et al. in 1998 demonstrated that the absence of HIF1-alpha resulted in reduced glucose transporter, glycolytic enzymes and VEGF RNA as well as decreased proliferation. HIF1-alpha negative embryos resulted in developmental arrest
and lethality at day 10, with notable neural tube defects, cardiovascular malformations and marked cell death within the cephalic mesenchyme. Comparable HIF1-alpha positive embryos showed increased HIF1-alpha expression at the same time-point (Iyer, Kotch et al. 1998; Ryan, Lo et al. 1998; Kotch, Iyer et al. 1999).

HIF-2 is expressed in the lung and neural crest derivatives in the embryo and is essential for embryonic vascular development (Ema, Taya et al. 1997; Tian, Hammer et al. 1998). In studies with HIF-2 negative mouse embryos lethality occurred between 9 and 14 days due to disorganisation of blood vessel growth within the embryo and yolk sac or later due to cardiorespiratory failure secondary to decreased lung surfactant or catecholamine production. However, this was rescued by reintroduction of HIF-2 (Tian, Hammer et al. 1998; Peng, Zhang et al. 2000; Compernolle, Brusselmans et al. 2002; Duan, Zhang-Benoit et al. 2005).

In HIF-1 / HIF-2 knockdown studies in mice, most genes were responsive only to HIF-1 knockdown, showing little or no effect to HIF-2 siRNA apart from EPO which appears to be HIF-2 specific. However the target gene specificity to either HIF-1 or HIF-2 can be overcome in certain cell types (Warnecke, Zaborowska et al. 2004; Chavez, Baranova et al. 2006).

In mouse embryo fibroblasts (MEFs) HIF-2 was detected within the cytoplasm regardless of oxygen tension, but was transcriptionally inactive until it was extrinsically overexpressed whereupon it translocated to the nucleus and initiated hypoxic gene expression (Park, Dadak et al. 2003). FIH selectively inhibits HIF-1 in low oxygen tensions, but not HIF-2 in MEFs, suggesting that the balance between HIF-1 and HIF-2 may allow cell-specific modulation of the hypoxic response (Park, Dadak et al. 2003).

Mice lacking HIF-2 that survive development demonstrate a phenotype of multiple organ pathologies, transformed gene expressions and biochemical
defects (including retinopathy, hepatic steatosis, cardiac hypertrophy, skeletal myopathy, hypocellular bone marrow, azoospermia, mitochondrial abnormalities, decreased transcriptional activation of the primary antioxidant enzymes and increased ROS). This suggests HIF-2 may play a role in the maintenance of mitochondrial and ROS homeostasis (Scortegagna, Ding et al. 2003).

HIF beta is ubiquitously expressed in the embryo. HIF beta negative embryos die at day 10 in a manner similar to HIF-1 negative embryos (Maltepe and Simon 1998; Cowden Dahl, Fryer et al. 2005). This is primarily due to yolk sac haemopoietic and angiogenic development problems. (Adelman, Gertsenstein et al. 2000; Ramirez-Bergeron and Simon 2001; Ramirez-Bergeron, Runge et al. 2006). These effects may be secondary to decreased VEGF production, which regulates endothelial cell production and vascular morphogenesis. (Ramirez-Bergeron, Runge et al. 2006)

1.4.2.9 HIF and Tumour Associated Macrophages

Large numbers of circulating monocytes are attracted to hypoxic tumours along chemotaxic gradients (Kelly, Davison et al. 1988) (Leek, Harris et al. 1994). They migrate across the vascular endothelium becoming Tumour-Associated Macrophages, TAMs - a process regulated to a large degree by intra-tumoural hypoxia. Their presence within tumours can be anti- or pro-tumourigenic due to their secondary effects on antigen presenting abilities to cytotoxic T-cells, or their ability to secrete tumour mitogens and angiogenic promoters (including HIF) (Leek and Harris 2002). This balance appears to depend on the tumour microenvironment, with high TAM infiltration in some tumour types being beneficial to survival (stomach, colon, and melanoma), whilst in others their presence is detrimental (breast, prostate, bladder, endometrial, kidney and lymphoma). (Lewis and Pollard 2006)
HIF activation within the tumour induces secretion of a number of factors that aid monocyte adhesion and extravasation, as well as TAM migration to the hypoxic centre of the tumour (Balkwill 2004) (Murdoch, Giannoudis et al. 2004). The most widely studied hypoxia-induced chemoattractant is VEGF, which not only triggers the VEGF-R1 receptor on TAM cell surface (Barleon, Sozzani et al. 1996), but may also initiate a positive feedback mechanism that amplifies the signal (Lewis, Landers et al. 2000).

Other important hypoxia induced chemoattractants include stromal cell-derived factor-1 (SDF-1) (Schioppa, Uranchimeg et al. 2003), Endothelin-1 (Grant, Loizidou et al. 2003), Endothelin-2 (Grimshaw, Wilson et al. 2002), transforming growth factor-alpha (TGF-alpha) (Gunaratnam, Morley et al. 2003) and EMAP II (endothelial-monocyte activating peptide II) (Matschurat, Knies et al. 2003). Macrophage migration inhibitory factor (MIF), a pleiotrophic cytokine, is also released in response to hypoxia and serves to retain the TAMs within the hypoxic environment by reducing further migration (Bacher, Schrader et al. 2003).

The phenotype of these macrophages changes upon migration into the tumour where they then function to promote angiogenesis (Leek, Lewis et al. 1996) (Lewis, Landers et al. 2000) (Miles, Happerfield et al. 1994), remodel the surrounding matrix (Petrella, Lohi et al. 2005) (Burke, Giannoudis et al. 2003) and suppress the adaptive immunity (Sica, Schioppa et al. 2006). This leads to a predominant pro-angiogenic phenotype in tumours with high a TAM component (Leek, Lewis et al. 1997).

1.4.2.10 HIF Regulated Gene Expression

As mentioned earlier in this chapter, EPO was the first target gene associated with HIF, and there are now many more (see table 1.2).
### Secreted Factors
- Adrenomedullin
- Angiopoietins
- Angiopoietin 2
- Angiopoietin like 4
- Connective Tissue Growth Factor, CTGF
- Endothelin 1
- Endothelin 2
- Erythropoietin, EPO
- Hepatocyte Growth Factor, HGF
- Interleukin-8, IL-8
- Inducible Nitric Oxide Synthase, iNOS
- ORP150 VEGF chaperone
- Osteopontin
- Platelet Derived Growth Factor beta, PDGF beta
- Stromal Cell-Derived Factor-1, SDF1 (CXCL12)
- Spingosine 1 Phosphate, S1P
- Stanniocalcin 1
- Stanniocalcin 2
- Transforming Growth Factor beta 2, TGF beta 2
- Transforming Growth Factor beta 3, TGF beta 3
- Tie2
- Vascular Endothelial Growth Factor, VEGF

### Tethered Ligand
- Delta-like 4, DLL4 - Notch signalling

### Cell Adhesion
- Integrins
- Tenascin (Hexabrachion)

### Transmembrane Receptors
- VEGF Receptor 1, VEGFR 1
- VEGF Receptor 2, VEGFR 2
- CXCR4, Fusin
- Transferrin receptors
- Adenosine receptors

### Copper Pathways
- Caeruloplasmin

### Extracellular Matrix Proteases
- Lysyl Oxidase, LOX
- Matrix Metalloproteinase 1, MMP 1
- Matrix Metalloproteinase 2, MMP 2
- Matrix Metalloproteinase 9, MMP 9
- Plasminogen activator inhibitor-1, PAI1
- Urokinase receptor

**Table 1.2** – HIF-1 alpha target genes
The methods that have been used for looking at HIF-dependent gene expression include screening for:

1. Genes with increased expression secondary to HIF activated cells (e.g. VHL mutated cells, hypoxic cells).
2. Genes with decreased expression secondary to targeted HIF inactivation.
3. Genes that contain a HIF binding site 5’-RCGTG-3’ at cis acting HREs.

There may be as many as 1-5% of all genes that can be expressed in response to hypoxia in a cell / tissue specific manner (Semenza 2003). This may be reflective of the fact that although the main role of HIF is the direct regulation of primary transcripts, there is also interaction with other signalling pathways that can amplify the hypoxic signal. Examples of this include HIF enhancing notch signalling which subsequently inhibits differentiation of certain cell types (Gustafsson, Zheng et al. 2005); HIF decreasing c-myc’s effect on p21 resulting in cell cycle arrest (Koshiji, Kageyama et al. 2004); as well as other effects on p53, c-Jun and NF-kappaB (reviewed in (Hickey and Simon 2006)).

1.4.2.11 HIF in Adult Tissue

Within physiologically normal adults, HIF-1 is important in erythropoiesis and pulmonary remodelling in response to chronic hypoxia (Yu, Shimoda et al. 1999), wound healing (Elson, Ryan et al. 2000), brain spatial memory function (Tomita, Ueno et al. 2003), breast tissue differentiation and lactation (Seagroves, Hadsell et al. 2003) and cardiac contractility (Huang, Hickey et al. 2004).

Pathophysiological angiogenesis, primarily through ischaemic injury causes induction of the HIF pathway and increased HIF-1 expression, as well as its associated target genes in many human tissues including the brain, heart, lung and eye (Yu, Frid et al. 1998; Bergeron, Yu et al. 1999; Ozaki, Yu et al. 1999; Lee, Wolf et al. 2000).
1.4.3 HIF in Cancer

1.4.3.1 HIF and VHL syndrome

VHL Syndrome is an autosomal dominant condition from an inherited mutation in one allele of the VHL gene located on the third chromosome. Subsequent mutation or loss of the remaining allele in somatic tissues leads to tumour development at that site. Features of this syndrome include angiomatosis (collections of disorganised capillaries in various organs), haemangioblastomas (tumours of the CNS, especially the cerebellum), phaeochromocytomas (tumours of the adrenal medulla), and clear cell tumours of the kidney. The VHL gene is also inactivated in a large percentage of haemangioblastomas and RCCs via mutation, large scale deletions or methylation (Kaelin 2002). An unanswered problem is why malignant tumours form in the kidney, but with the same mutations only haemangiomas form in the CNS.

The loss of the functional protein product of VHL leads to constitutive activation of HIF, with the resultant increase in expression of its target genes controlling angiogenesis, proliferation, glucose metabolism, and pH regulation.

That a hereditary cancer syndrome results directly in HIF upregulation has provided strong evidence for the role of HIF in cancer, but it is a puzzle as to why there are no mutations in HIF leading directly to its stabilisation. VHL has additional functions and partners that may be required, a subject of much investigation. Nevertheless, there is a striking correlation of genes induced by hypoxia and HIF and those regulated by VHL (Wykoff, Pugh et al. 2000).

1.4.3.2 HIF Protein Expression in Cancer

Identification of HIF alpha within normal and cancerous tissues with immunohistochemistry has shown that there is much variation between
different tissue types. Immunostaining is predominantly nuclear and generally strongest in perinecrotic regions (where presumably hypoxia is maximal) and there is often co-localisation of HIF expression and VEGF (Talks, Turley et al. 2000).

The majority of benign tissues do not express stabilised HIF-alpha; however it is commonly over-expressed in malignant tissues. The pattern of expression within tumours appears partly dependent upon the mechanism of activation. Diffuse expression is seen in tumours with mutations within the HIF degradation pathway such as clear cell RCC with its common mutation in the VHL gene. High and more localised expression is seen in peri-necrotic areas where the HIF activation is via hypoxia. (Krieg, Haas et al. 2000; Wykoff, Beasley et al. 2000; Zagzag, Zhong et al. 2000).

Hypoxia is the primary activator of HIF in tumours but other mechanisms such as mutations in tumour suppressor genes such as VHL, p53, PTEN can result in increased HIF (Maxwell, Wiesener et al. 1999; Ravi, Mookerjee et al. 2000; Zundel, Schindler et al. 2000). Also activation of oncogenes such as v-SRC, EGFR, and HER2 and subsequent signalling via the phosphatidylinositol-3-kinase (PI-3K) pathway and mitogen-activated protein kinase (MAPK) pathway increase or amplify HIF activation (Jiang, Agani et al. 1997; Chen, Pore et al. 2001; Laughner, Taghavi et al. 2001).

Interaction with the microenvironment and stroma is also important. HIF-1 deficient mouse astrocytoma cells when placed in a vessel-poor subcutaneous environment resulted in necrosis, poor growth and poor vessel development. However, when these same cells were placed in a vessel-rich environment (brain parenchyma) they grew quickly with extensive invasion. (Blouw, Song et al. 2003).
HIF-1 alpha protein is significantly increased in all human cancers studied (glioblastoma multiforme, prostate, breast, lung, pancreatic), and correlates with highly vascular tumours (Semenza 2002; Brahimi-Horn and Pouyssegur 2005). HIF-2 alpha can also be identified in these tumours, however its expression level is generally lower, apart from hepatocellular carcinoma where it is more pronounced (Bangoura, Yang et al. 2004) and in RCC where HIF-2 seems to predominate in more advanced tumours (Mandriota, Turner et al. 2002).

1.4.3.3 Xenograft studies
In-vivo studies determining the effect of HIF-1 in experimental xenograft tumours, demonstrate a HIF-1 dependent stimulatory effect on tumour growth and angiogenesis. The HIF dependent effect on angiogenesis is more distinct in studies on human cancer cells and is primarily due to VEGF production, although there is variation dependent upon the specific microenvironment and cell type. (Ryan, Lo et al. 1998) (Maxwell, Dachs et al. 1997) (Hopfl, Wenger et al. 2002) (Carmeliet, Dor et al. 1998) (Kung, Wang et al. 2000; Blouw, Song et al. 2003)

1.4.3.4 Prognosis
The net effect of HIF activation on outcome appears to vary depending upon the origin of the primary tumour. Positive correlation with pathological features and poor prognosis have been reported in brain (Korkolopoulou, Patsouris et al. 2004), breast (Schindl, Schoppmann et al. 2002), bladder (Theodoropoulos, Lazaris et al. 2004), ovarian (Birner, Schindl et al. 2001) and cervical tumours (Birner, Schindl et al. 2000), whilst HIF-1 over expression has been shown to be a predictor of better prognosis in kidney (Lidgren, Hedberg et al. 2005). This probably reflects the balance between HIF1 and HIF2, which is unique for this VHL mutated tumour. The majority of studies in lung cancer show high HIF-1 is associated with worse prognosis (Giatromanolaki, Koukourakis et al. 2001; Giatromanolaki,
Koukourakis et al. 2003; Kim, Rabbani et al. 2005) but there is an exception (Volm and Koomagi 2000). These differences may relate to the difficulties in reliability of HIF immunostaining of paraffin embedded tissues due to its inherent instability; the possible differential effects of HIF-1 and HIF-2 (Raval, Lau et al. 2005); or the variability of activity of HIF depending on its microenvironment (Blouw, Song et al. 2003).

Furthermore, overexpression of HIF in tumours has been shown to correlate with resistance to drug, radiation and hormone treatment as well as photodynamic therapy (Koukourakis, Giatromanolaki et al. 2001; Koukourakis, Giatromanolaki et al. 2002; Generali, Berruti et al. 2006; Koukourakis, Bentzen et al. 2006).

1.4.4 HIF-1 vs. HIF-2 - Differential Function

As discussed previously, HIF-1 and HIF-2 share 48% sequence homology, however their significant differences in their C-terminal sequences may explain why many RNAi knockdown studies have shown differential expression profiles of known hypoxia inducible genes by HIF-1 and HIF-2 (Sowter, Raval et al. 2003). A recent study on human RCC (VHL-defective) cell lines demonstrated that differential activation of HIF-1 or HIF-2 pathways has non-equivalent or even opposing effects on gene expression and experimental (mouse model) tumour growth (Raval, Lau et al. 2005). HIF-2 was shown to have positive effects on tumour growth, in this model, whilst HIF-1 had negative effects. Consistent with these observed effects on tumour growth were differential effects on the expression of specific genes with putative pro- and antitumourigenic effects. In particular, HIF-1 positively regulated BNIP3 but had no effect on CyclinD1, TGF-alpha and VEGF, whereas HIF-2 negatively regulated BNIP3 and positively regulated CyclinD1, TGF-alpha and VEGF.
Little is known of the effects of HIF-1 and HIF-2 on the overall survival of patients with primary Clear-Cell Renal Cell Carcinoma (CC-RCC), how their expression relates to their primary target gene products or the pathological characteristics of the tumour (Fuhrman grade, Stage or Tumour Size). It is also not known whether expression of HIF-1, within these VHL deficient tumours, is concomitant with expression of HIF-2, and whether co-expression (if present) leads to the preservation of the previously demonstrated HIF-isoform specific target gene products.

From the previous literature, there is a suggestion that HIF-1alpha is a prognostic marker of better overall survival in primary CC-RCC. Analysis of HIF-1 protein expression by western blot on 92 CC-RCCs was shown to be an independent prognostic factor (Lidgren, Hedberg et al. 2005), although subsequent Tissue Microarray (TMA) analysis only showed a trend towards HIF-1 being of prognostic significance (p=0.055) (Lidgren, Hedberg et al. 2006). CAIX, one of HIF-1’s principal target genes, has a more convincing case for prognostic significance, with high CAIX expression being an independent predictor of better prognosis (Bui, Visapaa et al. 2004).

There is a lack of data for the prognostic significance of HIF-2, although for HIF-2 dominant target gene products, GLUT-1 is positively correlated with worse tumour grade and poorer survival in breast, rectal and oral SCC tumours, although nothing has been shown in CC-RCC (Cooper, Sarioglu et al. 2003; Kunkel, Reichert et al. 2003; Vleugel, Greijer et al. 2005), and cyclin D1 is preferentially expressed in lower grade renal tumours (Hedberg, Davoodi et al. 1999).

1.5 Angiogenesis
Angiogenesis is the development of new branching vessels from existing vasculature, and has long been established as a hallmark of cancer and
essential for tumour development and metastasis. There are a number of different mechanisms involved including vessel sprouting and bridge formation, although both are dependent on endothelial cell migration and proliferation. Circulating endothelial progenitor cells derived from bone marrow are also recruited to sites of active angiogenesis by tumour-derived growth factors such as vascular endothelial growth factor (VEGF).

(Carmeliet and Jain 2000)

Other mechanisms that are involved in vascularisation of tumours include: intussusception, where interstitial tissue columns expand and insert into the lumen of an existing vessel; vasculogenesis, where major vessels are formed de novo from mesenchymal tissues (as in the developing embryo); vascular co-option, where tumour cells invade surrounding tissue and make use of existing blood vessels; and vascular mimicry, where vascular channels are formed without the presence of endothelial channels, by the tumour cells themselves organizing into a lumen. (Folberg and Maniotis 2004)

Angiogenic vessels show several differences from mature vessels. They have a disorganized and irregular structure, the interactions between cells, endothelial cells and pericytes are altered and the blood flow is abnormal. This is in contrast to the organized, regular structure and normal blood flow seen in mature vessels. The angiogenic vessels are leaky, aiding extracellular matrix signalling and metabolism, as well as contributing to tumour cell invasion and metastasis.

For angiogenesis to occur there must be a switch from the physiologic quintessence of endothelial cells to an ‘angiogenic’ phenotype. This may be a result of metabolic stresses such as hypoxia, acidosis, inflammation or immune-cell activation. Genetic mutations may also affect the equilibrium of the factors directly with oncogenes and tumour suppressor genes (such as H-ras, HER2 and p53) (Bergers and Benjamin 2003).
Tumour angiogenesis can be assessed by using an anti-endothelial-cell antibody to identify tumour vasculature. Microvessel density (MVD) can then be measured either as an average of counts over a number of randomly selected areas, termed the mean MVD; quantified in the densest areas of neovascularisation, termed hotspots; or the whole tumour section can be assessed (not hotspots), quantified as high, medium or low, termed Vessel Visual Index (VVI).

Marked angiogenesis is a key pathological feature of primary CC-RCC, due in part to the pre-mentioned VHL mutation and activation of the hypoxic pathway. The increasing array of non-cytokine based novel biological therapies that are available for the treatment of metastatic disease are primarily targeted against the vascular axis. However, angiogenesis is not the only determinant factor in tumour perfusion, as the composition and integrity of the proteoglycan basement membrane and the extent of pericyte coverage of the vessels are also known to be important factors in this regard.

The role of angiogenesis in CC-RCC tumourigenesis is well established, however, the role of these vessels in determining factors such as overall survival and tumour grade are yet to be fully elucidated. The majority of published papers support the intuitive direct relationship with angiogenesis, overall survival and Fuhrman grade (Anastassiou, Duensing et al. 1996; Rioux-Leclercq, Epstein et al. 2001; Sabo, Boltenko et al. 2001; Schraml, Struckmann et al. 2002; Imao, Egawa et al. 2004), however a few have demonstrated an inverse relationship (Yoshino, Kato et al. 1995; Nativ, Sabo et al. 1998; Joo, Oh et al. 2004), whilst others have failed to show any relationship (MacLennan and Bostwick 1995; Minardi, Lucarini et al. 2005). The earliest literature on these matters, were dogged by problems in methodology, namely the use of Von Williebrand Factor (vWF) as an endothelial marker. The discovery of CD31, CD34 and CD105 (endogolin) as endothelial markers, has rectified this satisfactorily and there have been a
number of reviews on MVD that summarize the growing body of evidence supporting MVD as a method to assess angiogenesis in urological malignancies, but that also show the pitfalls (Folkman 2004) (Kerbel and Folkman 2002) (Hlatky, Hahnfeldt et al. 2002) (Fox and Harris 2004). Additionally, it has recently been demonstrated that there is a strong positive correlation between these markers in CC-RCC, although a differential CD31/CD34 profile, namely CD34+/CD31-, has been shown to be indicative of an immature CC-RCC tumour vasculature and a worse prognosis in primary CC-RCC (Yao, Qian et al. 2007).

1.6 VEGF

VEGF is probably the most important HIF dependent target gene, and has a major role in hypoxia induced angiogenesis. It has been shown to have a significant role in many tumours including breast, colon, kidney, bladder, prostate, as well as many others.

It is a growth factor that is important in blood and vessels development due to its role in regulation of angioblast (endothelial progenitors) specification and vasculogenesis (Weinstein 1999). It exists in four forms of variable amino acid length, that each exerts a different mitogenic effect on vascular endothelial cells or tumour cells to promote angiogenesis. VEGF modulates its effects through its tyrosine kinase receptors VEGF receptor 1 (also known as fms-like tyrosine kinase receptor 1, or Flt-1) and VEGF receptor 2 (or kinase insert domain receptor, a product of the KDR gene). Further VEGF signal transduction occurs via other receptors including neuropilin-1 and -2.

In hypoxia, activation of HIF directly increases the VEGF transcript (Liu, Cox et al. 1995) (Forsythe, Jiang et al. 1996) and its receptor Flt-1 (Gerber, Condorelli et al. 1997), as well as enhancing the stability of VEGF mRNA via HIF activation of HuR protein, which binds to the VEGF 3’UTR (Levy,
Chung et al. 1998). VEGF translation is protected from the general reduction in protein synthesis secondary to hypoxia, by the presence of an internal ribosomal entry site within the 5’UTR of VEGF. This allows efficient cap-independent translation to be maintained (Stein, Itin et al. 1998).

Because HIF regulates many angiogenic pathways besides VEGF, (Harris 2002) the strong associations with VEGF may reflect these additional pathways, (Na, Wu et al. 2003) and may explain why VEGF has a better validated role in RCC compared to other urologic malignancies. (Edgren, Lennernas et al. 1999) VEGF is not expressed in renal tissue from patients with benign renal disease, but is increased in patients with conventional, papillary and chromophobe RCC (Jacobsen, Grankvist et al. 2004), particularly in clear cell tumours. High VEGF expression is a predictor of unfavourable survival and its expression level is proportional to both malignant potential of a tumour and its advancement, suggesting that it has a role as a prognostic marker in RCC.
1.7 MicroRNA

1.7.1 Introduction

As previously discussed, hypoxic stress decreases oxidative phosphorylation with subsequent reduction of ATP production and protein translation. However, the mechanisms involved in this selective transcriptional upregulation of certain proteins in a background of decreased ATP, is yet to be fully elucidated. There is some evidence to suggest that there is variable dependency of certain transcripts for the translational machinery, such as the internal ribosomal entry site within the 5’UTR of VEGF (Stein, Itin et al. 1998; Wek, Jiang et al. 2006). Another possibility, however, is that hypoxia induced microRNAs are regulating this specific protein translation. This is of particular interest in ccRCC with its known defect in the hypoxic pathway, and is explored later in this thesis.

1.7.2 Background

MicroRNAs are a recently discovered novel class of gene regulators. They are small (18-22 nucleotides) non-coding, single-stranded RNA. Despite their essential role in biological and disease processes, the first microRNA (lin-4) was only identified in 1993 (Lee, Feinbaum et al. 1993; Wightman, Ha et al. 1993). This remained an isolated example until 2000 when microRNA let-7 was also discovered in C. elegans, and found to be highly conserved in a wide range of organisms (Reinhart, Slack et al. 2000). Since this time, thousands of microRNAs have been cloned and characterised from a diverse range of organisms. There are currently 474 experimentally confirmed microRNAs listed in the miRBase database (Motzer, Hutson et al. 2007), representing approximately 1% of the human transcriptome.

However, computational approaches suggest that there may be over 1000 microRNAs in humans (Bentwich, Avniel et al. 2005; Berezikov, Guryev et al. 2005).
1.7.3 Synthesis

Most human microRNAs are encoded within introns of mRNAs, although others are located within exons or the 3’ UTR of mRNA (Rodriguez, Griffiths-Jones et al. 2004). MicroRNAs are transcribed as 5’- capped large polyadenylated transcripts (pri-microRNA). About 40% of all human microRNAs are co-transcribed as clusters of up to eight microRNAs per pri-microRNA. The pri-microRNA is cleaved within the nucleus by a microprocessor complex (including the RNaseIII-nuclease, Drosha), resulting in 60-70 nucleotide hairpin structure (pre-microRNA) which encodes for a single microRNA sequence. Exportin5 exports the pre-microRNA from the nucleus into the cytoplasm where it undergoes further cleavage by another RNaseIII-nuclease, Dicer. This forms a short-lived asymmetric duplex which is subsequently loaded into the miRISC complex in which Argonaut (Ago) proteins are the key effector molecules, producing an active mature microRNA (Schwarz, Hutvagner et al. 2003).

Figure 1.8 – MicroRNA Processing
1.7.4 Function
The primary function of microRNA is inhibition of protein translation. Their precise function though is complex and yet to be fully understood, although the outcome of mature microRNAs appears to be largely dependent upon the degree of complementarity between the microRNA and its target gene. In plants, microRNAs have near perfect homology to their targets and binding results in degradation of mRNA in a process similar to the siRNA pathway. Some human microRNAs are believed to act via a similar mechanism, however, the majority only have limited sequence homology to their targets, (commonly just a 6-8 nucleotide ‘seed’ region at the 5’ end), and inhibit protein translation without mRNA degradation (Yekta, Shih et al. 2004). The precise nature of this inhibition remains unclear, however suggested mechanisms include the prevention of elongation or degradation of developing translation products (Olsen and Ambros 1999); microRNA-miRISC sequestration in P-bodies, away from the translational machinery (Liu, Valencia-Sanchez et al. 2005); prevention of translational factors recognising the 5’ cap (Pillai, Bhattacharyya et al. 2005); or removal of the 5’ cap (Sen and Blau 2005). It is thought that each microRNA may target many hundreds of mRNAs, possibly accounting for 10-30% of all human genes (John, Enright et al. 2004; Gregory and Shiekhattar 2005).

1.7.5 MicroRNA and Cancer
1.7.5.1 Importance
MicroRNAs have been shown to be vital in many cellular processes including development, differentiation, apoptosis and proliferation (Bartel 2004; Harfe 2005). Mutations within, or abnormal expression of microRNAs can allow them to dysregulate these processes and function indirectly as oncogenes or tumour-suppressor genes (Esquela-Kerscher and Slack 2006). Furthermore, several microRNAs have also been proven to interact with known oncogenic or tumour-suppressor pathways. (See Table 1.3)
1.7.5.2 Diagnosis

The use of microRNAs in differentiating specific tumour types and their level of differentiation is an interesting area of future development. For all cancer types investigated so far, specific microRNA profiles have been identified. These include colon (Michael, SM et al. 2003), breast (Iorio, Ferracin et al. 2005), leukaemia (Calin, Ferracin et al. 2005), liver (Murakami, Yasuda et al. 2006), lung (Yanaihara, Caplen et al. 2006), pancreatic (Volinia, Calin et al. 2006) and thyroid cancer (He, Jazdzewski et al. 2005).

Two sizeable studies have looked at microRNA profiles from a large number of different tumour types and both have been able to accurately cluster their microRNA expression profiles according to their developmental lineage and state of differentiation, and to a far greater accuracy than compared to standard gene expression arrays (Lu, Getz et al. 2005; Volinia, Calin et al. 2006). Interestingly these studies have shown that global expression of microRNA is reduced in tumours compared to normal tissue, although individual microRNAs may be up or down regulated, and appears to be quite tumour specific. This general downregulation may be a reflection of the state of cellular differentiation, and there is some evidence to show that microRNA expression in poorly differentiated tumours is similar to early embryonic developmental (Song and Tuan 2006).

It may be possible in the future to use microRNA profiles as a diagnostic tool from clinical samples such as blood, urine, serum or biopsy specimens. This is clearly an exciting possibility in Urology where accurate biological classification of small kidney lesions, early diagnosis or surveillance bladder cancer and Prostate Specific Antigen (PSA) testing in prostate cancer are ongoing difficulties.
1.7.5.3 Prognosis

In lung cancer high expression of miR-155 has been shown to correlate with poor prognosis (Yanaihara, Caplen et al. 2006). A decreased level of let-7 has also been demonstrated to be an independent prognostic factor in a separate study in lung cancer from Japan (Takamizawa, Konishi et al. 2004). The significance of decreased let-7 may be due to its known inhibition of the Ras oncogene (Johnson, Grosshans et al. 2005).

There have been no studies yet analysing microRNAs and their prognostic significance in urological malignancies, although clearly this could be beneficial if it were to highlight patients that would benefit from more aggressive treatment.

1.7.5.4 Treatment

Although patient safety and drug efficacy clearly need to be established, it may be possible to inhibit many oncogenic genes via modified microRNA molecules. Developments of potential delivery systems are in an early stage (e.g. anti-microRNA oligonucleotides (Weiler, Hunziker et al. 2006), locked nucleic acid oligonucleotides (Orom, Kauppinen et al. 2006), and antagomirs (Krutzfeldt, Rajewsky et al. 2005)), although it is possible that microRNAs may offer a novel method of gene manipulation in a clinical setting, in the near future.
### Table 1.3 - MicroRNA with known cancer associations

<table>
<thead>
<tr>
<th>MicroRNA</th>
<th>Cancer Association</th>
<th>Reference</th>
<th>MicroRNA Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-15a, miR-16</td>
<td>Negatively regulates anti-apoptotic gene BCL2</td>
<td>(Cimmino, Calin et al. 2005)</td>
<td>Tumour-Suppressor</td>
</tr>
<tr>
<td>Let-7 family members</td>
<td>Negatively regulates the RAS oncogene + Decreased in Lung Ca</td>
<td>(Eder and Scherr 2005; Johnson, Grosshans et al. 2005; Akao, Nakagawa et al. 2006)</td>
<td>Tumour-Suppressor</td>
</tr>
<tr>
<td>miR-143, miR-145</td>
<td>Decreased in Colon, Breast, Prostate, Cervical, and Lymphoid Ca</td>
<td>(Michael, SM et al. 2003; Akao, Nakagawa et al. 2006)</td>
<td>Tumour-Suppressor</td>
</tr>
<tr>
<td>miR-17-92 cluster (17-5, 17-3, 18a, 19a, 20a, 19b-1, 92-1)</td>
<td>Negatively regulates the MYC oncogene</td>
<td>(Hayashita, Osada et al. 2005; Tagawa, Karube et al. 2007)</td>
<td>Tumour-Suppressor</td>
</tr>
<tr>
<td>miR-155</td>
<td>Increased in Breast Ca and lymphomas</td>
<td>(Iorio, Ferracin et al. 2005; Kluiver, Haralambieva et al. 2006; Tam and Dahlberg 2006)</td>
<td>Pro-Tumourigenic</td>
</tr>
<tr>
<td>miR-21</td>
<td>Anti-apoptotic + Upregulated in Glioblastomas and Breast Ca</td>
<td>(Chan, Krichevsky et al. 2005; Iorio, Ferracin et al. 2005; Si, Zhu et al. 2007)</td>
<td>Pro-Tumourigenic</td>
</tr>
</tbody>
</table>

1.7.6 MicroRNA and Hypoxia

A recent paper by Kulshreshtha et al. has assessed the effect of hypoxia on tumour cell lines, and analysed for differential effects, based on HIF induction (Kulshreshtha, Ferracin et al. 2007). They showed that there is a reliable hypoxic signature of nine microRNAs (termed hypoxia regulated microRNAs, HRMs), and that HIF induced this increased expression under hypoxia. (See Table 1.4)

Interestingly HIF-1 was shown to have a more dominant effect on miR-24 and miR-181, whilst HIF-2 had a greater effect on miR-26. Although the potential targets or functions of these microRNAs are unknown, it adds weight to the idea that HIF-1 and HIF-2 have differential functions.
### Hypoxia Regulated MicroRNA

<table>
<thead>
<tr>
<th>MicroRNA</th>
<th>HIF Isoform Induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>23</td>
<td>HIF-1</td>
</tr>
<tr>
<td>24</td>
<td>HIF-2</td>
</tr>
<tr>
<td>26</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td></td>
</tr>
<tr>
<td>103</td>
<td></td>
</tr>
<tr>
<td>107</td>
<td></td>
</tr>
<tr>
<td>181</td>
<td>HIF-1</td>
</tr>
<tr>
<td>210</td>
<td></td>
</tr>
<tr>
<td>213</td>
<td></td>
</tr>
</tbody>
</table>

Table 1.4 – Experimentally confirmed Hypoxia regulated MicroRNAs

### 1.7.7 MicroRNA and VHL

Very little is known about the relationship between microRNA expression and VHL, although it could be hypothesised that microRNA specific to VHL binds to the 3’ or 5’ ends of VHL mRNA blocking its translation. In addition the regulation of transcription factor (e.g. HIF) stability by VHL in normoxia and hypoxia could potentially result in differences in microRNA expression. (See Figure 1.9)

![Figure 1.9 - Hypothesised interaction of microRNA with VHL and hypoxia pathway](diagram.png)
1.8 Hypotheses
The differential regulation of HIF dependent target genes demonstrated in-vivo is a reflection of the biology within clinical ccRCC. Furthermore, high expression of the pro-tumourigenic HIF2 isoform within tumours is associated with poor prognosis, whilst high expression of HIF1 is associated with better prognosis. This differential expression of the HIF isoforms is regulated by microRNA.

1.9 Primary topics addressed in this thesis
Initial focus is given to the assessment and analysis of all nephrectomies performed at our institution, with the aim to understand the nature of changing incidence and clinico-pathological variables of renal tumours. The establishment of a clinical database has enabled this assessment and analysis, as well as allowing an appropriate cohort of tumours to be selected for further investigation.

Further chapters, using a series of patient derived primary tumours from the clinical database, investigate the observed differential effects of HIF-1 and HIF-2 on HIF target gene expression and tumour growth, in the VHL defective cell lines and the in-vivo mouse model, demonstrated by Raval et al. (Raval, Lau et al. 2005).

Assessment of expression of HIF isoforms attempts to establish what the relationship is between HIF-1 and HIF-2 and whether these correlate to clinico-pathological variables, including patient survival. Furthermore, this thesis assesses the utility of HIF isoforms and their target gene products, as prognostic markers in primary ccRCC.

Further work in this thesis explores the role of microRNA in a VHL defective tumour model, and examines whether specific microRNAs are
differentially regulated according to the functionality of the hypoxic pathway, and HIF isoform expression.

Therefore, the research in this thesis is primarily directed at determining the nature of the HIF pathway, its regulation and thus its role in renal tumours.
Chapter 2:
Materials and Methods
Chapter 2: Material and Methods

2.1 Clinico-Pathological Variables in RCC

2.1.1 Relational database

Clinical, pathology and outcome data on all urological malignancies within Oxford since 1983, available in electronic format, was amalgamated, structured and cross-referenced into a single relational database called CRUD© (Charlesworth Research Uro-Oncology Database), using Filemaker Pro v6 (Apple Inc, USA) (See Figure 2.1). Prospective data was initiated in October 2006 for all urological malignancies in Oxford with electronic links to the Pathology archives (Pathology Department, Oxford Radcliffe Hospitals NHS Trust), the Oxford Cancer Intelligence Unit (OCIU), and the Infoflex data system for collection of all urology patients discussed at the weekly Multi-Disciplinary Team Meeting (Chameleon Information Management Services Ltd, UK). This has organised and pooled a large quantity of retrospective data and has enabled extensive prospective data collection on all urological patients in Oxford, with minimal data entry.

Further electronic links are in development with the Oncology Department (Oxford Radcliffe Hospitals NHS Trust) databases for chemotherapy, radiotherapy, clinical trials, the Radiology department (Oxford Radcliffe Hospitals NHS Trust) databases for diagnostic imaging, as well as blood result indices from the Haematology and Biochemistry departments. (See Figure 2.2)

CRUD© has also developed into an expanding collaborative resource for all urological research in Oxford, and has associated modules that monitor all urological tissue, blood and urine specimen collection, storage and use. Furthermore there are additional modules in development that assist the Urological cancer surgeons in collecting British Association of Urological Surgeons (BAUS) Cancer Registry compatible data.
2.1.2 Retrospective Kidney Cancer Data Collection

All kidney cancer pathology samples have been logged within the archives of the Pathology Department (Oxford Radcliffe Hospitals NHS Trust) since 1983. The pathology reports were available in electronic format and were downloaded into the relational database CRUD©. The inclusion criteria for
the subsequent data analysis within this thesis were all nephrectomies (Radical, Partial or Laparoscopic) with documented cancer involvement, reported by the Pathology department from January 1983 to May 2007. Pathological data from these cases was assessed within the CRUD© system to extract pathological tissue diagnosis, tumour T-stage (reclassified according to 2002 criteria), Fuhrman grade, and maximum tumour diameter (cm).

Each tumour was related to available clinical data (age, sex and date of operation) and patient survival data from the Oxford Cancer Intelligence Unit (OCIU), within the CRUD© system. Survival data from the OCIU originates from death certificates, with death from Kidney Cancer being classified as being 1a. The death status of patients was classified by Alive / Dead, Date of death, Cause of Death (Kidney Cancer / Other).

2.1.3 Ethical Approval

Prior to amassing data retrospectively and establishing protocols for prospective data collection we ascertained proof of our ethical approval from the Central Office for Research Ethics Committees (main REC – Oxfordshire). We also had to fulfil the Caldicott regulations for data protection, anonymisation, proliferation and security when establishing our database relationships (Health; Health 1997).

2.1.4 Tissue Collection

In Oxford we have excellent procedures for the collection of fresh kidney and bladder cancer tissue as well as blood and urine samples for research purposes and have many hundreds of samples within the urology liquid nitrogen bio-banks. With the introduction of the Human Tissue Act we have had a responsibility to ensure that we meet these rigorous criteria.
2.1.5 Human Tissue Act 2004

In 2001 the Chief Medical Officer issued recommendations to the government regarding the removal, storage and use of human organs and tissue from post-mortem examination (Health 2004). Subsequently the government introduced legislation in November 2004 regulating these matters (Human Tissue Act 2004) (Health 2004). The Human Tissue Authority (a DOH ‘arm’s length body’) was also established to oversee and regulate the institutional collection and retention of human tissues, with each institution required to apply for an HTA licence by April 2006 (Authority 2006). Under the University of Oxford licence, collection of each sample has to have specific ethical approval and patient consent. The storage of the sample has to be in an approved bio-bank in an anonymous manner. The eventual use of samples can only be within the boundaries of the original ethical approval and all sample usage needs to be auditable retrospectively.

We have developed a system that fulfils all of these criteria with minimal data-entry at the point of tissue collection or utilisation. (See Figure 3.3)

Figure 2.3 – CRUD© Urology Tissue – Data Entry Page
2.1.6 Project Tracking

To protect the patients on whom we collect data and tissue we have established strict mechanisms to only allow access to the data/tissue to personnel that fulfill the criteria of their and our regional ethics committees. We have done this by only allowing access to the data/tissue to researchers/doctors with unique project tracking numbers. These are only issued upon receipt of a completed project tracking request form, which has to pass through the CRUD© Committee and satisfy the essential ethical and legal criteria. The additional benefit of this is that the use of all data and tissue is tracked in real-time and fully auditable retrospectively.

To maintain Oxford’s ethical and legal obligations in this regard we have developed a module adjoined to CRUD© that anonymises, tracks and protects all of our tissue collections and storage. (See Figure 2.4)

<table>
<thead>
<tr>
<th>Sample #:</th>
<th>Project Name</th>
<th>Amount</th>
<th>Gender</th>
<th>Sample Type</th>
<th>Date of Birth</th>
<th>Age</th>
<th>Date of Collection</th>
<th>Date of Death</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>Project 1</td>
<td>1</td>
<td>Male</td>
<td>Tissue</td>
<td>01/01/2020</td>
<td>30</td>
<td>20/03/2020</td>
<td>20/12/2020</td>
<td>Notes</td>
</tr>
<tr>
<td>P2</td>
<td>Project 2</td>
<td>2</td>
<td>Female</td>
<td>Tissue</td>
<td>02/02/2020</td>
<td>28</td>
<td>21/04/2020</td>
<td>21/12/2020</td>
<td>Notes</td>
</tr>
<tr>
<td>P3</td>
<td>Project 3</td>
<td>1</td>
<td>Male</td>
<td>Tissue</td>
<td>03/03/2020</td>
<td>31</td>
<td>22/05/2020</td>
<td>22/12/2020</td>
<td>Notes</td>
</tr>
</tbody>
</table>

Figure 2.4 – CRUD© Urology Tissue – Project Tracking page
2.1.7 Data Analysis
Statistical analysis and graphs were generated using Graphpad Prism v4.03. The log-rank test was used to perform a univariate analysis for survival and the survival curves were estimated by Kaplan-Meier method. P values were considered statistically significant when P<0.05.

2.2 Assessment of Angiogenesis and Hypoxia in CC-RCC prognosis
2.2.1 Introduction
Clear cell RCC specimens were stained using Immunohistochemistry (IHC) for HIF-1 and HIF-2 antibodies, as well as some of their target genes highlighted as having differential regulation by the Raval et al.. Tissue MicroArray technology was used to increase the throughput and number of samples analysed. Clinical and pathological data was collected from the CRUD© relational database and cross-analysed with the IHC data.

2.2.2 Selection Criteria
Selection criteria for inclusion into the tissue microarrays (TMAs) used in this study were all registered primary nephrectomies for clear cell renal carcinoma from 1983 to 1999, performed at the Churchill or John Radcliffe Hospitals, Oxford. The first TMA to be constructed, TA38, included all primary CC-RCC sourced from the pathology archives from 1983 to 1993, and included a total of 93 cases. The second TMA, TA71 used all primary CC-RCCs from 1994-1999 and included 77 cases. There were no records available for 1994 due to a lack of pathology data, and therefore no samples have been included into the TMAs from that year.

2.2.3 Ethical Approval
Use of all samples was under the guidelines laid out by the Human Tissue Authority (HTA) and with the approval of the Central Office of Research
Ethics Committee (COREC) covering the samples at the Churchill Hospital and John Radcliffe Hospital, Oxford, UK.

2.2.4 Data Storage
The aforementioned relational database ‘Charlesworth Research Uro-Oncology Database’ (CRUD©) was used to collect, organise and store the clinical and pathological data on the tumour samples contained within the TMAs.

2.2.5 Pathology Validation
2.2.5.1 Tumour Tissue Diagnosis
The archived formalin-fixed and paraffin-embedded blocks of tumour tissue were retrieved from the pathology stores and 5µm sections, from all cases, were cut on a microtome (Anglia Scientific, model 0325). These were reassessed by a Consultant Pathologist using Haematoxylin & Eosin (H&E) stained whole tumour sections at x100 magnification, to confirm the tissue diagnosis of clear cell renal cancer.

2.2.5.2 Fuhrman grade
Fuhrman grade was also re-assessed according to Fuhrman et al (Fuhrman, Lasky et al. 1982), with the grade assigned being the highest grade present in each tumour..

2.2.5.3 Tumour Stage
Using the original pathology reports, all cases were re-staged using the 2002 TNM classification (Cancer 2002). It is however worth noting that the renal sinus was commonly not assessed in 1983-1999 and therefore many stage 1 and 2 tumours could be under-staged according to modern criteria. Also, nodal and metastatic status was indeterminate for many of the samples due to the pathological data available; therefore it was not included in the T-
staging for analysis. Maximum tumour diameter (cm) was also obtained from the pathology report at the same time as re-staging.

2.2.6 Patient Survival

Patient outcome was assessed via the Oxford Cancer Intelligence Unit archives, which monitors cancer deaths by analysis of death certificate. Outcome data was classified as alive, deceased (non-cancer), deceased (other cancer – not kidney) or deceased (kidney cancer). The patients categorized as alive were cross-referenced against the NHS hospital appointments system to confirm date last seen.

2.2.7 Tissue Microarray Construction

Two tissue cores, each 1.5 mm in diameter were taken from morphologically representative regions of the paraffin blocks, excluding areas of necrosis or non-tumoural tissue. The tissue cores were then inserted into recipient paraffin blocks to construct the two TMAs (TA38 and TA71), using a tissue arrayer (Beecher Instruments). Tissue cores of non-neoplastic renal tissue from ten cases were also included in the microarray as controls. The construction of the TMAs was performed by Dr Leticia Campo, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, Oxford.

Figures 2.5a and 2.5b – Example of TMA block (left) and TMA 4micrometer slice stained with H&E.
2.2.8 Immunohistochemistry

The TMAs were sectioned into 4 micrometre slices, deparaffinised in citroclear and rehydrated in a series of gradient ethanol, then rinsed in distilled water. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxidase for 5 min.

Please refer to Table 2.1 for the antibody source, the antigen retrieval method and the dilutions used. The mouse and rabbit anti-human primary antibodies were probed with their respective secondary antibodies using the Chemate polymer Envision-HRP kit. For the goat anti-sheep secondary antibody (Abcam, Ely, UK) a dilution of 1:200 for 30 minutes at room temperature was used to label the LDH5 primary antibody and rabbit anti-goat (DAKO, Ely, UK) was used at a dilution of 1:200 for 30 minutes at room temperature for Oct-4.

The DAB signal was generated in all cases by mixing the chromagen substrate with the appropriate buffer provided for in the Chemate kit and applying the resultant solution onto the tissue sections for between 2-5 minutes dependant upon the epitope load within the tumour. All sections were subsequently counterstained with haematoxylin.

As will be discussed later in the results section of chapter 4, the antigenicity of HIF-1 and HIF-2 subjectively appeared to be decreased on the older tumour samples during scoring. All TMA slides used for HIF-1 or HIF-2 were cut freshly off the arrays and stained at the same time to minimise any other variable that would alter staining intensities.

All immunohistochemistry performed on the TMAs was performed by Dr Helen Turley and Dr Leticia Campo, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, Oxford.
Table 2.1 - Primary antibodies used on TA38 and TA71.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Antigen Retrieval</th>
<th>Antibody Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>HIF-1 alpha</em></td>
<td>Mouse monoclonal, Clone 54 BD Biosciences Pharmingen</td>
<td>1 mM EDTA pH 8.0</td>
<td>1:100 8 hours 4 degrees</td>
</tr>
<tr>
<td><em>HIF-2 alpha</em></td>
<td>Mouse monoclonal, EP190; Oxford University</td>
<td>1 mM EDTA pH 8.0</td>
<td>Neat application 8 hours 4 degrees</td>
</tr>
<tr>
<td><em>BNIP3</em></td>
<td>Mouse monoclonal, ANa40; Sigma, UK</td>
<td>Sodium Citrate pH 6.0</td>
<td>1:200 2 hours Room temperature.</td>
</tr>
<tr>
<td><em>CAIX</em></td>
<td>Mouse monoclonal, M75; Albert Einstein CM, New York, USA</td>
<td>Sodium Citrate pH 6.0</td>
<td>1:50 2 hours Room temperature.</td>
</tr>
<tr>
<td><em>CD31</em></td>
<td>Mouse monoclonal, JC70A; Oxford University.</td>
<td>Sodium Citrate pH 6.0</td>
<td>1:5 30 minutes Room temperature.</td>
</tr>
<tr>
<td><em>Cyclin D1</em></td>
<td>Mouse monoclonal, DCS-6; Dako, Ely, UK</td>
<td>Tris-EDTA pH 9.0</td>
<td>1:50 1 hour Room temperature.</td>
</tr>
<tr>
<td><em>GLUT-1</em></td>
<td>Rabbit polyclonal, DAKO, UK</td>
<td>Sodium Citrate pH 6.0</td>
<td>1:200 1 hour Room temperature.</td>
</tr>
<tr>
<td><em>LDH5</em></td>
<td>Sheep polyclonal; Abcam, UK</td>
<td>No antigen retrieval</td>
<td>1:400 2 hours Room temperature.</td>
</tr>
<tr>
<td><em>Oct-4</em></td>
<td>Goat polyclonal (C-20); Santa-Cruz, USA</td>
<td>Sodium Citrate pH 6.0</td>
<td>1:200 2 hours Room temperature.</td>
</tr>
<tr>
<td><em>VEGF</em></td>
<td>Rabbit monoclonal, SP28; Abcam, UK</td>
<td>Sodium Citrate pH 6.0</td>
<td>Pre-diluted 10 minutes Room temperature.</td>
</tr>
</tbody>
</table>

2.2.9 TMA Scoring

The TMAs were reviewed by two people and a consensus determined. A score was obtained for each stain using all optical fields, in the relevant cell compartment (see Table 3.2). Staining was scored according to the intensity and percentage of tumour cells stained. The intensity was scored 0 (no staining); 1 (mild); 2 (moderate) and 3 (strong staining). Percentage staining
was scored as 0 (no staining); 1 (1-10%); 2 (11-50%); 3 (51-90%) and 4 (91-100%). An expression ‘Histoscore’ was then calculated for analysis using the product of the two scores, giving a resultant score of 0-12.

2.2.10 Angiogenesis Quantification

To quantify angiogenesis we used the Vessel Visualisation Index (VVI) at x200 magnification, as it is known to positively correlate with Microvessel Density (MVD) and Chalkley count and has recently been shown to correlate positively between TMA staining and whole tumour section staining for CD31 (Yao, Qian et al. 2007). VVI was determined by looking at each core as a whole (not vascular hotspots) and angiogenesis was scored as ‘high’, ‘moderate’ and ‘low’. Subsequently, for statistical purposes, this was further delineated into either of two scores: 1= high vessel tumours and 0 = moderate or low vessel tumours.

<table>
<thead>
<tr>
<th>Stain</th>
<th>Cell Compartment Scoring (used in analysis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-1 alpha</td>
<td>Nucleus</td>
</tr>
<tr>
<td>HIF-2 alpha</td>
<td>Nucleus</td>
</tr>
<tr>
<td>BNIP3</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>CAIX</td>
<td>Membrane</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>Nucleus</td>
</tr>
<tr>
<td>GLUT-1</td>
<td>Membrane</td>
</tr>
<tr>
<td>LDH5</td>
<td>Nucleus</td>
</tr>
<tr>
<td>Oct-4</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>VEGF</td>
<td>Cytoplasm</td>
</tr>
</tbody>
</table>

Table 2.2 - Cell compartment scored for each stain

2.2.11 Statistical Analyses

Statistical analysis and graphs were generated using Graphpad Prism v4.03 and the Stata package release 8.1 (Stata Corporation, Texas, USA).

For non-continuous analysis high / low cut-offs were determined by calculating the median expression histoscore (See Table 4.2, Chapter 4). Contingency tables were analysed using the Pearson's chi-squared test. Correlation between continuous variables was assessed by Spearman Rank
correlation. The log-rank test was used to perform a univariate analysis for survival and the survival curves were estimated by Kaplan-Meier method. Prognostic factors for survival were evaluated in multivariate analyses by Cox proportional hazards regression. P values were considered statistically significant when P<0.05.

2.3 MicroRNA in Renal Clear Cell Carcinoma and its relationship to the Von-Hippel Lindau tumour-suppressor gene and hypoxia pathway

2.3.1 Cell Lines
A panel of clear cell renal cancer cells lines were chosen for the in-vitro section of this work. All cell lines were ordered via Cancer Research UK Central Cell Services, London, UK. 768-O and RCC4 cell lines were selected for consistency with the previous in-vitro and mouse model work as discussed in the introduction to this thesis (Raval, Lau et al. 2005). A further cell line, UMRC2 (a gift from EA Maher) was also used and was available from CRUK central cell services. All cell lines originate from a clear cell RCCs with an inherent non-functional VHL gene, and were available with a paired identical cell line with stably transfected functional wild-type VHL gene. Therefore six cell lines were used for my experiments, 3 x identical pairs with only their VHL status as a variable.

Stable VHL transfection and HIF-1 / HIF-2 expression has been validated for these cell lines 768-0 (Maxwell, Wiesener et al. 1999; Raval, Lau et al. 2005; Yuen, Cockman et al. 2007), RCC4 (Maxwell, Wiesener et al. 1999; Raval, Lau et al. 2005; Yuen, Cockman et al. 2007) and UMRC2 (Maxwell, Wiesener et al. 1999; Jung, Isaacs et al. 2003). Furthermore UMRC2 and RCC4 cells have been shown to express both HIF-1alpha and HIF-2alpha, while 768-0 cells only express HIF-2alpha (Maxwell, Wiesener et al. 1999).
HIF subunits were constitutively expressed in normoxia (21% Oxygen) as well as hypoxia (0.1% Oxygen) in the VHL mutant cells (UMRC2/VHL-ve, RCC4/VHL-ve and 768-0/VHL-ve), but were only detectable in hypoxia in the VHL functional cells (UMRC2/VHL-ve, RCC4/VHL+ve and 768-0/VHL+ve) (Maxwell, Wiesener et al. 1999; Raval, Lau et al. 2005; Yuen, Cockman et al. 2007).

2.3.2 Cell Culture

All the cell lines were maintained using Dulbecco’s Modified Eagle’s Medium (DMEM) in polystyrene dishes (Becton Dickinson, UK) and flasks with vented caps (Falcon, UK), supplemented with 10% heat-inactivated fetal calf serum (FCS) (Globepharm, UK), 2mM L-glutamine, 50 IU/ml penicillin and 50 µg/ml streptomycin. The 768-O, RCC4 and UMRC2 cells were stably transfected with VHL, as previously described (Clifford, Cockman et al. 2001; Zatyka, da Silva et al. 2002; Craven, Hanrahan et al. 2006), and maintained with 1mg/ml of G418 (GIBCO-BRL).

Cells were cultured in a humidified atmosphere of 5% CO2 at 37 degrees Celsius. They were passaged by washing in phosphate buffered saline (PBS – 150mM NaCl and 150mM NaH2PO4), detached with trypsin-EDTA solution (4mg/ml trypsin, 0.2mM EDTA in PBS) (GIBCO-BRL), and replated to the required density (dependent upon experiment).

All cells were routinely screened for mycoplasma infection by CRUK central cell services, as per laboratory protocol. For cryopreservation, cultured cells were harvested, spun down (GS-6R Centrifuge, Beckman Instruments, UK), re-suspended in 20% dimethyl sulfoxide (DMSO) 80% FCS, and aliquoted into cryovials. These aliquots were then slowly cooled to minus 80 overnight, prior to placement in liquid nitrogen for storage. For revival, cells were quickly thawed with pre-warmed medium, and re-plated.
For experimental purposes, when cells reached 80% confluence within the Falcon flasks, they were passaged, diluted by 1 in 10 with medium and plated onto 10cm polystyrene tissue culture dishes (Falcon, Becton Dickinson, UK). Cells were then allowed to rest in normoxia for 8 hours prior to incubation in normoxia (21% Oxygen) or in hypoxia (0.1% Oxygen) in a Galaxy R CO2 incubator (RS Biotech, UK) for 16 hours.

2.3.3  RNA Extraction

For RNA extraction, cells were removed from the incubator after 16 hours and cell culture medium removed. Cells were lysed directly in the culture dish by addition of 1ml of TRI reagent (Sigma-Aldrich, UK) and passed several times through a pipette. After five minutes, to allow complete dissociation of the nucleoprotein complexes, 0.2ml of chloroform was added, and the sample was vortexed (Labinc Vortex, Netherlands) for 15 seconds. After storage for 15 minutes at room temperature, the sample was centrifuged at 12 000g for 15 minutes at 4 degrees Celsius (Mikro 22R, Hettrich Zentrifugen, Netherlands). The RNA containing colourless upper aqueous phase was then removed, placed in a fresh tube and the RNA precipitated using 0.5ml of isopropanol. After a further centrifugation at 12000g for 8 minutes (4 degrees Celsius), the liquid isopropanol was removed and the precipitated RNA pellet briefly allowed to air-dry. It was then resuspended in 0.2ml RNAse free-water (Qiagen, UK), and quantified using a ND-100 Spectrophotometer (NanoDrop Technologies, USA) at extinction co-efficient of 40, and a 2100 Bioanalyser (Agilent, UK). RNA samples were subsequently stored at minus 80 degree Celsius until further use.

2.3.4  MicroRNA isolation

MicroRNA was isolated from the RNA samples using the RNeasy Mini Kit (Qiagen, UK). A starting concentration of 45 micrograms of RNA was used (optimum starting concentration as per Qiagen protocol).
The RNA was initially placed onto RNAeasy – mini spin column (Qiagen, UK) with 80% ethanol, and centrifuged at 8000g for 15 seconds. The concentration of ethanol used precipitated out large RNA which subsequently collected on the glass-fibre filter of the column, but was insufficient to precipitate out small RNA (including microRNA), which passed through the filter and was collected in a separate container. The total RNA excluding microRNA remaining on the column filter was then subjected to a series of washes, using RW1 and RPE buffers (supplied in RNeasy Mini Kit, Qiagen, UK). 30 microlitres of pre-heated RNAase free water was subsequently passed through the column to re-dissolve the precipitated RNA (excluding the microRNA).

980µl of 100% ethanol was added to the flow-through (containing the microRNA) from the first RNeasy mini spin column. This ethanol concentration was sufficient for microRNA precipitation and succeeding addition to the RNAeasy – mini elute column (Qiagen, UK), resulted in the trapping of the precipitated microRNA onto the finer glass fibre filters of this column. Washes of the precipitated microRNA were performed using 0.5ml RPE buffer and 80% ethanol, each centrifuged through the column at 8000g for 15 seconds.

2.3.5 MicroRNA MicroArrays

2.3.5.1 Introduction

The microRNA microarrays (µRNA™ Microarray, University of Oxford) used were made available on a collaborative basis with Dr C Lawrie, Nuffield Department of Clinical Laboratory Sciences, University of Oxford. Each chip contained microRNA sense probes for all known human microRNAs, as well as control ‘spike-in’ sequences, for scanning and image analysis normalisations.
2.3.5.2 Hybridisation

The microarrays and lifterslips (M-series 25x42, VWR, UK) were prepared prior to hybridisation via cleaning with 100% ethanol; rapidly dried using high pressure nitrogen; and incubated at 65 degrees Celsius for 20 minutes in pre-hybridisation solution (35mls 20x SSC (3M NaCl, 0.3M Sodium Citrate) (Ambion, UK); 20mls Bovine Serum Albumin solution (BSA 100mg/ml) (Sigma-Aldrich, UK); 2mls 10% SDS (Sodium Dodecyl Sulfate, Ambion, UK)).

Two micrograms of purified microRNA was used from each one of the original paired cell line samples, and diluted into a volume of 13.5µl with RNase-free water (Qiagen, UK). The MicroRNA samples were used as a template to produce complimentary microRNA by reverse transcription (RT), using Superscript II kit (Invitrogen, UK). A mastermix of the superscript components including spike-in controls (Invitrogen,UK) were added prior to labelling each pair of the cell line with 2µl Cy3 or Cy5 RT primers (MicroRNA labelling kit, Genesphere, UK), whereupon the samples were incubated for 10 minutes at 65°C. The paired samples, Cy3 or Cy5 respectively, were mixed together and added to 25µl of hybridisation buffer (MicroRNA labelling kit, Genesphere, UK), and loaded onto the prepared microRNA microarrays contained within sterilised hybridisation chambers (Cambio, UK). The chambers were then incubated at 52°C for 16 hours, to allow the tagged complimentary microRNA sequences to hybridise to the sense probes on the microarray.

Following hybridisation, the microarrays were removed from the chambers and washed in filter-sterilised 2 x SSC, 0.2% SDS (Ambion, UK) with shaking for 15 minutes at 42°C. Further washes were then performed with 2 x SCC and 0.2 x SSC, with shaking for 15 minutes each, before the slides were rapidly dried using nitrogen as before.
A mastermix containing 2.5µl of Cy3 and Cy5 3DNA capture reagents (MicroRNA labelling kit, Genesphere, UK) with hybridisation buffer was then prepared for each chip, and loaded carefully onto each using fresh lifterslips. This was performed in limited light conditions due to the light sensitivity of the capture reagents. The microarrays were then incubated for a further 4 hours at 62°C, followed by washing and drying as previously described.

2.3.5.3 MicroArray Scanning
The microarrays were subsequently scanned using a ScanArray SA4000 (GSI, Lumonics, Packard / Perkin Elmer, USA). Focusing and PMT gain setting were automatically adjusted via the preset optimised protocol. Resultant Cy3 and Cy5 images were saved and transferred to the image analysis software as high resolution ‘.tiff’ files.

2.3.5.4 Quality control
The threshold of expression intensity varied for each cell line, due to cell line variability in microRNA expression as well as experimental efficiency of microRNA binding to the array. Therefore subjective assessment of array quality was made after scanning, with only high quality arrays being included in the analysis. (See Figure 2.6). Microarrays were repeated as necessary to complete three biological replicates per cell line comparison.
Figure 2.6 – Cell line RCC4 (VHL+ve Cy3: VHL-ve Cy5). Typical example of MicroRNA Expression MicroArray quality.

2.3.5.5 Image Analysis

Image analysis was performed using the software package, BlueFuse for MicroArrays version 3.3 (BlueGnome, UK).

The individual Cy3 and Cy5 images were imported into the software, as well as the microarray ‘.gal’ file, containing an overlay grid for the location of all microRNAs on the array. The .gal file overlay was performed automatically by the software but was also manually checked prior to image quantification. A protocol was written in the software to exclude low intensity and background hybridisation, although all arrays were manually
checked to confirm correct inclusion / exclusion criteria. (See Figure 2.7). Figures 2.8 and 2.9 demonstrate individual Cy3 and Cy5 images with composite image (combined Cy3 and Cy5). Cy3 and Cy5 pixel intensities within each hybridised spot were quantified by the BlueFuse software.

Subsequent post-processing (also performed by the BlueFuse software) resulted in image global lowess normalisation based on controls and hybridised microRNAs. Weighted medians were automatically generated for the microRNA quadruplicates and fused to produce a single Excel spreadsheet (Windows Microsoft Office 2003) of all expressed microRNAs on the array.

2.3.5.6 Data Analysis
Further data manipulation and analysis was performed using GeneSpring v7 (Agilent, USA), GraphPad Prism v4.03 and Filemaker Pro v7 (Apple Inc, USA).

Figure 2.7 - MicroRNA Expression MicroArray with overlying .gal file in BlueFuse software (BlueGnome, UK), used to identify each expressed ‘spot’ of microRNA.
Figure 2.8 - Demonstration of individual pixel intensity analysis of Cy3 and Cy5 together with Cy5/Cy3 composite.

Figure 2.9 - Three examples of variable Cy3 / Cy5 hybridisation: Image 1 (left) shows high Cy3 intensity giving an overall green composite image; Image 2 (centre) shows high Cy5 intensity giving an overall red composite image; Image 3 (right) shows saturated Cy3 and Cy5 from a ‘spike-in’ control.
2.3.6  Polymerase Chain Reaction (PCR)

2.3.6.1  Introduction
Quantitative analysis of mature microRNA was performed using two-step real-time polymerase chain reaction (RT-PCR) (Taqman MicroRNA Assays, Applied Biosystems).

2.3.6.2  Reverse Transcription
In the reverse transcription (RT) step, cDNA was reverse transcribed from the RNA samples using specific microRNA looped RT-primers (miR-23a RT-primer and RNU6B RT-primer, Taqman MicroRNA Assays, Applied Biosystems) and the reagents from the Taqman MicroRNA Reverse Transcription kit, as described in the manufacturer’s instruction protocol.

2.3.6.3  PCR
In the PCR step, PCR products were amplified from the cDNA samples (from the RT step) using the Taqman MicroRNA Assay together with the Taqman Universal PCR Master Mix, as described in the manufacturer’s instruction protocol. This was performed using a LightCycler 480 Real-Time PCR system (Roche Applied Science). The resultant data was transferred to Excel (Windows Microsoft Office 2003) and GraphPad Prism v4.03 for analysis.
Chapter 3:
Renal Cancer Prognosis and Clinico-Pathological Variables
CHAPTER 3: Renal Cancer Prognosis and Clinico-Pathological Variables

3.1 Introduction
Kidney Cancer is a common condition in the UK accounting for 2% of all cancers. Treatment has changed very little over the past few decades for primary locally confined disease, however patient survival has improved significantly over this time. The mechanisms of this observed change are yet to be fully understood although tumour size at diagnosis, surgical caseload or a change in disease distribution may be important.

3.2 Results
3.2.1 Clinico-Pathological and Survival Analysis of all cancer nephrectomies in Oxford, January 1983 to May 2007
A total of 750 cancer nephrectomies were performed at the John Radcliffe or Churchill Hospitals, from January 1983 to May 2007. 664 (89%) were for Renal Cell Carcinoma (RCC) (See Table 3.1 and 3.2), with the remaining 11% mainly Transitional cell Carcinoma (TCC). The 1, 5 and 10-year survival of patients post-nephrectomy for RCC was 89%, 73% and 63% respectively (See Figure 3.2 and Table 3.4).
Table 3.1 – Age, Sex and Survival Distribution of all cancer nephrectomies (all tumour types) in Oxford, January 1983 to May 2007.

<table>
<thead>
<tr>
<th>All Kidney Tumour Types</th>
<th>Cancer Nephrectomies in Oxford</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
</tr>
<tr>
<td><strong>Year of Nephrectomy</strong></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>1983-2007</td>
</tr>
<tr>
<td><strong>Total No of Cases</strong></td>
<td>750</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>490</td>
</tr>
<tr>
<td>Female</td>
<td>260</td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>64</td>
</tr>
<tr>
<td>Range</td>
<td>10-91</td>
</tr>
<tr>
<td><strong>End State</strong></td>
<td></td>
</tr>
<tr>
<td>Alive</td>
<td>437</td>
</tr>
<tr>
<td>Death from Kidney Cancer</td>
<td>191</td>
</tr>
<tr>
<td>Death from other cause</td>
<td>122</td>
</tr>
</tbody>
</table>

Table 3.2 – Distribution of Pathology Tissue Diagnoses of all cancer nephrectomies for RCC in Oxford, January 1983 to May 2007.
**Figure 3.1** – Kaplan Meier Survival Curve (Death from Kidney Cancer) of all cancer nephrectomies (all tumour types) in Oxford Jan 1983 to May 2007.

**Figure 3.2** - Kaplan Meier Survival Curve (Death from Kidney Cancer) of all cancer nephrectomies for RCC in Oxford January 1983 to May 2007.
<table>
<thead>
<tr>
<th>All RCCs</th>
<th>Cancer Nephrectomies in Oxford</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Year of Nephrectomy</th>
<th>1983-2007</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total No of Cases</td>
<td>664</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>429</td>
</tr>
<tr>
<td>Female</td>
<td>235</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>64</td>
</tr>
<tr>
<td>Range</td>
<td>18-87</td>
</tr>
<tr>
<td>Tumour T Stage</td>
<td></td>
</tr>
<tr>
<td>1a</td>
<td>116</td>
</tr>
<tr>
<td>1b</td>
<td>115</td>
</tr>
<tr>
<td>2</td>
<td>84</td>
</tr>
<tr>
<td>3a</td>
<td>103</td>
</tr>
<tr>
<td>3b</td>
<td>233</td>
</tr>
<tr>
<td>3c</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Fuhrman Grade</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>89</td>
</tr>
<tr>
<td>2</td>
<td>297</td>
</tr>
<tr>
<td>3</td>
<td>179</td>
</tr>
<tr>
<td>4</td>
<td>57</td>
</tr>
<tr>
<td>Max Tumour Diameter (cm)</td>
<td></td>
</tr>
<tr>
<td>&lt;5</td>
<td>222</td>
</tr>
<tr>
<td>5-7</td>
<td>128</td>
</tr>
<tr>
<td>7-10</td>
<td>168</td>
</tr>
<tr>
<td>&gt;10</td>
<td>121</td>
</tr>
<tr>
<td>End State</td>
<td></td>
</tr>
<tr>
<td>Alive</td>
<td>397</td>
</tr>
<tr>
<td>Death from Kidney Cancer</td>
<td>167</td>
</tr>
<tr>
<td>Death from other cause</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 3.3 – Clinico-Pathological Variables of all cancer nephrectomies for RCC in Oxford, January 1983 to May 2007.
Table 3.4 – 1, 5 and 10-year survival patients undergoing nephrectomy for all tumour types of kidney cancer and just RCC, in Oxford January 1983 to May 2007.

3.2.2 Renal Cancer Survival – Trends over Time

To assess how prognosis from kidney cancer has changed over time, all RCC nephrectomies were divided into six cohorts of four inclusive years, based upon their date of operation. Figure 3.3 below shows a clear change in kidney cancer specific survival over the past 24 years (logrank test $p<0.0001$), with 1-year survival improving from 74% (1983-1986) to 95% (2003-2007), and 10-year survival changing from 17% (1983-1986) to 70% (1995-1998) (See Table 3.5). Further analyses later in this chapter focus on the nephrectomies from 2002 to 2007 as survival during this period is relatively stable.
Figure 3.3 - Kaplan Meier Survival Curve (Death from Kidney Cancer) of all cancer nephrectomies for RCC in Oxford January 1983 to May 2007, split into 4-year cohorts.

Table 3.5 - 1, 5 and 10-year survival patients undergoing nephrectomy for RCC, in Oxford January 1983 to May 2007, split in 4-year cohorts.
3.2.3 Cancer Nephrectomy Caseload

The number of RCC nephrectomies performed each year has increased by a factor of 10 over the past 24 years, and has a significant association with 4-year survival from kidney cancer (Chi-squared p<0.0001).

**Figure 3.4** – Number of Nephrectomies for RCC in Oxford per year, January 1983 to May 2007.

**Figure 3.5** - Number of Nephrectomies for RCC in Oxford per 4 years vs. 4 year Survival, January 1983 to May 2007. (Bars on left represent number of Nephrectomies per year / Bars on right represent % 4-year survival)
3.2.4 Operative Mortality

For all cancer nephrectomies, operative mortality as measured by death within 30 days of operation is 2.3%, which has not changed significantly over the past 24 years.

Figure 3.6 – Percentage 30-day mortality following Nephrectomy for RCC in Oxford per 4-year cohort, January 1983 to May 2007.

Table 3.6 – Number and percentage 30-day mortality following Nephrectomy for RCC in Oxford per 4-year cohort, January 1983 to May 2007.
3.2.5 Age at Operation

The mean age at operation for all cancer nephrectomies has not changed significantly over the past 24 years. (See Figure 3.7)

![Variation of Age at Nephrectomy over Time](image)

**Figure 3.7** – Mean age at Nephrectomy for RCC in Oxford per 4-year cohort, January 1983 to May 2007.

3.2.6 Fuhrman Grade

Nuclear Fuhrman grade shows clear univariate survival significance (logrank test p<0.0001), with Fuhrman grade 4 tumours having the shortest survival (See Figure 3.8). Survival per Fuhrman grade category is not significantly altered when the analysis is focused upon the most recent nephrectomies (2002-2007) compared to the earlier nephrectomies (1983-2001) (See Figure 3.9 a & b). Fuhrman grade 3 and 4 tumours continue to have similar survival with 5-year survival approximately 20%, and 65% respectively. In comparison, Fuhrman grade 1 and 2 tumours have significantly improved survival with 5-year survival rates improving from approximately 85% to 95% and 100% respectively (See Figure 3.9b). The distribution of Fuhrman grade per year has not changed significantly over this time (See Figure 3.10).
Figure 3.8 - Kaplan Meier Survival Curve (Death from Kidney Cancer) of all cancer nephrectomies for RCC in Oxford January 1983 to May 2007, split into 4 Fuhrman Grade categories.

Figure 3.9a - Kaplan Meier Survival Curve (Death from Kidney Cancer) of all cancer nephrectomies for RCC in Oxford January 1983 to 2001, split into 4 Fuhrman Grade categories.
Figure 3.9b - Kaplan Meier Survival Curve (Death from Kidney Cancer) of all cancer nephrectomies for RCC in Oxford January 2002 to May 2007, split into 4 Fuhrman Grade categories.

Figure 3.10 – Percentage Distribution of Fuhrman Grade of RCC Nephrectomy specimen, January 1983 to May 2007.
3.2.7 Tumour T-stage

Tumour T-stage is an important univariate prognostic factor (logrank test \( p<0.0001 \)). Analysis of the prognostic significance of stage after 2002, demonstrates consistent 5-year survival for T3 and T4 tumours, but an improved survival in T2 tumours from 85% to 97%, and an improved survival in T1 tumours from 93% to 100% (See Figure 3.12 a & b). The distribution of T-stage has not changed over time (See Figure 3.13).

![Kaplan Meier Survival Curve](image)

**Figure 3.11** - Kaplan Meier Survival Curve (Death from Kidney Cancer) of all cancer nephrectomies for RCC in Oxford, January 1983 to May 2007, split into 4 Tumour Stage categories.
CHAPTER 3

Figure 3.12a - Kaplan Meier Survival Curve (Death from Kidney Cancer) of all cancer nephrectomies for RCC in Oxford, 1983 to 2001, split into 4 Tumour Stage categories.

Figure 3.12b - Kaplan Meier Survival Curve (Death from Kidney Cancer) of all cancer nephrectomies for RCC in Oxford, January 2002 to May 2007, split into 4 Tumour Stage categories.
Figure 3.13 – Percentage Distribution of Tumour Stage of RCC Nephrectomy specimen, January 1983 to May 2007.

3.2.8 GS Product Score

A simple continuous variable of the product of Fuhrman grade and T-stage for RCCs was generated for prognostic significance, and termed GS product score. This was subsequently divided into four cohorts to aid interpretation. This demonstrated increased prognostic significance compared to Fuhrman grade or T-stage alone. Patients with a GS product score of 4 or less showed a 5-year survival of 100%, whilst increased score showed progressively poorer prognosis (See Figure 3.14 and Table 3.7).
Figure 3.14 - Kaplan Meier Survival Curve (Death from Kidney Cancer) of all cancer nephrectomies for RCC in Oxford, January 2002 to May 2007, split into 4 cohorts of Grade and Stage Product.

<table>
<thead>
<tr>
<th>GS Product Score</th>
<th>1 year survival</th>
<th>5 year survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;4</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>6</td>
<td>95%</td>
<td>86%</td>
</tr>
<tr>
<td>8-9</td>
<td>93%</td>
<td>54%</td>
</tr>
<tr>
<td>&gt;12</td>
<td>62%</td>
<td>20%</td>
</tr>
</tbody>
</table>

Table 3.7 - 1 and 5-year survival of patients undergoing nephrectomy for RCC, in Oxford, January 2002 to May 2007, split in 4 cohorts of Grade and Stage Product.
3.2.9 Tumour Size - Trend over time

Mean tumour size has not changed over the past 24 years (See Figure 3.15). Further analysis of tumour size was performed by assessing the percentage of cases per centimetre. Figures 3.16 and 3.17 show the distribution of tumour sizes in 2 cohorts (1983-1998 and 1999-2007) with a subtle shift to smaller tumours in later years. To investigate this further the size of tumour was sub-categorised into three similarly sized cohorts (A: <6cm, B: 6-8cm and C: >8cm) tumours. Figure 4.18 shows a 5 fold increase in the number of size A tumours, with a corresponding decrease in size B tumours. Of note is that there is no change in size C tumours over the past 24 years. This sub-categorisation of maximum tumour diameter is a significant univariate prognostic marker (logrank test p<0.0001), with size C tumours having the poorest survival (See Figure 3.19).

<table>
<thead>
<tr>
<th>GS Product Score</th>
<th>Stage x Grade</th>
<th>Number of Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;4</td>
<td>1x1</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>1x2</td>
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</tr>
<tr>
<td>&gt;12</td>
<td>3x4</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>4x3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>4x4</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 3.8 - Distribution of patients to each 4 cohorts of Grade and Stage Product.
CHAPTER 3

Nephrectomies: 1983-1998 (n=237)

Tumour Size

Percentage of Nephrectomies

Figure 3.15 – Mean Maximum Tumour Diameter vs. Year of Nephrectomy, of all cancer nephrectomies for RCC in Oxford, January 1983 to May 2007.

Variation of Tumour Size at Nephrectomy over time

Nephrectomies: 1999-2007 (n=405)

Tumour Size

Figure 3.16 – Percentage distribution of Maximum Tumour Diameter, of all cancer nephrectomies for RCC in Oxford, January 1983 to December 1998.

Figure 3.17 – Percentage distribution of Maximum Tumour Diameter, of all cancer nephrectomies for RCC in Oxford, January 1999 to May 2007.
Figure 3.18 – Percentage distribution of Maximum Tumour Diameter per 4-year cohort according to size sub-category (A-C), of all cancer nephrectomies for RCC in Oxford, January 1999 to May 2007.

Figure 3.19 - Kaplan Meier Survival Curve (Death from Kidney Cancer) of all cancer nephrectomies for RCC in Oxford, January 2002 to May 2007, split into 3 cohorts of Size sub-category (A-C).
3.3 Discussion

3.3.1 Survival
Overall patient survival in Oxford compares well to national data, with 5-year survival rates nationally being 42% (Office for National Statistics, 2007) whilst in Oxford they are 73%. This was certainly not the case 20 years ago when the 5-year survival in Oxford was only 43%. The trend towards improved survival over the years has been progressive, with gradually increasing survival seen for every four year cohort analysed. The cause of this significant change is likely to be multifactorial, and the breakdown of caseload, Fuhrman grade, T-stage, tumour size and operative mortality goes some way to explain this phenomenon.

3.3.2 Incidence
The number of nephrectomies performed has risen by a factor of 10, which correlates to the improving survival. It is established that high throughput cancer centres with experienced Urological surgeons performing large numbers of operations is beneficial to patients (National Institute for Clinical Excellence 2002) and therefore it is one of the many changes in provision of cancer services within the UK in recent years. It is unclear whether this alone can result in the dramatic increase demonstrated, although it is likely to be a contributing factor.

3.3.3 Operative Mortality
The stable rate in operative mortality over the past 24 years partially negates the idea that the rise in cancer nephrectomy caseload is the sole reason for the changes in survival. It would be expected that if the change was only due to improved operative experience, then a similar trend would be seen in death within 30 days of operation. However, the concentration of cancer operations within cancer centres in the UK and the development of co-ordinated multi-disciplinary teams for the selection and management of operative cases may well have an influence on outcome.
3.3.4 Age at Operation

It could be hypothesised that in an aging population, that the age of patients at the time of their nephrectomy has changed over the past 24 years. This was not supported by the analysis which demonstrated that there has been no change over this time. This suggests that age is a less important risk factor in RCC that previously thought.

3.3.5 Fuhrman Grade

The observation that Fuhrman grade is a prognostic marker in RCC is not surprising, and is well documented in the literature. However, the improved survival in grade 1 and 2 tumours in the past 20 years, with unchanged grade 3 and 4, suggests that the nature of disease may have changed, and the increased incidence is generating lower grade tumours that hence have improved survival. Alternatively it may be that the increased specialisation of reporting pathologists has resulted in a change in distribution of grade.

3.3.6 T-stage

It is also unsurprising that tumour T-stage is a prognostic marker in RCC, however, the same shift towards improved survival for low stage tumours in recent years, is demonstrated in T-stage as it was in tumour grade. The compounding factor with T-stage is the change in the TNM classification in 2002. The inclusion of the renal sinus into pathological assessment has meant that many T2 and some T1 tumours prior to 2002 would now be classified as T3. This goes some way to explain the demonstrated improved prognosis for T1 and T2 tumours. It is possible that the previously assumed non-invasive tumours (that were in actual fact invading the renal sinus), have been shifted into the T3 category, where the decreased survival of this cohort represents them better.
3.3.7 GS Product Score
The combination of grade and stage to produce a single prognostic factor is not unique. The SSIGN prognostic algorithm developed by the Mayo clinic has much greater prognostic power, based on grade, stage, necrosis and patient performance status (Frank, Blute et al. 2002). However, the simple product of grade and stage may prove to be of value clinically, particularly with regards follow up post nephrectomy. Many clinicians would discharge a low grade and low stage tumours early, however if a patient had a T2 tumour with Fuhrman grade 3, early discharge may not be warranted as their 5-year survival is only 85%. Furthermore, added confidence is gained in the knowledge that even in the presence of vascular invasion (T3), if Fuhrman grade was 1 then early discharge and patient reassurance may be possible.

Additionally the cohort of patients with GS Product Score of >12, do dramatically worse than the other cohorts. This may highlight the category of patients to include in future research trials, as they are the patients most likely to progress.

3.3.8 Size
With the increased quality and quantity of diagnostic imaging available, a greater number of incidental (pre-symptomatic) tumours are being detected (Chow, Devesa et al. 1999). The true significance of these tumours is currently a hotly debated subject in Urology. It is difficult to know what the true clinical course of these tumours would be if left undetected, and whether the removal of all of them is warranted.

It is also not clear cut whether the increased detection of these renal tumours is solely due to improved diagnostic imaging or whether there is a natural pattern of increasing incidence of RCC from another source. The increase in detection rates has also highlighted locally advanced and metastatic renal tumours, which does suggest that the increased detection of localised
tumours is not the sole reason for the observed improvement in patient survival.

My data shows that there has been no overall decrease in the mean size of tumour at time of nephrectomy. However, on further evaluation of tumour size by sub-division into three size cohorts (A: <6cm, B: 6-8cm and C: >8cm) it is clear that there has been a significant increase in the number of tumours <6cm and a corresponding decrease in tumours 6-8cm. This may relate to the trend of progressively improving survival, as the 5-year survival for size A tumours is 95% whilst for size B tumours it is 85%. However, this change would only seem to account for a 10% increase in 5-year survival, and does not completely explain the changes documented.

The consistent proportion of size C tumours undergoing nephrectomy is interesting and seems to go against the changes seen in tumours ≤8cm. A possible explanation may be a change in referral patterns coinciding with a shift towards earlier detection of renal masses. Within Oxford there has been a subjective increase in the number of difficult cases involving large tumours referred from other centres, although I have no data to analyse this objectively. An alternative possibility is that the cohort of large tumours represents an alternative tumour biology that grows rapidly, avoiding early detection and curative nephrectomy. Later in this thesis I explore this possibility further with analysis of the relationship of tumour size with markers of angiogenesis and the hypoxic pathway, including the HIF isoforms.
Chapter 4: Angiogenesis and Hypoxia in Primary Clear Cell Renal Carcinoma and its effect on patient prognosis
CHAPTER 4: Angiogenesis and Hypoxia in Primary Clear Cell Renal Carcinoma and its effect on patient prognosis

4.1 Introduction

A recent study on human RCC (VHL-defective) cell lines demonstrated that despite close similarities between the HIF isoforms, differential activation of HIF-1 or HIF-2 pathways has non-equivalent or even opposing effects on gene expression and experimental (mouse model) tumour growth (Raval, Lau et al. 2005). HIF-2 was shown to have positive effects on tumour growth, in this model, whilst HIF-1 had negative effects. Consistent with these observed effects on tumour growth were differential effects on the expression of specific genes with putative pro- and anti-tumourigenic effects. In particular, HIF-1 positively regulated BNIP3 but had no effect on CyclinD1, TGF-alpha and VEGF, whereas HIF-2 negatively regulated BNIP3 and positively regulated CyclinD1, TGF-alpha and VEGF.

With a paucity of literature on the effects of both HIF-1 and HIF-2 isoforms as regards overall survival and primary tumour parameters such as grade and stage in primary ccRCC, it is not yet known whether these in-vitro and in-vivo observations by Raval et al. are reflective of the biology within clinical samples of ccRCC. Similarly, there is a lack of data as regards HIF target genes on the overall survival in primary ccRCC and their prognostic utility when combined with HIF isoforms and gross macroscopic tumour parameters. Indeed, there are no published studies exploring the effects of many of these factors in a single large cohort of patients with primary clear-cell renal cancer (ccRCC).

It is the aim of this chapter to investigate the utility of the HIF isoforms and their target gene products, as prognostic markers in primary ccRCC using a series of patient derived primary tumours.
4.2 Results

4.2.1 Clinico-Pathological Variables of CC-RCC TMAs

Table 4.1 shows the distribution of age, sex, stage, grade, tumour size and patient outcome across the two TMAs used, TA38 and TA71 (Refer to Section 2.2.2). Figures 4.1, 4.2 and 4.3 show the graphical distribution of stage, grade and size of the CC-RCC specimens within the arrays.

**Figure 4.1** – Distribution of Stage of tumour specimens within TA38 and TA71 TMAs.

**Figure 4.2** – Distribution of Grade of tumour specimens within TA38 and TA71 TMAs.

**Figure 4.3** – Distribution of Size of tumour specimens within TA38 and TA71 TMAs.
<table>
<thead>
<tr>
<th>Variable</th>
<th>TA38</th>
<th>TA71</th>
<th>TA38 + TA71</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td><strong>Year of Nephrectomy</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total No of Cases on Array</strong></td>
<td>93 (55%)</td>
<td>77 (45%)</td>
<td>170</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>63</td>
<td>51</td>
<td>114</td>
</tr>
<tr>
<td>Female</td>
<td>30</td>
<td>24</td>
<td>54</td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>62</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>Range</td>
<td>36-84</td>
<td>27-87</td>
<td>27-87</td>
</tr>
<tr>
<td><strong>Tumour T Stage</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1a</td>
<td>4</td>
<td>10</td>
<td>14</td>
</tr>
<tr>
<td>1b</td>
<td>23</td>
<td>10</td>
<td>33</td>
</tr>
<tr>
<td>2</td>
<td>19</td>
<td>5</td>
<td>24</td>
</tr>
<tr>
<td>3a</td>
<td>16</td>
<td>27</td>
<td>43</td>
</tr>
<tr>
<td>3b</td>
<td>29</td>
<td>25</td>
<td>54</td>
</tr>
<tr>
<td>3c</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Fuhrman Grade</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>14</td>
<td>5</td>
<td>19</td>
</tr>
<tr>
<td>2</td>
<td>46</td>
<td>39</td>
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<tr>
<td>3</td>
<td>15</td>
<td>23</td>
<td>38</td>
</tr>
<tr>
<td>4</td>
<td>17</td>
<td>9</td>
<td>26</td>
</tr>
<tr>
<td><strong>Max Tumour Diameter (cm)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;5</td>
<td>16</td>
<td>21</td>
<td>37</td>
</tr>
<tr>
<td>5-7</td>
<td>27</td>
<td>15</td>
<td>42</td>
</tr>
<tr>
<td>7-10</td>
<td>28</td>
<td>17</td>
<td>45</td>
</tr>
<tr>
<td>&gt;10</td>
<td>18</td>
<td>21</td>
<td>39</td>
</tr>
<tr>
<td><strong>Patient Outcome</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alive</td>
<td>25</td>
<td>37</td>
<td>62</td>
</tr>
<tr>
<td>Death from Kidney Cancer</td>
<td>52</td>
<td>21</td>
<td>73</td>
</tr>
<tr>
<td>Death from other cause</td>
<td>16</td>
<td>19</td>
<td>35</td>
</tr>
</tbody>
</table>

**Table 4.1** – Clinico-Pathological Variables (age, sex, stage, grade, size and survival) of tumour specimens within TA38 and TA71 TMAs.
4.2.2 Distribution of Expression Histoscores

Table 4.2 shows the number of specimens per expression histoscore for each of the immunohistochemical stain used on the arrays. For non-continuous analysis high / low cut-offs were determined by calculating the median expression histoscore.

<table>
<thead>
<tr>
<th>Histoscore</th>
<th>HIF1</th>
<th>HIF2</th>
<th>BNIP3</th>
<th>CAIX</th>
<th>CyclinD1</th>
<th>GLUT1</th>
<th>OCT4</th>
<th>LDH5</th>
<th>VEGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>25</td>
<td>36</td>
<td>100</td>
<td>10</td>
<td>16</td>
<td>33</td>
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<td>33</td>
<td>3</td>
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<td>29</td>
<td>18</td>
<td>1</td>
<td>0</td>
<td>7</td>
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<td>6</td>
<td>24</td>
<td>22</td>
<td>3</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>8</td>
<td>15</td>
<td>5</td>
<td>14</td>
<td>13</td>
<td>2</td>
<td>2</td>
<td>13</td>
</tr>
<tr>
<td>4</td>
<td>25</td>
<td>24</td>
<td>14</td>
<td>19</td>
<td>23</td>
<td>17</td>
<td>25</td>
<td>20</td>
<td>54</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
<td>20</td>
<td>5</td>
<td>28</td>
<td>25</td>
<td>27</td>
<td>7</td>
<td>97</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>8</td>
<td>0</td>
<td>2</td>
<td>121</td>
<td>36</td>
<td>53</td>
</tr>
<tr>
<td>7</td>
<td>8</td>
<td>4</td>
<td>9</td>
<td>14</td>
<td>27</td>
<td>17</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>69</td>
<td>7</td>
<td>9</td>
<td>2</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>162</td>
<td>164</td>
<td>159</td>
<td>167</td>
<td>166</td>
<td>158</td>
<td>164</td>
<td>166</td>
<td>159</td>
</tr>
</tbody>
</table>

Table 4.2 - Distribution of Expression Histoscore. Median histoscore was taken as the delineation between high and low scores. Grey highlight represents high score, unshaded represents low score.

4.2.3 Prognostic significance of the pathological characteristics of the tumour

4.2.3.1 Fuhrman Grade

In keeping with the analysis from Chapter 3, Fuhrman grade 1-3 group together with significantly (P<0.0001) better prognosis than patients with Fuhrman grade 4. For statistical analysis with other parameters, from the TMAs, Fuhrman grade was split into high grade (Fuhrman grade 4) and low grade (Fuhrman grade 1-3). (See Figures 3.8 and 4.4)
Figure 4.4 - Kaplan Meier Survival Curve (Death from Kidney Cancer) of all CC-RCC specimens in TA38 and TA71, split by Fuhrman Grade.

4.2.3.2 Tumour Stage
Increasing stage shows significantly worse prognosis (p=0.0006), (no nodal or metastatic data included). (See Figure 4.5)

Figure 4.5 - Kaplan Meier Survival Curve (Death from Kidney Cancer) of all CC-RCC specimens in TA38 and TA71, split by Tumour Stage (no nodal or metastatic data included).
4.2.3.3 Maximum Tumour Diameter (cm)

Maximum tumour diameter was divided into three equal size cohorts, A (<6cm), B (6-8cm) and C (>8cm). Category A tumours have significantly better survival (p<0.0001) than B or C tumours. (See Figure 4.6)

![Death from Kidney Cancer vs Size](Image)

**Figure 4.6** - Kaplan Meier Survival Curve (Death from Kidney Cancer) of all CC-RCC specimens in TA38 and TA71, split by tumour size category.

4.2.4 Time-distributed death cohorts

For the purposes of further analyses (see later in chapter), I divided all patients who had died of kidney cancer into three equally sized cohorts. I labelled these ‘Early Death’ <11 months; ‘Intermediate Death’ 11-27 months; and ‘Late Death’ >27 months. Naturally, these groups were significantly different when plotted on Kaplan-Meier survival curves. (See Figure 4.7 and Table 4.3)
Figure 4.7 - Kaplan Meier Survival Curve (Death from Kidney Cancer) of all CC-RCC specimens in TA38 and TA71, split by Early (<11 months), Intermediate (11-27 months) and Late Death (>27 months).

<table>
<thead>
<tr>
<th></th>
<th>Logrank Test p-value</th>
<th>Hazard Ratio (95% Confidence Interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early vs Inter</td>
<td>&lt;0.0001***</td>
<td>5.4 (13-85)</td>
</tr>
<tr>
<td>Early vs Late</td>
<td>&lt;0.0001***</td>
<td>5.5 (15-94)</td>
</tr>
<tr>
<td>Intermediate vs Late</td>
<td>&lt;0.0001***</td>
<td>5.4 (14-84)</td>
</tr>
</tbody>
</table>

Table 4.3 – Kaplan-Meier survival curve analysis (See Figure 4.7) for Early, Intermediate and Late Death from CC-RCC.

4.2.5 Change in Kidney Cancer Specific Survival over time.

As per the previous analysis in Chapter 3, cohorts of ‘year of nephrectomy’ were generated, and a similar trend was demonstrated (see comparable analysis Figure 3.3). Patients undergoing nephrectomy most recently (1995-1999) had the best overall survival (10-year survival = 71%) whilst the patients in the earliest cohort (1983-1986) had the worst prognosis (10-year survival = 28%). (See Figure 4.8 and Table 4.4)
Figure 4.8 - Kaplan Meier Survival Curve (Death from Kidney Cancer) of all CC-RCC specimens in TA38 and TA71, split into 4-year cohorts to assess survival changes over time.

<table>
<thead>
<tr>
<th></th>
<th>Logrank Test p-value</th>
<th>Hazard Ratio (and 95% Confidence Intervals)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1983-1986 vs 1995-1999</td>
<td>0.0018**</td>
<td>2.6 (1.6-7.1)</td>
</tr>
<tr>
<td>1987-1990 vs 1995-1999</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>1991-1994 vs 1995-1999</td>
<td>0.28</td>
<td></td>
</tr>
<tr>
<td>1983-1986 vs 1987-1990</td>
<td>0.062</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.4 – Kaplan-Meier survival curve analysis (See Figure 4.8) for 4-year cohort (year of Nephrectomy)

4.2.6 Change in antigenicity of nuclear HIF-1 and HIF-2 over time

As discussed during the materials and methods section of this chapter, it was subjectively noticed that the intensity of the HIF-1 and HIF-2 antibodies appeared decreased on the older tumour samples. It was found on analysis that the nuclear expression of HIF-1 and HIF-2 changes significantly depending upon the age of the paraffin block (HIF-1 alpha: p < 0.0001;
HIF-2 alpha: p < 0.0001, Mann-Whitney tests). This was recapitulated using the respective HIF alpha isoform Histoscores for each individual year between 1983 and 1999, for both HIF-1 alpha and HIF-2 alpha, using one-way ANOVA analysis (Kruskal-Wallis tests). (See Figures 4.9 and 4.10). Therefore for all further analyses within this thesis only TA71, where HIF staining was stable, was used. This chronological alteration in histoscores was not recapitulated for any of the HIF target gene products of BNIP3, CAIX, CD31, cyclin D1, GLUT-1, LDH5, Oct-4 and VEGF.

<table>
<thead>
<tr>
<th>Variable</th>
<th>TA38</th>
<th>TA71</th>
<th>TA38 + TA71</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td><strong>Nuclear HIF-1 alpha</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>27</td>
<td>46</td>
<td>89</td>
</tr>
<tr>
<td>Low</td>
<td>61</td>
<td>28</td>
<td>73</td>
</tr>
<tr>
<td><strong>Nuclear HIF-2 alpha</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>29</td>
<td>29</td>
<td>69</td>
</tr>
<tr>
<td>Low</td>
<td>58</td>
<td>48</td>
<td>95</td>
</tr>
</tbody>
</table>

**Table 4.5** – Distribution of HIF Expression Histoscores for each TMA and both combined.

**Figure 4.9** – Median HIF-1 alpha histoscore per age of paraffin block

**Figure 4.10** – Median HIF-2 alpha histoscore per age of paraffin block
4.2.7 HIF-1 and HIF-2 in primary CC-RCC prognosis.

4.2.7.1 Individual HIF effect on prognosis

No correlation was found between the nuclear or cytoplasmic expression of HIF-1 and HIF-2, and individually, neither nuclear expression of HIF-1 or HIF-2 had any effect on overall survival. (See Figures 4.11 and 4.12).

Interestingly, alternative survival analysis using the ‘time-distributed death cohorts’ revealed that nuclear expression of HIF-1 is reduced in patients that die within the intermediate group (11-27 months) when compared to the early or late death cohorts. HIF-2 shows no significant difference between groups (See Figures 4.13 and 4.14 & Tables 4.6 and 4.7).

![Figure 4.11](image-url) - Kaplan Meier Survival Curve (Death from Kidney Cancer) of all CC-RCC specimens in TA71, split by HIF-1 high / low expression histoscore.
Figure 4.12 - Kaplan Meier Survival Curve (Death from Kidney Cancer) of all CC-RCC specimens in TA71, split by HIF-2 high / low expression histoscore.

Figure 4.13 – Mean HIF-1 expression histoscore per Time-distributed death cohort.

<table>
<thead>
<tr>
<th></th>
<th>Mann-Whitney p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early Death</td>
<td>Intermediate Death</td>
</tr>
<tr>
<td>Early Death</td>
<td>Late Death</td>
</tr>
<tr>
<td>Intermediate</td>
<td>Late Death</td>
</tr>
</tbody>
</table>

Table 4.6 – Mann-Whitney T-test analysis of differences between Mean HIF-1 expression histoscore per Time-distributed death cohort.
4.2.8 HIF-1 and HIF-2 and their primary target genes in CC-RCC prognosis

Seven primary target genes of HIF-1 and HIF-2 were also assessed on the CC-RCC TMAs and were analysed along with HIF-1 and HIF-2. They were: BNIP3, CAIX, CyclinD1, GLUT-1, LDH5, Oct-4 and VEGF.

4.2.8.1 Correlation with HIF-1 alpha

HIF-1 was positively correlated to CyclinD1, and showed no significant correlation with BNIP3, CAIX, GLUT-1, LDH5, Oct-4 or VEGF. (See Table 4.8).
4.2.8.2 Correlation with HIF-2 alpha

HIF-2 also was only positively correlated to CyclinD1, and showed no significant correlation with BNIP3, CAIX, GLUT-1, LDH5, Oct-4 or VEGF. (See Table 4.8).

4.2.8.3 Individual Prognostic Significance of HIF Target Genes

Taken individually none of the seven factors had any correlation on overall survival. (See Figures 4.15 -4.21)

<table>
<thead>
<tr>
<th>HIF Isoform</th>
<th>Protein / Target Gene</th>
<th>R value</th>
<th>Spearman Rank p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-1</td>
<td>HIF-2</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>HIF-1</td>
<td>BNIP3</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>HIF-1</td>
<td>CAIX</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>HIF-1</td>
<td>CyclinD1</td>
<td>0.35</td>
<td>0.0026*</td>
</tr>
<tr>
<td>HIF-1</td>
<td>GLUT1</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>HIF-1</td>
<td>LDH5</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>HIF-1</td>
<td>Oct-4</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>HIF-1</td>
<td>VEGF</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>HIF-2</td>
<td>BNIP3</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>HIF-2</td>
<td>CAIX</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>HIF-2</td>
<td>CyclinD1</td>
<td>0.38</td>
<td>0.0007</td>
</tr>
<tr>
<td>HIF-2</td>
<td>GLUT1</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>HIF-2</td>
<td>LDH5</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>HIF-2</td>
<td>Oct-4</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>HIF-2</td>
<td>VEGF</td>
<td></td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 4.8 – Correlations of HIF-1 and HIF-2 to their primary target genes BNIP3, CAIX, CyclinD1, GLUT1, LDH5, Oct-4 and VEGF.
Figure 4.15 - Kaplan Meier Survival Curve (Death from Kidney Cancer) of all CC-RCC specimens in TA38 and TA71, split by BNIP3 high / low expression histoscore.

Figure 4.16 - Kaplan Meier Survival Curve (Death from Kidney Cancer) of all CC-RCC specimens in TA38 and TA71, split by CAIX high / low expression histoscore.
Figure 4.17 - Kaplan Meier Survival Curve (Death from Kidney Cancer) of all CC-RCC specimens in TA38 and TA71, split by CyclinD1 high / low expression histoscore.

Figure 4.18 - Kaplan Meier Survival Curve (Death from Kidney Cancer) of all CC-RCC specimens in TA38 and TA71, split by GLUT1 high / low expression histoscore.
Figure 4.19 - Kaplan Meier Survival Curve (Death from Kidney Cancer) of all CC-RCC specimens in TA38 and TA71, split by LDH5 high / low expression histoscore.

Figure 4.20 - Kaplan Meier Survival Curve (Death from Kidney Cancer) of all CC-RCC specimens in TA38 and TA71, split by Oct-4 high / low expression histoscore.
**Figure 4.21** - Kaplan Meier Survival Curve (Death from Kidney Cancer) of all CC-RCC specimens in TA38 and TA71, split by VEGF high / low expression histoscore.
4.2.9 Fuhrman Grade in primary CC-RCC and its relationship with hypoxic pathway proteins, macroscopic pathological tumour parameters and prognosis

As demonstrated earlier in this section, high grade (Fuhrman 4) CC-RCC tumours have worse overall survival compared to low grade tumours (Fuhrman 1-3) (p<0.0001, logrank test). (Refer to Figure 4.4). Fuhrman grade was significantly correlated with T-stage, Maximum Tumour Diameter, BNIP3 and Oct-4. (See Table 4.9).

Although there was no direct correlation between Fuhrman grade and either of the HIF isoforms, HIF-1 and HIF-2 were both split by Fuhrman grade on Kaplan-Meier survival curve analysis. Nuclear expression of both HIF-1 and HIF-2 are not able to further differentiate the high and low tumour grade survival curves. (See Figures 4.22 and 4.23 & Tables 4.10 and 4.11)

<table>
<thead>
<tr>
<th>Comparative Factor</th>
<th>R value</th>
<th>Spearman Rank p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-Stage</td>
<td>0.3555</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td>Maximum Tumour Diameter</td>
<td>0.2928</td>
<td>0.0002***</td>
</tr>
<tr>
<td>BNIP3</td>
<td>0.2178</td>
<td>0.0061**</td>
</tr>
<tr>
<td>CyclinD1</td>
<td>-0.3412</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td>Oct-4</td>
<td>0.1595</td>
<td>0.0426*</td>
</tr>
</tbody>
</table>

Table 4.9 – Correlations of Fuhrman Grade to hypoxic pathway proteins and macroscopic pathological tumour parameters
Figure 4.22 - Kaplan Meier Survival Curve (Death from Kidney Cancer) of all CC-RCC specimens in TA71, split by high / low – Fuhrman Grade / HIF1 expression histoscore.

Table 4.10 - Kaplan-Meier survival curve analysis (See Figure 4.23) all CC-RCC specimens in TA71, split by high / low – Fuhrman Grade / HIF1 expression histoscore.
Figure 4.23 - Kaplan Meier Survival Curve (Death from Kidney Cancer) of all CC-RCC specimens in TA71, split by high / low – Fuhrman Grade / HIF2 expression histoscore.

Table 4.11 - Kaplan-Meier survival curve analysis (See Figure 4.23) all CC-RCC specimens in TA71, split by high / low – Fuhrman Grade / HIF2 expression histoscore.
4.2.10 Angiogenesis in primary CC-RCC and its relationship with hypoxic pathway proteins, Fuhrman grade, macroscopic pathological tumour parameters and prognosis.

4.2.10.1 Angiogenesis and CC-RCC Prognosis

Angiogenesis quantified by the Vessel Visualisation Index (VVI) with CD31 antibody staining has prognostic significance, with high levels of angiogenesis conferring a good overall survival ($p=0.0003$, logrank test; Hazard Ratio 0.3823 (95% CI: 0.2119-0.6312)). (See Figure 4.24).

Angiogenesis was found to vary significantly across the three time-distributed death cohorts (as previously defined). Significantly lower VVI was demonstrated in the early death cohort compared to the late death cohort ($p=0.0360$, Mann-Whitney test). (See Figure 4.25 and Table 4.12).

![Angiogenesis (CD31) and Death from Kidney Cancer](image)

**Figure 4.24** - Kaplan Meier Survival Curve (Death from Kidney Cancer) of all CC-RCC specimens in TA38 and TA71, split by high / low Vessel Count (VVI).
Table 4.12 – Mann-Whitney T-test analysis of differences between Mean Vessel Count (VVI) per Time-distributed death cohort.

<table>
<thead>
<tr>
<th>VVI</th>
<th>Mann-Whitney p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early Death vs. Interim Death</td>
<td>0.4527</td>
</tr>
<tr>
<td>Early Death vs. Late Death</td>
<td>0.0360*</td>
</tr>
<tr>
<td>Intermediate Death vs. Late Death</td>
<td>0.1669</td>
</tr>
</tbody>
</table>

4.2.10.2 Angiogenesis and Fuhrman Grade

VVI split Fuhrman grade such that low grade tumours could be differentiated into two distinct prognostic categories. Patients with low grade tumours with high VVI survived significantly longer than patients with low grade tumours with low VVI (p=0.0017). High or low VVI in high grade tumours had no discriminatory survival effect. (See Figures 4.26 and 4.27 & Table 4.13). Specific Fuhrman Grade was found to have a strong negative correlation with CD31+ VVI (chi-squared: p=0.0026; spearman rank correlation: p=0.0005 (r value -0.2941)).
Figure 4.26 - Kaplan Meier Survival Curve (Death from Kidney Cancer) of all CC-RCC specimens in TA38 and TA71, split by high / low – Fuhrman Grade / Vessel Count (VVI)

<table>
<thead>
<tr>
<th>Fuhrman Grade split by Angiogenesis:</th>
<th>Fuhrman Grade</th>
<th>Angiogenesis</th>
<th>Fuhrman Grade</th>
<th>Angiogenesis</th>
<th>Logrank Test p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>High High vs High Low</td>
<td>High</td>
<td>High</td>
<td>High</td>
<td>Low</td>
<td>0.0865</td>
</tr>
<tr>
<td>Low High vs Low Low</td>
<td>Low</td>
<td>High</td>
<td>Low</td>
<td>Low</td>
<td>0.0017**</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Angiogenesis split by Fuhrman Grade:</th>
<th>Fuhrman Grade</th>
<th>Angiogenesis</th>
<th>Fuhrman Grade</th>
<th>Angiogenesis</th>
<th>Logrank Test p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>High High vs Low Low</td>
<td>High</td>
<td>High</td>
<td>Low</td>
<td>High</td>
<td>0.0044**</td>
</tr>
<tr>
<td>High Low vs Low Low</td>
<td>High</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
<td>0.0004***</td>
</tr>
</tbody>
</table>

Table 4.13 - Kaplan-Meier survival curve analysis (See Figure 4.26) all CC-RCC specimens in TA38 and TA71, split by high / low – Fuhrman Grade / Vessel Count (VVI).
4.2.10.3 Angiogenesis, Maximum Tumour Diameter and hypoxic pathway proteins

VVI was found to negatively correlate with maximum tumour diameter (p=0.0210, r value -0.1992) as well as Oct-4 (p=0.0020, r value -0.2624), and was found to positively correlate with CAIX (p=0.0130, r value 0.2100). (See Table 4.14)

<table>
<thead>
<tr>
<th>VVI</th>
<th>Comparative Factor</th>
<th>R value</th>
<th>Spearman Rank p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Maximum Tumour Diameter</td>
<td>-0.1992</td>
<td>0.0210*</td>
</tr>
<tr>
<td></td>
<td>Oct-4</td>
<td>-0.2624</td>
<td>0.0020**</td>
</tr>
<tr>
<td></td>
<td>CAIX</td>
<td>0.2100</td>
<td>0.0130*</td>
</tr>
<tr>
<td></td>
<td>HIF-1</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HIF-2</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.14 – Correlations of Vessel Count (VVI) with hypoxic pathway proteins and macroscopic pathological tumour parameters (excluding Fuhrman Grade).
4.2.10.4 Angiogenesis and HIF

When VVI was compared to the two HIF isoforms there was no correlation seen, however, both HIF-1 and HIF-2 survival curves were split with VVI high/low. (See Figures 4.28 and 4.29). Interestingly it was low HIF-1 tumours that was differentiated by VVI (low HIF-1 with high VVI had better prognosis (p=0.0004)), whilst in HIF-2 it was high HIF-2 together with high VVI that conferred the better prognosis (p=0.0005). Furthermore, both isoforms of HIF demonstrated better prognosis when high, taken together with high VVI, when compared to low HIF and low VVI (HIF-1 p=0.0054; HIF-2 p=0.0059). (See Tables 4.15 and 4.16).

![HIF-1, Angiogenesis and Death from Kidney Cancer](p=0.0022)

**Figure 4.28** - Kaplan Meier Survival Curve (Death from Kidney Cancer) of all CC-RCC specimens in TA71, split by high / low –Vessel Count (VVI) / HIF-1 expression histoscore.
| HIF-1 split by Angiogenesis: | | | | | |
|---|---|---|---|---|
| High | High vs High | Low | 0.2985 | |
| Low | High vs Low | Low | 0.0004*** | 0.2786 (0.1214-0.5444) |

| Angiogenesis split by HIF-1: | | | | | |
|---|---|---|---|---|
| High | High vs Low | High | 0.6918 | |
| High | Low vs Low | Low | 0.1229 | |
| High | High vs Low | Low | 0.0054** | 0.3460 (0.1681-0.7343) |

Table 4.15 - Kaplan-Meier survival curve analysis (See Figure 4.28) all CC-RCC specimens in TA71, split by high / low –Vessel Count (VVI) / HIF-1 expression histoscore.

**Figure 4.29** - Kaplan Meier Survival Curve (Death from Kidney Cancer) of all CC-RCC specimens in TA71, split by high / low –Vessel Count (VVI) / HIF-2 expression histoscore.
### Table 4.16 - Kaplan-Meier survival curve analysis (See Figure 4.29) all CC-RCC specimens in TA71, split by high / low –Vessel Count (VVI) / HIF-2 expression histoscore.

<table>
<thead>
<tr>
<th>HIF-2 split by Angiogenesis:</th>
<th>HIF-2</th>
<th>Angiogenesis</th>
<th>HIF-2</th>
<th>Angiogenesis</th>
<th>Logrank Test p-value</th>
<th>Hazard Ratio (and 95% Confidence Intervals)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High High vs High Low</td>
<td>0.0005***</td>
<td>0.2073 (0.04821-0.4242)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low High vs Low Low</td>
<td>0.1496</td>
<td>0.1521</td>
<td>0.2171</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**4.2.11 VEGF in primary CC-RCC and its relationship with hypoxic pathway proteins, Fuhrman grade, macroscopic pathological tumour parameters and prognosis.**

**4.2.11.1 VEGF and Prognosis**

VEGF was not demonstrated to effect patient overall survival when using Kaplan–Meier survival curves. (See Figure 4.30). However, when the mean cytoplasmic histoscores where compared across the three time-distributed death cohorts, the late death cohort had significantly lower expression levels of VEGF compared to either early death or intermediate death (p=0.0058 and 0.0080 respectively). (See Figure 4.31 and Table 4.17).
Figure 4.30 - Kaplan Meier Survival Curve (Death from Kidney Cancer) of all CC-RCC specimens in TA38 and TA71, split by high / low VEGF expression histoscore.

Figure 4.31 - Mean VEGF expression histoscore per Time-distributed death cohort.

<table>
<thead>
<tr>
<th>VEGF</th>
<th>Mann-Whitney p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early Death vs Intermediate Death</td>
<td>0.3411</td>
</tr>
<tr>
<td>Early Death vs Late Death</td>
<td>0.0058**</td>
</tr>
<tr>
<td>Intermediate Death vs Late Death</td>
<td>0.0080**</td>
</tr>
</tbody>
</table>

Table 4.17 – Mann-Whitney T-test analysis of differences between Mean VEGF expression histoscore per Time-distributed death cohort.
4.2.11.2 VEGF, macroscopic tumour markers and other Hypoxic Pathway Proteins including HIF

Using Spearman Rank, VEGF was found to correlate positively with BNIP3 (p<0.0001, r value 0.4053). No correlation was found with HIF-1, HIF-2 or any other measured marker of the tumour or hypoxic pathway. (See Table 4.18). On Kaplan-Meier survival curve analysis HIF-1 or HIF-2 added no further prognostic significance when used to split VEGF. (See Figures 4.32 and 4.33).

<table>
<thead>
<tr>
<th>VEGF</th>
<th>Comparative Factor</th>
<th>R value</th>
<th>Spearman Rank p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HIF-1</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>HIF-2</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>BNIP3</td>
<td>0.4053</td>
<td>&lt;0.0001***</td>
</tr>
</tbody>
</table>

Table 4.18 – Correlations of VEGF with other hypoxic pathway proteins including HIF (non-significant comparisons not shown).

**Figure 4.32** - Kaplan Meier Survival Curve (Death from Kidney Cancer) of all CC-RCC specimens in TA71, split by high / low –VEGF / HIF-1 expression histoscore.
4.2.12 Multivariate Analysis

When multivariate analysis was performed with a Cox proportional hazards test, high angiogenesis (CD31+ VVI) was shown to be the most significant prognostic marker of increased overall kidney cancer specific survival. Additionally, a low Fuhrman grade and small tumour size (maximum tumour diameter) demonstrated better overall survival in primary CC-RCC. Neither HIF isoform nor any of their six target genes investigated here, had any prognostic utility. (See Table 4.19)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard Ratio</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD31+ VVI</td>
<td>0.3800</td>
<td>0.2100-0.7012</td>
<td>0.0020</td>
</tr>
<tr>
<td>Fuhrman’s Grade</td>
<td>1.5211</td>
<td>1.0413-2.2194</td>
<td>0.0317</td>
</tr>
<tr>
<td>Tumour Size</td>
<td>1.0885</td>
<td>1.0000-1.1735</td>
<td>0.0421</td>
</tr>
</tbody>
</table>

Table 4.19 - Multivariate analysis with a Cox proportional hazards test on all Clinical, Pathological, Angiogenic and Immunohistochemical analyses of TMAs TA38 and TA71.
4.3 Discussion

4.3.1 Change in antigenicity of nuclear HIF-1 and HIF-2 over time

It would appear that the antigenicity of HIF deteriorates with the age of the CC-RCC paraffin-embedded block from which the TMA core is derived. This phenomenon has previously been described for p53 and EGFR detection in human tissues (Olapade-Olaopa, Ogunbiyi et al. 2001), although this is the first time it has been shown for HIF isoforms. This may be due to alterations in the antigenicity of the HIF molecule, possibly due to loss of tertiary configuration caused by the paraffin embedding process (this would alter the antibody affinity for cognate epitope) or degradation of HIF protein itself caused by paraffin embedding.

If this phenomenon were true then it could cast into significant doubt previous literature about HIF-1 immunostaining in human cancers using this particular HIF-1 antibody (HIF-1α mouse monoclonal, Clone 54 BD Biosciences Pharmingen). Similarly, the reliability of HIF-2 immunostaining in archival tumour samples with the antibody used within this study (HIF-2 mouse monoclonal, EP190, Oxford University), has also to be questioned.

However, it is possible that the deterioration may be due to variations in the conditions in which the two TMAs were constructed, and therefore reflect a global decrease in staining efficacy in the original array. Although the median histoscores for HIF suggest a gradual and progressive reduction in HIF staining over time, the possibility of array to array variation should be excluded. This could be achieved by taking whole tumour sections from the original blocks and assessing HIF staining in them compared to age of specimen. Unfortunately this is beyond the scope of this thesis.
4.3.2 **HIF-1 and HIF-2 in primary CC-RCC prognosis.**

Analysis of nuclear expression of HIF-1 and HIF-2 did not demonstrate a correlation. This may not be reflective of the true biology, but due to the limited power of the analysis secondary to the unexpected loss of 93 cases with deterioration in their HIF antigenicity. Further work will be required using a larger number of cases from newer specimens to answer this satisfactorily, which is unfortunately beyond the scope of this thesis.

Regarding the prognostic significance of either HIF isoform, it is apparent that neither affects overall survival in CC-RCC, and therefore this does not support the previous literature that HIF-1 expression has prognostic significance in metastatic CC-RCC (Lidgren, Hedberg et al. 2005), or that high HIF-2 expression confers a reduced patient survival, as has been demonstrated in paediatric neuroblastoma (Nilsson, Jogi et al. 2005; Holmquist-Mengelbier, Fredlund et al. 2006). It is possible that when both HIF alpha isoforms are co-expressed, as may be expected in a VHL depleted tumour model such as cc-RCC, the functional effects of one mitigate the other, possibly due to competition for HIF-1beta. However, these results must again be questioned due to the low numbers in the analysis secondary to the unexpected loss of TA38.

Furthermore, it is also worth commenting that Kaplan-Meier survival analysis uses cut-offs for high/low HIF expression. These were calculated in this case using the median histoscore; however this may not represent a functional difference in high / low expression of HIF. However, alternative cut-off values were used which also failed to reach significance.

Finally, the lack of utility of both HIF isoforms as markers of disease prognosis within this ccRCC TMA study may be a reflection of experimental methodology. In breast cancer (although HIFα isoforms are hypoxia-induced), using whole tumour sections, diffuse expression of
nuclear HIF-1 seems to denote less aggressive disease with subsequent improved outcome on analysis (Koukourakis, Giatromanolaki et al. 2002). Therefore, it would be useful to perform similar experiments on matched CC-RCC whole tumour sections and use laser microdissection to extract cDNA (for HIF transcriptional products) from intra-tumoural areas that have differential HIF isoform expression levels.

4.3.3 Time-Distributed Death Cohorts
An alternative method of survival analysis using only patients that had died from CC-RCC shows clear differences in HIF-1 and HIF-2 expression levels between the three cohorts of early, intermediate and late tumour-related deaths. This is particularly clear in tumours with low levels of HIF-1, which are associated with an intermediate death group status. The equivalent levels of HIF-1 expression amongst the early and late death groups may indicate that nuclear HIF-1 interacts with different molecular partners in differing patient groups, which ultimately influences disease prognosis. However, it is possible that this finding is simply due to chance, and a result of the multiple analyses performed.

4.3.4 HIF1 / HIF-2 and Fuhrman Grade
Analysis of patient survival versus tumour grade demonstrates that Fuhrman grade 4 tumours have significantly worse prognosis in comparison to Fuhrman grades 1 to 3, which all have similar outcome. Differentiation between high and low grade was made on this result with grade 4 determined as high grade, and grade 1-3 as low grade.

Spearman rank correlation demonstrated positive correlations of grade with stage, maximum tumour diameter, BNIP3 and Oct-4. The relationships with stage and tumour size are of little surprise, given their established co-dependence and utility as independent prognostic markers in many epithelial tumours. It is possible that the positive correlations with BNIP3 and Oct-4
may be independent of the hypoxic pathway and actually be due to genetic changes (such as the loss of p53) as the tumour growths and becomes less differentiated.

Sub-division of grade by HIF-1 and HIF-2 showed no additional differentiation of prognosis on survival curve analysis. This is in contrast to a recent analysis of neuroblastomas where high HIF-2 expression could differentiate the low-grade tumours into distinct prognostic categories and overall, HIF-2 was considered a marker of neuroblastoma aggressiveness (Holmquist-Mengelbier, Fredlund et al. 2006). This may be reflective of the difference in VHL functionality between the two tumour types, such that in VHL sufficient tumours such as neuroblastoma, HIF-2 is induced primarily by hypoxia, compared to CC-RCC where it is activated mainly due to lack of pVHL.

Multivariate analysis clearly shows tumour grade to be an independent prognostic marker in primary CC-RCC with high grade on either a high or low HIF-1 background conferring a worse survival than low grade. No similar effects are seen with HIF-2 possibly indicating that the biological factors controlling grade are superior to the effects of differential HIF-1 signalling, although this is not the case for HIF-2 signalling.

4.3.5 HIF1 / HIF-2 and Apoptosis / Autophagy

As discussed in the introduction to this thesis, BNIP3 has been shown to be positively correlated with HIF-1, and negatively correlated with HIF-2 in a CC-RCC mouse model. In these clinical samples however, BNIP3 was not correlated with either HIF isoform and expression of BNIP3 had no overall effect on survival. This suggests that the regulation of BNIP3 is more complex in-vivo and may be model dependent. This could be due to the possible contrasting effects upon BNIP3 regulation by each HIF isoform or possibly that there is co-regulation of BNIP3 by binding partners such as
BCL-2. However, it may well be due again to the lack of cases represented in this analysis due to the loss of 93 cases from HIF antigen degradation.

### 4.3.6 HIF1 / HIF-2 and pH regulation

In-vitro experiments show that HIF-1 is the sole positive regulator of CAIX, but this is not recapitulated in these clinical CC-RCC samples. Whether this is due to the lack of samples failing to reach significance or alternative regulatory mechanisms of CAIX expression is unknown.

### 4.3.7 HIF1 / HIF-2 and Proliferation

Previous in vitro / in vivo experiments suggest that cyclin D1 is a predominant target gene of HIF, is support by this clinical series of primary CC-RCCs, where a strong positive correlation was demonstrate with both HIF-1 and HIF-2.

My analysis shows that cyclin D1 has no significant influence on CC-RCC disease survival; however I did show that cyclin D1 expression has negative correlations with grade, stage and tumour size. This suggests that it may have a tumour suppressive role. A possible explanation of this phenomenon may be that high levels of cyclin D1 have a tumour suppressive effect via decreased proliferation rather than increased. This has certainly been the case in a number of epithelial tumours, including bladder and colorectal (Palmqvist, Stenling et al. 1998; Suwa, Takano et al. 1998), where cyclin D1 has been shown to have an inverse correlation with Ki-67. This may be due to an inhibition of glycolytic enzymes such as LDH5 as has been demonstrated in an inducible-cyclin D1 system in a mouse model of breast cancer (Yu, Geng et al. 2001).

### 4.3.8 HIF1 / HIF-2 and Aerobic Glycolysis

The two proteins selected to explore HIF dependent aerobic glycolysis in CC-RCC are located at different ends of the cell’s glucose pathways,
GLUT-1 (distal) and LDH5 (proximal). The in-vitro data would suggest a positive correlation with HIF-1; however neither HIF isoforms correlated with GLUT-1 or LDH5. This again may be due to the low numbers in the final analysis, or may reflect a general difficulty in assessing specific isoform correlations within a tumour where both HIF isoforms are likely to be ubiquitously expressed. Neither GLUT-1 nor LDH5 effected the overall disease survival in CC-RCC in contrast to other epithelial tumours where membrane expression of GLUT-1 and nuclear LDH5 have been associated with worse overall survival outcomes and/or higher tumour grades (Medina and Owen 2002; Koukourakis, Giatromanolaki et al. 2006; Colgan, Mukherjee et al. 2007).

4.3.9 HIF1 / HIF-2 and Differentiation

Immunohistochemical analysis of Oct-4 staining demonstrated that it was only differentially expressed within the cytoplasm, with constitutive expression within the nucleus. The cytoplasmic localization of Oct-4 within this study is contrary to the differential nuclear localization of Oct-4 observed in invasive primary bladder cancer, using the same antibody (Atlasi, Mowla et al. 2007). This would suggest that different Oct-4 isoforms may be expressed in different epithelial urological tumours with distinct oncogenic functions.

Within these CC-RCC samples nuclear expression failed to correlate with either of the HIF isoforms, but did positively correlate with Fuhrman grade. The failure of Oct-4 to correlate with HIF-2 goes against strong in-vitro data suggesting HIF-2 specific binding to Oct-4’s promoter region. However this may be related to the difference in cellular localisation as previously discussed. The correlation with grade may be explained by Oct-4 having a putative role as a growth factor signalling protein and indeed this function has been proposed for cytoplasmic Oct-4 before (Covello, Kehler et al. 2006).
4.3.10 HIF1 / HIF-2 and Angiogenesis

The regulation of angiogenesis within primary human CC-RCC is highly complex. This is highlighted by the fact that the methodology of vessel quantification has been shown previously to change the prognostic value of angiogenesis (Sabo, Boltenko et al. 2001; Fox and Harris 2004; Yao, Qian et al. 2007). This is also the case in this analysis where the chosen method of CD31+ vessel quantification, VVI, did not correlate with either HIF isoform or VEGF. Furthermore, neither HIF-1 nor HIF-2 correlated to VEGF expression.

Interestingly, there is a strong positive correlation between VEGF and cytoplasmic BNIP3. Both proteins can be positively regulated by HIF-1 in vitro, although this was not recapitulated in vivo within this clinical series. As well, in vitro experiments have shown that they share marked similarities in transcriptional regulation (HIF-induced or otherwise), as well as similarities in protein stability – poorly stable with high affinities for proteosomal degradation (Guo, Searfoss et al. 2001). Therefore, it is possible that the positive correlation between VEGF and BNIP3 may reflect their overlapping regulatory mechanisms at a transcriptional level.

Differential levels of angiogenesis, as measured by the CD31+ VVI, had a marked effect on overall disease survival in primary CC-RCC, as well as demonstrating an effect within the patients who died of CC-RCC. High levels of CD31+ VVI conferred a better overall disease prognosis when compared to low CD31+ VVI and higher levels were also expressed within the late death cohort patients when compared to their early death counterparts. There was a strong negative correlation between VVI and tumour grade, and this, together with the fact that high VVI conferred a better prognosis than low VVI in the low-grade tumours, underlies the utility of the VVI as a prognostic marker within low-grade CC-RCC.
Additionally, it demonstrates that a high VVI cannot rescue patients with high-grade tumours from their worse prognostic fate. Indeed, the CD31$^+$ VVI was confirmed as the most powerful independent prognostic factor on multivariate analysis.

At first appearance, a high VVI conferring a better overall survival may seem counterintuitive, but the functionality of angiogenesis may be determinant here. Given that CD31$^+$ endothelial cells may represent either undifferentiated or differentiated endothelium, it is possible that the CD31$^+$ vessels identified within this study are functional, such that a high VVI is related to good tumour microcirculation and ultimately better tumour perfusion. This would enable enhanced removal of aerobic glycolysis end-products, such as lactate and H$^+$. Cancer cells within these high VVI tumours are probably not exposed to sustained periods of ischaemia, unlike the low VVI group, from which hypoxia death-resistant clones could arise facilitating tumour metastasis.

Furthermore, it is possible that high CD31$^+$ angiogenesis represents ordered new vessels growth, resembling normal tissue. Low CD31$^+$ VVI may therefore represent disorganised angiogenesis, itself a reflection of many angiogenic tumour stimuli and pathways. It maybe that it is this difference in angiogenic stimulation that truly represents the discrepancy in patient outcome seen.

As Fuhrman tumour grade increases, the degree of angiogenesis decreases. Mechanistically, it could be proposed that a reduction in angiogenesis with subsequent increase in tumour ischaemia / hypoxia drives morphological dedifferentiation. This may, in turn, drive an increase in tumour proliferation, (as demonstrated by the negative correlation between tumour size and the VVI). Sustained ischaemia could subsequently select for cancer cells that
have comparatively lower levels of cyclin D1, facilitating tumour proliferation.

As regards the lack of any relationship between each HIF isoform and angiogenesis, this may not be too surprising given that both HIF isoforms are constitutively expressed in CC-RCC as opposed to other solid tumours. Indeed, the literature that has shown positive correlation between HIF-2 and angiogenesis is in tumours where HIF expression is induced via hypoxia, such as neuroblastoma and teratoma (Covello, Simon et al. 2005; Covello, Kehler et al. 2006; Holmquist-Mengelbier, Fredlund et al. 2006), as opposed to VHL deficiency.

Recent literature also suggests a differential role of CD31 and CD34 in endothelial cell function, such that CD34+ endothelium is thought to be mature and CD31+ CD34- endothelium is immature with poor functionality, as manifest by poor pericyte coverage (identified by α-SMA staining). Therefore, it would be prudent to co-label the TMAs or whole tumour sections with CD31 and α-SMA (+/- CD34), using double (or triple) labelling immunochemistry confocal microscopy. It would be hypothesised that if the CD31 vessels identified in this CC-RCC TMA study are indeed functional, they are more likely to co-express α-SMA and CD34.

The finding of high CD31+ VVI being indicative of better overall survival in primary CC-RCC does potentially highlight one problem as regards anti-angiogenic therapies. Novel agents such as bevacizumab and sorafenib are potential candidates for both neo-adjuvant (pre-nephrectomy) and adjuvant (post-nephrectomy) therapies in primary CC-RCC. However, one would also need to ascertain whether the angiogenesis within the primary tumour was functional or not, as this data implies that if CD31 angiogenesis were reduced by such drugs, patient survival may be detrimentally affected.
Indeed, the success of both bevacuzimab and tyrosine kinase inhibitors sorafanib or sunitinib in Phase II/III clinical trials in metastatic CC-RCC may indicate that the angiogenic phenotype changes with disease progression. In primary CC-RCC, given this data, the vasculature is mature due to endothelial differentiation, but within metastatic tumour sites, the endothelium is poorly functional and subsequently immature. Therefore, a comparative serial study of primary verses secondary CC-RCC, for analysis of the angiogenic functional phenotype, may highlight important differences.
Chapter 4:
Supplementary Pictures
CyclinD1 High

CyclinD1 Low
CHAPTER 4 – Supplemental Pictures

GLUT1 High

GLUT1 Low
HIF1 High

HIF1 Low
CHAPTER 4 – Supplemental Pictures

LDH5 High

LDH5 Low
CHAPTER 4 – Supplemental Pictures

Oct 4 High

Oct 4 Low
PHD2 High

PHD2 Low
Chapter 5:
MicroRNA in Clear Cell Renal Carcinoma and its relationship with VHL
Chapter 5: MicroRNA in Clear Cell Renal Carcinoma and its relationship with VHL

5.1 Introduction
MicroRNAs are a recently discovered novel class of gene regulators, some of which have been shown to interact with known oncogenic or tumour-suppressor pathways. Nine microRNAs are expressed in hypoxic conditions under HIF-1 induction (MiR 23, 24, 26, 27, 103, 107, 181, 210 and 213) (See Table 1.4); however the expression of microRNA in CC-RCC, a cancer with known reliance on the hypoxia / HIF axis, and its relationship with VHL functionality are yet to be explored.

5.2 Results
5.2.1 MicroRNA isolation
As described in the Materials and Methods section of this thesis, MicroRNA was purified from total RNA from these cell lines. Analysis of size within the differing portions of RNA, using a 2100 Bioanalyser (Agilent, UK), allowed assessment of sample quality.

Three samples were run in parallel: total RNA, total RNA excluding microRNA and purified microRNA. Figure 5.1 demonstrates a typical example of the quality of samples attained from microRNA purification. The three overlapping curves show total unpurified RNA (green); filtered RNA with microRNA extracted (red); and purified microRNA (blue). This typical graph represents good quality starting RNA with only small RNA (i.e. microRNA) contained within the purified fraction.
Figure 5.1 – Graph generated from Bioanalyser (Agilent, UK) showing MicroRNA isolation. Three samples run in parallel (total RNA, total RNA excluding microRNA and purified microRNA), from 768-0/VHL+ve cell line.

5.2.2 MicroRNA MicroArrays

5.2.2.1 Quality control

As stated in Chapter 2 of this thesis (Materials and Methods) subjective assessment of array quality was made after scanning, with only high quality arrays being included in the analysis. (See Figure 2.6). Microarrays were repeated as necessary to complete three biological replicates per cell line comparison.

Non-specific binding of the Cy3 or Cy5 dyes was excluded computationally by comparing nucleotide sequences of the Cy tags to the known sequences.
of the microRNAs. Furthermore, two additional microarrays were performed on identical samples. This showed no difference in hybridisation between Cy3 and Cy5 tagged cDNA.

5.2.2.2 Graphical representation of data
All graphical data below represents a change in the VHL mutated cells (VHL-ve) over the VHL functional cell (VHL+ve). This corresponds to a fold change of microRNA expression in the VHL mutated cells, compared to microRNA expression in the VHL functional cells. For consistency, the hypoxic experimental data is also represented in a fold change of hypoxia over normoxia.

5.2.2.3 VHL+ve vs. VHL-ve
MicroRNA expression microarrays were performed for each cell line, comparing differential microRNA expression between the VHL mutated cells (VHL-ve) and the VHL functional cells (VHL+ve). Within each microarray, individual microRNAs were replicated 4 times, and each array was repeated three times in biological triplicate, giving a total of 12 replicates per microRNA per cell line. (See Table 5.1).

Differential expression of microRNAs was assessed by comparison of microRNA expression across all cell lines in VHL-ve / VHL+ve experiments. Figure 5.2 demonstrates the considerable variability between the expression of different microRNAs between cell lines, with many being up or down regulated, as well as those with no differential expression.

To focus the results and improve the reliability of the data, only those microRNAs that were expressed in all three replicates, in all three cell lines were included. (See Figure 5.3). This demonstrates that five microRNAs are robustly expressed in CC-RCC cell lines: miR-23a, miR-15b, miR-103, miR-24 and miR-21.
To further investigate which of these microRNAs are reliably differentially regulated by the presence of a functional VHL gene, only those microRNAs that had differential expression in all three of the cell lines was included. The cut-off criterion for significant differential expression was taken as a fold change of 1.35. (See Figure 5.4). These strict inclusion criteria narrowed the results to highlight only one microRNA, miR-23a that was reliably differentially expressed.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Paired Variable</th>
</tr>
</thead>
<tbody>
<tr>
<td>768-0</td>
<td>768-0/VHL+ve vs. 768-0/VHL-ve</td>
</tr>
<tr>
<td>RCC4</td>
<td>768-0/VHL+ve vs. 768-0/VHL-ve</td>
</tr>
<tr>
<td>UMRC2</td>
<td>768-0/VHL+ve vs. 768-0/VHL-ve</td>
</tr>
<tr>
<td>7680/VHL+ve</td>
<td>Normoxia (21% O2) vs. Hypoxia (0.1% O2)</td>
</tr>
<tr>
<td>RCC4/VHL+ve</td>
<td>Normoxia (21% O2) vs. Hypoxia (0.1% O2)</td>
</tr>
<tr>
<td>UMRC2/VHL+ve</td>
<td>Normoxia (21% O2) vs. Hypoxia (0.1% O2)</td>
</tr>
</tbody>
</table>

**Table 5.1** – Paired variables per cell line for MicroRNA expression microarrays. All performed in biological triplicate.

**Figure 5.2** - VHL-ve / VHL+ve: All microRNAs expressed in the three cell lines (768-0 (left); RCC4 (centre); and UMRC2 (right)), represented as expression of all microRNAs median fold change (relative to VHL-ve) with error bars, across the three cell lines.
**Figure 5.3** - VHL-ve / VHL+ve: All microRNAs expressed in all three biological replicates, in all three cell lines, represented as expression of all microRNAs median fold change (relative to VHL-ve) with error bars, across the three cell lines.

**Figure 5.4** - VHL-ve / VHL+ve: All differentially expressed microRNAs in all three biological replicates, in all three cell lines, represented as expression of all microRNAs median fold change (relative to VHL-ve) with error bars.
5.2.2.4 Hypoxia vs. Normoxia (VHL+ve)

To validate that the observed differentially expressed microRNAs were specific to the HIF / hypoxia axis, further microarrays were performed on the VHL functional cells (VHL+ve) after exposure to 16 hours of normoxia (21% O2) versus hypoxia (0.1% O2). No expression arrays were performed for VHL mutated cell (VHL-ve) in normoxia versus hypoxia as no differentiation hypoxia induced expression would be expected, due to the constitutive expression of HIF in VHL-ve cells (Maxwell, Wiesener et al. 1999). As with the previous experiments (VHL-ve vs. VHL+ve), microRNA expression microarrays were performed for each cell line, in biological triplicate. Differential microRNA expression was assessed between the VHL functional cell, cultured in hypoxic conditions and the same cells cultured in normoxia. As before, within each microarray, individual microRNAs were replicated 4 times, resulting in a total of 12 replicates per microRNA per paired cell line. (See Table 5.1).

All microRNAs expressed in the first batch of arrays (VHL-ve / VHL+ve) were cross-referenced with the results from this second set of microRNA microarrays (VHL+ve Hypoxia vs. VHL+ve Normoxia). Figure 6.5 shows that all of the five ubiquitously expressed microRNAs identified in the first batch of arrays were also expressed in the VHL functional cells exposed to normoxia and hypoxia. Furthermore, the only microRNA that was differentially expressed in all these arrays was miR-23a. (See Figure 5.6).

5.2.2.5 MicroRNA 23a expression in MicroRNA microarrays

MicroRNA 23a was therefore identified to be differentially expressed in all cell lines. The direction of regulation was consistent with the hypoxic pathway, as upregulation was seen in VHL mutated cells compared to VHL functional cells, and in VHL functional cells subjected to hypoxia compared to the same cells cultured in normoxia. (See Figure 5.7).
Figure 5.5 - VHL+ve Hypoxia vs. Normoxia: All five ubiquitously expressed microRNAs from first batch of arrays (VHL-ve / VHL+ve), expressed in all three biological replicates, in all three cell lines (VHL+ve Hypoxia / Normoxia). Represented as expression of all microRNAs median fold change (relative to VHL+ve Hypoxia) with error bars.

Figure 5.6 - VHL+ve Hypoxia vs. Normoxia: All differentially expressed (fold change >1.35) microRNAs in all three biological replicates, in all three
cell lines (VHL+ve Hypoxia / Normoxia). Represented as expression of microRNA median fold change (relative to VHL+ve Hypoxia) with error bars.

**Figure 5.7** – Summary of differential expression of microRNA 23a across all microarray experiments. Represented as fold change of VHL-ve or VHL+ve Hypoxia over VHL+ve or VHL+ve Normoxia.

**5.2.3 Quantitative Real-Time Polymerase Chain Reaction (PCR) Validation**

Validation of the expression of miR-23a was investigated by qRT-PCR, (RT-PCR assay kit (Taqman, Applied Biosystems)). The small ribosomal RNA, RNU6B was used as an internal control, as its expression is consistent across cell lines (Lawrie, Soneji et al. 2007).

As described in detail in the materials and methods section of this thesis, 5ng of total RNA was probed with miR-23a and RNU6B, and cycled on a Roche LightCycler 480. Biological triplicate samples were used throughout.

MicroRNA 23a was found to be expressed in all the cell lines. Figure 5.8 and Table 5.2 show its differential expression across all paired cell lines.

**Figure 5.8** – Summary of quantitative real-time PCR data showing expression of miR-23a in all cell lines. Represented as fold change of VHL-ve or VHL+ve Hypoxia over VHL+ve or VHL+ve Normoxia.
The results are consistent with the microarray data for the RCC4 and UMRC2 cell lines, with microRNA-23a confirmed as being upregulated in VHL mutated cells and VHL +ve hypoxic cells. However there was a reversed trend for the 768-0 cells, where microRNA-23a was found to be down regulated.

**Figure 5.8** – qRT-PCR median expression levels of microRNA 23a represented as fold-change (relative to mutated VHL (VHL-ve) or VHL+ve hypoxia).

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Variable</th>
<th>Median Fold Change</th>
<th>Fold Change Range</th>
<th>P value (t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>768-0</td>
<td>VHL-ve / VHL+ve</td>
<td>-1.7</td>
<td>-1.5 - -1.8</td>
<td>0.0001</td>
</tr>
<tr>
<td>RCC4</td>
<td>VHL-ve / VHL+ve</td>
<td>4.6</td>
<td>3.1 – 9.1</td>
<td>0.027</td>
</tr>
<tr>
<td>UMRC2</td>
<td>VHL-ve / VHL+ve</td>
<td>2.4</td>
<td>1.3 – 3.6</td>
<td>0.027</td>
</tr>
<tr>
<td>768-0</td>
<td>Hypoxia / Normoxia</td>
<td>-1.7</td>
<td>-1.1 - -1.8</td>
<td>0.021</td>
</tr>
<tr>
<td>RCC4</td>
<td>Hypoxia / Normoxia</td>
<td>1.3</td>
<td>1.3 – 1.6</td>
<td>0.0060</td>
</tr>
<tr>
<td>UMRC2</td>
<td>Hypoxia / Normoxia</td>
<td>2.0</td>
<td>1.5 – 2.2</td>
<td>0.010</td>
</tr>
</tbody>
</table>

**Table 5.2** - Median expression levels of MicroRNA 23a represented as fold-change (relative to mutated VHL or VHL+ve hypoxia).
5.3 Discussion

5.3.1 Optimisation

Significant optimisation of the available methodology was required to achieve the results presented in this chapter. This was primarily because isolation of microRNA and its subsequent hybridisation had not previously been performed on cell line samples at the commencement of this research, within the institution where it was performed. Following this work many more microRNA experiments have been performed at this institution, and the optimised protocols as described in Chapter 2 of this thesis, have allowed improved quality and efficiency of results.

5.3.2 MicroRNA Isolation

The isolation of microRNA from total RNA is a vital first step in the generation of microRNA microarrays. The commercially available microRNA separation kits rely upon size discrepancies between standard RNA and microRNA. The generation of good quality (i.e. unfragmented) total RNA from the cell line cultures results in a high percentage of microRNA within the small RNA fraction. This subsequently decreases non-specific binding of RNA fragments to the microarray, together with increasing the actual quantity of microRNA used for the experiment. Figure 5.1 demonstrates the quality of total RNA used in a typical experiment and highlights the minimal total RNA fragmentation within these experiments.

5.3.3 MicroArrays

It became clear during the analysis of my arrays that there was a reasonable amount of variability in the number of microRNAs expressed in different cells lines, and also between different experiments on the same cell line. This is an inherent problem due to the complex methodology of microarray hybridisation. Every effort was made to improve this sensitivity by optimising the methodology, and by having a low threshold for repeating the microarray if the sensitivity was poor. However, it is clear from the
results that not all microRNA expression is demonstrated using these methods. Furthermore, there may well be functional microRNAs that are yet to be discovered and are therefore not represented in these arrays.

However, although my arrays may have failed to produce a complete list of all expressed microRNAs in these cells, the low sensitivity improves the confidence that the microRNAs that were highlighted are reliably expressed.

Reliability in results was further improved by strict inclusion criteria, with only microRNAs expressed in all three biological replicates, across all three cell lines, included in analysis. This stringent method of exclusion may well have ignored many microRNAs that could be functionally important; however, I felt that the robustness of the results was of greater importance than quantity. Therefore, the completeness of microRNA expression in CC-RCC may not be answered by these results, but it is my hope that the most important individual microRNAs have been highlighted.

5.3.4 RT-PCR

RT-PCR is established as a more accurate measure of expression, compared to microarray analysis. I was unable to validate all of my results by this method, only the microRNA that was highlighted as the most interesting, miR-23a. Validation of the others (miR-15b, miR-103, miR-24 and miR-21) would be of value and interest in the future.

MiR-23a was highlighted as the only robustly expressed microRNA in CC-RCC, which was consistently differentially regulated. The RT-PCR confirmed the microarrays in proving that miR-23a is expressed in all the cell lines; however the direction of regulation was different in 768-0 when compared to the arrays. It did confirm a significant up-regulation in RCC4 and UMRC2 cell lines.
Assuming the PCR data to be the true reflection of the biology, it is possible that the differential expression of miR-23a is reflective more of the dominant HIF isoforms expressed. 768-0 does not express HIF-1alpha, only HIF-2alpha, whilst the other two cells express both HIF isoforms. Therefore it is possible that HIF-1 and HIF-2 have a reciprocal effect on miR-23a expression. Dependent upon the potential targets of miR-23a this may induce pro-tumourigenic or anti-tumourigenic effects, in keeping with the hypothesis from chapter 4 of this thesis, and the results of Raval et al. (Raval, Lau et al. 2005).

5.3.5 MicroRNA 23a

5.3.5.1 Background
MicroRNA 23a (AUCACAUUGCCAGGGAUUUCC) is a 21 nucleotide microRNA that was first identified in 2001 (Lagos-Quintana, Rauhut et al. 2001). It is highly conserved (expressed in human, rhesus monkey, mouse, dog, horse, platypus, lizard and chicken) (University of California Santa Cruz - Genome BioInformatics). Kawasaki and Taira reported that miR-23 regulates the transcriptional repressor Hairy enhancer of split (HES1) (Kawasaki and Taira 2003). However, this finding was later retracted after the discovery that the regulated gene was human homolog of ES1 (HES1), whose function is unknown.

5.3.5.2 Potential Targets
MiRBase (Sanger Institute (Olapade-Olaopa, Ogunbiyi et al. 2001; miRBase Database)) highlights 1227 potential targets for miR-23a. Ranked according probability of regulation (via miRBase algorithm), Lactate Dehydrogenase-B (LDH-B) is registered as 25th on the list, with a p-value of 0.000006.
5.3.5.3 Lactate Dehydrogenase (LDH)
LDH catalyses interconversion of lactate to pyruvate. Two genes LDH-A and LDH-B encode five LDH isoenzymes, each with variability of localisation within different human viscera and differential effects on regulation of aerobic glycolysis. LDH-A is a known target of HIF-1, and has greater efficiency in aerobic glycolysis in hypoxic conditions (Koukourakis, Giatromanolaki et al. 2005). Loss of LDH-B expression will lead to greater LDH-A expression and increased malignant potential of cells (Leiblich, Cross et al. 2006). LDH-B has been shown to be repressed by hypoxia in CC-RCC cells (Maxwell, Wiesener et al. 1999) and its promoter has been shown to be hypermethylated in prostate cancer (Leiblich, Cross et al. 2006).

LDH-B is a potentially interesting target for miR-23a as it is repressed by hypoxia compared to LDH-A, a HIF-1 target. It may be possible that the expression level of miR-23a is reflective of LDH-B or inhibits it? Therefore, in a hypoxic tumour environment LDH-A is upregulated by HIF-1 binding to its HRE, whilst LDH-B is repressed by an interaction with miR-23a. This would give the tumour cell greater aerobic glycolytic efficiency and encourage tumour cell survival.

This differential expression in tumour cells that express either HIF-2 only or both HIF isoforms suggests that miR-23a’s function is likely to be regulated by HIF rather than interacting directly with pVHL in CC-RCC.

5.3.6 Future Work
Differential protein expression levels of LDH-A and LDH-B within these cell lines in normoxia and hypoxia would be of value in examining the interaction between hypoxia, HIF, miR-23a and LDH. Furthermore it would be of great interest to explore the expression patterns of miR-23a in cells that have had either HIF-1 or HIF-2 knocked down, as well as the change in
gene expression if miR-23a was knocked down or over expressed. I believe it is the plan for my research successors in my institution to explore these, and hopefully the function and true significance of miR-23a will be elucidated in CC-RCC.
Chapter 6: Final Discussion and Conclusions
Chapter 6: Final Discussion and Conclusions

6.1 Final Discussion

The analysis of all the RCC nephrectomies from Oxford (1983-2007) in this thesis has clearly demonstrated that patient survival has dramatically improved over this time period. The most important driving influences behind this trend appear to be the decrease in tumour size at time of operation, and the number of operations performed. This would certainly fit with the increased quality and quantity of diagnostic imaging, and the greater number of incidental (pre-symptomatic) tumours that are being detected worldwide (Chow, Devesa et al. 1999). Detection of renal tumours whilst they are locally confined and amenable to curative nephrectomy, clearly affords the patient a survival benefit.

Further to this, analysis of Fuhrman grade within these tumours has shown that 5-year survival has significantly improved from grade 1 and 2 tumours, yet survival of grade 3 and 4 tumours is unchanged (See figures 3.8 and 3.9). Additionally, analysis of maximum tumour diameter at nephrectomy demonstrated a stable incidence rates of tumours >8cm, whilst tumours <8cm have a shift in their distribution towards smaller maximal diameters. Taken together this suggests that although there has been increased detection of tumours of a smaller size, there has also been a change in the biology of these tumours. It is possible that Fuhrman grade increases as the tumour grows, which again may be due to changes in the molecular pathways of the tumour as a response to hypoxia or other stimuli. Alternatively the increased detection of renal tumours by imaging may be causing Urological Surgeons to remove renal tumours that may not have proved clinically significant to the patient. However, without further understanding of the different molecular pathways and natural clinical courses in these two possible alternative renal tumours, it is impossible to
withhold known curative treatment, for patients diagnosed with a renal tumour by imaging.

Although this thesis has failed to identify clear correlations between the HIF isoforms or the primary hypoxic target genes (except cyclinD1) is has successfully managed to highlight many other interesting areas.

This thesis has clearly demonstrated that the antigenicity of HIF does deteriorate with the age of the paraffin-embedded block from which the TMA core is derived. Although this phenomenon has previously been described it is the first time it has been shown for HIF isoforms, and is a very important finding since it casts into significant doubt previous literature about HIF-1 and HIF-2 immunostaining in human cancers using these particular antibodies.

The subsequent lack of significance of the HIF isoforms with each other or their target genes is surely related to the loss of over 50% of the clinical samples in this series. However it would have been incorrect to include known erroneous data into the analysis. Further work will be required assessing the relationship of the HIF isoforms in ccRCC, with larger numbers of samples from fresher specimens, although unfortunately this would extend beyond the scope of this thesis.

An additional factor is the methodology used to assess this HIF isoforms in these tumours. TMAs have certainly allowed a large number of tumours to be assessed together, adding statistical weight to the correlations shown in this thesis. The real situation however may be more complex within the tumour as a whole. The TMA cores have only sampled a representative histological area; however the remaining tumour may be significantly different when pertaining to dominant HIF isoform staining. Further work on these TMAs is currently being conducted to examine HIF-1 and HIF-2
within the whole tumour section. Correlation between HIF staining on the TMAs and whole sections will be important to ascertain whether this is truly significant.

Further work in this thesis has demonstrated that differential levels of angiogenesis, as measured by the CD31\(^+\) VVI, have a significant prognostic value in ccRCC. High levels of CD31\(^+\) VVI conferred a better overall disease prognosis when compared to low CD31\(^+\) VVI and higher levels were also expressed within the late death cohort patients when compared to their early death counterparts.

Although at first appearance, a high VVI conferring a better overall survival seems counterintuitive, it may be that CD31\(^+\) endothelial cells represent either undifferentiated or differentiated endothelium, and the CD31\(^+\) vessels identified in this thesis are functional. This may mean that a high VVI is related to good tumour microcirculation and ultimately better tumour perfusion. This would enable reduction in sustained periods of ischaemia, unlike the low VVI group, from which hypoxia death-resistant clones could arise facilitating tumour metastasis.

Furthermore, it is possible that high CD31\(^+\) angiogenesis represents ordered new vessels growth, resembling normal tissue. Low CD31\(^+\) VVI may therefore represent disorganised angiogenesis, itself a reflection of many angiogenic tumour stimuli and pathways. It maybe that it is this difference in angiogenic stimulation that truly represents the discrepancy in patient outcome seen.

Additionally, the negative correlation of angiogenesis (CD31\(^+\)) to Fuhrman grade and tumour size suggests that hypoxic pathway changes as a tumour increases in size. Although no specific correlations to HIF have been identified in this thesis, it may be that tumours >8cm have alterations in
their angiogenic pathways leading to a poorly differentiated and aggressive phenotype.

The finding of high CD31\(^+\) VVI being indicative of better overall survival in primary CC-RCC does potentially highlight one problem as regards anti-angiogenic therapies. Novel agents such as bevacizumab and sorafenib are potential candidates for both neo-adjuvant (pre-nephrectomy) and adjuvant (post-nephrectomy) therapies in primary CC-RCC. However, one would also need to ascertain whether the angiogenesis within the primary tumour was functional or not, as this data implies that if CD31\(^+\)angiogenesis were reduced by such drugs, patient survival may be detrimentally affected.

Due to the complex nature of microRNA microarrays a complete pattern of microRNA expression has been impossible to demonstrate. However the emergence of microRNA 23a from the array data, and its subsequent validation via PCR has highlighted an interesting target for further study. This is particularly the case due to the possible differential effect of HIF-1 and HIF-2 expression of miR-23a.

Target prediction remains a difficult problem within the microRNA field, with many thousands of targets commonly predicted by the best available algorithms. Functional studies will eventually overcome this difficulty, but it will take time for these results to emerge. MicroRNA-23a is currently under further investigation within the institution where this work was performed, and hopefully its role in the hypoxic pathway will become clear in the near future.

6.2 Conclusion
In conclusion, this thesis has shown kidney cancer to be an aggressive and lethal condition that is becoming increasingly prevalent. Our current management is working well with patient prognosis increasing; however, it
is through the further understanding of the biological nature of the disease that we will be able to develop novel treatments targeted against it. This will undoubtedly be to the benefit of patients, and I hope that the work contained within this thesis has brought that closer.


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