MOLECULAR CHARACTERISATION OF RENAL CELL CARCINOMA AND RELATED DISORDERS

by

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A thesis submitted to
the University of Birmingham
for the degree of
DOCTOR OF PHILOSOPHY

School of Clinical and Experimental Medicine
College of Medical and Dentistry
September 2015
ABSTRACT

Over the last two decades genetic advances have provided novel insights into the molecular basis of familial and sporadic cancers and provided the basis for the development of novel therapeutic approaches. For example, the identification of the gene for von Hippel Lindau disease provided seminal insights into its role in most clear cell renal carcinomas (RCC) and led to new treatments for RCC.

In this thesis I investigated three related genetic aspects of neoplasia. Firstly, I analyzed the results of genetic testing for inherited phaeochromocytoma and investigated how clinical features could be used to stratify patients and improve the cost effectiveness of genetic testing. Secondly, I sought to identify novel causes of inherited neoplasia. Through exome sequencing of familial RCC kindreds, CDKN2B was identified as a novel familial RCC gene. The role of CDKN2B mutations in neoplasia was evaluated in familial and sporadic RCC and phaeochromocytoma. In vitro assays confirmed that germline CDKN2B mutations associated with inherited RCC caused an abrogation of tumour suppressor function. Finally, I explored how a gene-based strategy might be used to identify novel therapeutic strategies. Thus, using a siRNA library screen, in RCC cells with inactivated VHL, potential candidate targets (e.g. PLK1/STK-10) were identified for selectively decreasing the viability of RCC cells with inactivated VHL.
ACKNOWLEDGMENTS

Firstly I would like to acknowledge God for his many blessings. My mother, Kishwar Jafri has been an immense support to me throughout my life and is a passionate believer in the value of education. During my research, my mother, along with my sister and brother have always stepped in without complaining to look after my two young children, Yusuf and Idris, when I was working late or at weekends or when my experiments ‘over-ran’. Their love and support have not only enabled me to achieve my goals but also is a great comfort to the children who have not missed their mother. I am also thankful to my father who although he passed away many years ago, instilled me with a sense of curiosity and desire for self-improvement. My husband has also had to endure many weekends and bank holidays of me working in the laboratory and not being in the home. His patience has been amazing.

I am very grateful to staff in the Department of Medical Genetics, they welcomed me into the department and became my friends. In particular, I’d like to thank, Mr Dean Gentle, Dr Dewi Astuti and Dr Malgosia Zatyka who provided me with friendship and technical support.

Lastly, I would like to thank my two supervisors. Professor Eamonn Maher had infectious enthusiasm for science and modesty regarding his many achievements and Professor Farida Latif who was equally modest and taught me a lot about having an inquiring scientific mind. I really enjoyed my training in this rapidly developing field and hope it will hold me in good stead for the rest of my career. Most importantly, I hope it will enable me to help my patients, who suffer with cancer, to have more effective and less toxic treatments.
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<tr>
<td>ACMG</td>
<td>American College of Medical Genetics</td>
</tr>
<tr>
<td>aPCA</td>
<td>adrenal phaeochromocytoma</td>
</tr>
<tr>
<td>BAP1</td>
<td>BRCA associated protein 1</td>
</tr>
<tr>
<td>BRAF</td>
<td>B-Raf proto-oncogene</td>
</tr>
<tr>
<td>BRCA</td>
<td>breast cancer 1</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin dependent kinase</td>
</tr>
<tr>
<td>CPG</td>
<td>cancer predisposition genes</td>
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<tr>
<td>CRISPR</td>
<td>clustered regularly spaced short palindromic repeats</td>
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<td>double distilled water</td>
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<td>eFPGL</td>
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</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>EMT</td>
<td>epithelial-mesenchymal transition</td>
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<td>fumarate hydratase</td>
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<td>FISH</td>
<td>fluorescence in situ hybridization</td>
</tr>
<tr>
<td>GWAS</td>
<td>genome wide association studies</td>
</tr>
<tr>
<td>HIF</td>
<td>hypoxia inducible factor</td>
</tr>
<tr>
<td>HLRCC</td>
<td>hereditary leiomyomatosis associated renal cell carcinoma</td>
</tr>
<tr>
<td>HNPGL</td>
<td>head and neck paraganglioma</td>
</tr>
<tr>
<td>HR</td>
<td>homologous recombination</td>
</tr>
<tr>
<td>HRPC</td>
<td>hereditary papillary renal cancer</td>
</tr>
<tr>
<td>IDH1</td>
<td>isocitrate dehydrogenase 1</td>
</tr>
<tr>
<td>KIF1B</td>
<td>kinesin family member 1B</td>
</tr>
</tbody>
</table>
MAX MYC associated factor X
MEN2 multiple endocrine hyperplasia type 2
MLPA multiplex ligation dependent probe amplification
MTC medullary thyroid cancer
MTOR mechanistic target of rapamycin also known as mTOR (mammalian target of rapamycin)
NF1 neurofibromatosis 1
NGS next generation sequencing
OS Overall survival
PARP Polyadenosine diphosphate [ADP] ribose polymerase
PD-1 programmed death 1
PDL1 programmed death ligand 1
PHD2 Also known as EGLN1 egl-9 family hypoxia inducible factor
PBRM1 polybromo 1
PBS phosphate buffered saline
PCR polymerase chain reaction
PFS progression free survival
PGL paraganglioma
PPGL phaeochromocytoma and paraganglioma
PHD HIFα prolyl hydroxylases
PLK1 Polo-like kinase 1
PNMT phenylethanolamine N-methyl transferase
PPGL phaeochromocytoma and paraganglioma
PTEN phosphatase and tensin homologue deleted from chromosome 10
RB1 retinoblastoma 1
RCC renal cell carcinoma
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>RET</td>
<td>ret proto-oncogene</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RYK</td>
<td>receptor-like tyrosine kinase</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SDHB</td>
<td>Succinate Dehydrogenase B subunit gene</td>
</tr>
<tr>
<td>SDHD</td>
<td>Succinate Dehydrogenase D subunit gene</td>
</tr>
<tr>
<td>SDHx</td>
<td>succinate dehydrogenase</td>
</tr>
<tr>
<td>SDHAF2</td>
<td>succinate dehydrogenase complex assembly factor 2</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphisms</td>
</tr>
<tr>
<td>SPRI</td>
<td>Solid Phase Reversible Immobilisation Beads</td>
</tr>
<tr>
<td>STK-10</td>
<td>serine threonine kinase 10</td>
</tr>
<tr>
<td>TALENs</td>
<td>transcription activator like effector nucleases</td>
</tr>
<tr>
<td>TCGA</td>
<td>The Cancer Genome Atlas</td>
</tr>
<tr>
<td>TMEM127</td>
<td>transmembrane protein 127</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>TNM</td>
<td>tumour, nodes, metastases</td>
</tr>
<tr>
<td>TP53</td>
<td>tumour protein p53</td>
</tr>
<tr>
<td>TSG</td>
<td>tumour suppressor gene</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VHL</td>
<td>Von Hippel Lindau (VHL represents Von Hippel Lindau gene)</td>
</tr>
<tr>
<td>v/v</td>
<td>volume of solute/volume of soluble</td>
</tr>
<tr>
<td>WES</td>
<td>whole exome sequencing</td>
</tr>
<tr>
<td>WGS</td>
<td>whole genome sequencing</td>
</tr>
<tr>
<td>WMRGS</td>
<td>West Midlands Regional Genetics Service</td>
</tr>
</tbody>
</table>
CHAPTER 1: INTRODUCTION

1.1 Current clinical management of cancers
Cancer is defined by the World Health Organisation as ‘the uncontrolled growth and spread of cells’ (1). Until recently, the diagnosis and treatment of cancer was tissue specific. Diagnosis was largely achieved by biopsying areas of potentially cancerous tissue and evaluating them under the microscope. Histopathological features such as nuclear grade and cell type enabled diagnoses with the aid of immunohistochemical markers (2). Treatment of metastatic solid organ tumours rested predominantly on empirical chemotherapeutic agents which targeted proliferating cells. Such treatments are associated with significant systemic toxicity, and when used in metastatic adult solid organ cancers with the exception of some cancers such as testicular cancer and lymphoma, are infrequently associated with cure (3). Response rates to chemotherapeutic agents can be modest and vary depending on context and disease stage for example in advanced breast cancer can be between 18 and 30% (4).

The initial sequencing of the human genome in 2000 (5) has greatly facilitated the field of genomics (the study of genes and their function). There has been much interest in the study of the molecular basis of cancer and it has been described as a disease driven by genetic changes. (6) This research project was undertaken in the context of an evolving paradigm shift in the treatment of patients with cancer away from ‘site specific treatments’ to treatments based on specific genetic variants in tumours of individuals with cancer. Therefore, the work presented in this thesis has taken place in an exciting time for translational oncology where important improvements have taken place in patient care due to close collaboration between laboratory scientists and clinicians managing individuals with cancer. This change has in no small part been achieved by the work of the previous three decades; understanding important
cancer genes, signaling pathways and importantly the developments in cancer genetics attributed to the human genome project. Thus, in the introduction to this thesis both the clinical and basic scientific background to current cancer management and research is discussed. The aim of this research fellowship is to better understand the molecular pathology of cancer in order to facilitate more personalized cancer care in the future.

1.1.1 Clinical management of solid organ cancers
Despite a decade of improvements in the management of cancers described in section 1.4, in the case of adult cancers, with the exception of lymphoma and testicular cancers, unless tumours are detected and treated at a local stage (i.e. without evidence of metastatic spread) they tend to be incurable. The main treatment of solid organ tumours is either surgical resection alone, a combination of radiotherapy and surgery or in some cases radiotherapy at a radical (‘curative’) dose. These treatments aim to remove all macroscopic disease and thus eradicate the complications of local tissue invasion and spread. However, in the treatment of many cancers despite there being no clinical evidence of disease post radical treatment (surgery and/or radiotherapy), patients can succumb to metastatic disease many years later. This is presumed to be due to the presence of micro-metastases known as the ‘seed’, which move via the blood and/or lymphatics away from the primary site and once in the appropriate microenvironment or ‘soil’ develop into macroscopic deposits if they receive adequate nutrition. These metastatic deposits are responsible for the significant morbidity and mortality associated with cancer (7). Metastatic disease if localized to specific areas can be treated with local therapies such as; surgical resection, radiotherapy and radiofrequency ablation. However, widespread recurrent disease requires systemic treatment. To reduce the chance of metastases developing, patients can receive adjuvant or neo-adjuvant treatment to
help eradicate micro-metastases. These are adjuncts to radical treatments and typically comprise either chemotherapy, hormonal therapy or both dependent on the tumour type (8, 9).

1.1.2 The development of chemotherapy as a treatment for cancer

Until recently the commonest method of treating disseminated disease was chemotherapy. Chemotherapy is usually either administered as oral tablets or more commonly intravenously. Although the term chemotherapy was first coined in the early 1900s, its development has been partly serendipitous. Different agents have differing mechanisms of action summarized in Table 1.1.2. The first chemotherapy agents were developed when it was noted that individuals exposed to mustard gas experienced depletion of the bone marrow and lymph nodes (10). Mustard compounds were subsequently used in lymphoma. Mustard compounds are alkylating agents which form covalent links with DNA thus inhibiting DNA replication. Numerous other alkylating agents have been produced such as; chlorambucil and cyclophosphamide. Alkylating agents effect proliferating cells and are thus unable to differentiate between rapidly dividing normal cells and malignant cells. This can lead to toxicities affecting the gastrointestinal, haemopoetic systems and alopecia. Despite these toxicities, these agents were responsible for the first durable responses demonstrated in the treatment of lymphoma. (10) Platinum agents such as cisplatin also cause adducts to DNA primarily within the same strand (11). The adducts cause DNA damage which result in cellular apoptosis (11). Cisplatin effects proliferating cells and can cause toxicity, classically; peripheral neuropathy, ototoxicity and nephropathy in addition to nausea, vomiting and alopecia. Cisplatin is commonly used today. In germ cell tumours cisplatin is responsible for cure (12). Platinum agents produce durable responses in epithelial ovarian cancer (13), and
are used in the adjuvant/neoadjuvant setting in a wide range of cancers including lung (14) and breast cancer (15).

Antimetabolites were the next class of drug identified in the treatment of cancer, folic acid is required for bone marrow function and methotrexate, an inhibitor of folate metabolism was shown to cause remissions in paediatric leukaemias.(10) Methotrexate continues to be used today and also affects normal proliferating cells. Pemetrexed is a multi-targeted folate antagonist which is active in mesothelioma and non-small cell lung cancer.(16) In the 1950s, rat hepatomas were found to use more uracil than normal cells. Thus, 5-FU was produced, it could be taken up by tumours and would replace the uracil reducing cell growth. 5-FU remains the key agent in the management of colorectal cancer.(10)

Another common mode of action of anti-cancer drugs is targeting tubule formation required for cell division. There are two main anti-microtubule agents; vinca alkaloids derived from the periwinkle (e.g. vincristine and vinblastine) and taxanes derived from the Pacific Yew Tree (docetaxel and paclitaxel). These function by inhibiting microtubule formation and thus mitosis.(17) These agents are also associated with toxicity due to their non-selective action and cause alopecia and neuropathy in addition to gastrointestinal and haemopoietic toxicity. Taxanes are used in a wide range of malignancies including breast (18), lung (19) and oesophago-gastric cancers (20). The vinca alkaloids are used in a wide range of paediatric and adult cancers including leukaemia, lymphoma and sarcoma(21).

Chemotherapy is given on a cyclical basis and scheduling is important. This is because at any given dose of drug, a constant fraction of cells are killed ‘the Cell Kill hypothesis’(10) Thus, the number of remaining cancer cells is influenced by the number of cells present at the beginning of a cycle. Treatment is given repeatedly at a time where there is an equipoise
between toxicity to ‘normal tissues’ and prevention of cancer cell growth. Combining different agents into a regimen enables more cancer cells to be killed at any one time by utilizing a number of varied anti-cancer mechanisms (22).

Over five decades in the last century, medical oncology has developed using chemotherapeutic agents to treat cancer. These drugs were used to influence rapidly dividing cells which causes some associated toxicity to normal tissues. Thus, there was a need for targeted therapies and these were established through better understanding of the pathology of cancer. Currently available targeted therapies will be described in section 1.4, however the basic science of tumourigenesis will be described first.

Table 1.1.2. A summary of the mechanism of action of different chemotherapy drugs.

<table>
<thead>
<tr>
<th>Drug Class</th>
<th>Example</th>
<th>Mechanism of action</th>
<th>Clinical uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vinca alkaloids</td>
<td>Vincristine/vinblastine</td>
<td>Microtubule inhibitor</td>
<td>Haematological malignancies/sarcoma</td>
</tr>
<tr>
<td>Taxanes</td>
<td>Docetaxel/paclitaxel</td>
<td>Microtubule inhibitor</td>
<td>Breast/lung/upper GI/prostate</td>
</tr>
<tr>
<td>Antimetabolite</td>
<td>5FU/methotrexate/pemetrexed</td>
<td>Antimetabolites</td>
<td>Colorectal, breast, lung cancer</td>
</tr>
<tr>
<td>Alkylating agent</td>
<td>Chlorambucil/cyclophosphamide</td>
<td>Covalent links across DNA stopping DNA replication</td>
<td>Haematological malignancies</td>
</tr>
<tr>
<td>Platinum agents</td>
<td>cisplatin</td>
<td>Adduct formation causing DNA damage and apoptosis</td>
<td>Germ cell, lung, upper GI and breast cancers</td>
</tr>
</tbody>
</table>
1.2 Genomics and cancer medicine

1.2.1 Overview of the genetics of cancers

1.2.1.1 Hallmarks of cancer: pathways to human cancer
Cancer has been eloquently described by Hanahan and Weinberg (23) in their seminal review as a ‘disease involving dynamic changes in the genome’. This definition encompasses many diseases with different characteristics but at whose heart is genetic alteration. Cancers grow and cause morbidity because of their ability to adapt to differing milieu such as inadequate blood supply by gaining new genetic alterations allowing them to evolve. Thus, it is important to reflect on the general pathophysiological processes that occur in normal cells over a period of time in order for them to become malignant. These processes have been termed the ‘Hallmarks of cancer (23)’ and are described in figure 1.2.1. These characteristics have been summarized below, however for a more detailed explanation the reviews by Hanahan and Weinberg are recommended.(23, 24) In order for a cancer to develop, changes occur not only in the cancer cells but also in surrounding stromal cells, thus these hallmark traits involve the tumour microenvironment in addition to the tumour cells themselves.(24)

1.2.2 The step-wise process of cancer development and accumulation of genetic alterations
Cancer is a step-wise process. Cancer cells gain genetic alterations that enable them to achieve the processes described as the ‘Hallmarks of Cancer’. These are: unlimited proliferation potential, resistance to apoptosis, self-sufficiency in growth signals, angiogenesis, immune evasion and the ability to metastasize.(25) The cancer phenotype is achieved by ‘re-wiring’ pre-existing signaling pathways within the cell. This is achieved by genetic alterations in key genes. Cancer develops as cells with alterations involving such pathways survive and grow faster producing a clone which can grow more efficiently than other cells in the same situation where other cells would not.(25) pRb was the first tumour
suppressor gene (TSG) (see section 1.2.3 for a more detailed explanation) to be described, it remains unique as inactivation of pRb is both necessary and sufficient for the development of retinoblastoma (26). Most cancers need between four and seven rate limiting genetic events to develop (27). Pathological examinations of the colonic tract have identified pre-cancerous lesions that progress to become cancers (the adenoma to carcinoma sequence described in colorectal cancer)(28). 70% of colorectal adenoma contain a mutation in APC, then subsequently mutations occur in KRAS and P53. These mutations allow cell growth and decreased DNA repair. There is associated chromosomal instability, thus progressive mutations enable the adenoma to develop in to a carcinoma by gaining the ‘hallmarks of cancer’, being able to have a proliferative advantage and invade other tissues.(28) These transitional steps in cancer are exploited in screening programmes for cervical and colorectal cancer. Thus, cancer develops in a process analogous to Darwinian evolution where successive genetic changes allow cells to gain growth advantages, leading to the progressive conversion of normal cells to cancer cells.(23).

1.2.2.1 The hallmarks of cancer

Key Characteristic 1- Sustaining Proliferative Signaling
In healthy tissue there is strict control of growth by careful control of the production and release of growth promoting signals which allow cell cycle entry and progression. Growth factors bind to receptors typically containing intracellular tyrosine kinases which activate branched intracellular signaling pathways that regulate cellular growth and cell cycle progression. This control is abrogated in cancer cells, allowing cells to become more autonomous and replicate at higher rates. Increased intracellular signaling can also influence cell survival and energy metabolism. Sustained signaling can occur by: (i) altered growth factor production, (ii) deregulated receptor expression (iii) structural changes in the receptors
leading to ligand independent signaling and (i.v.) constitutive activation of downstream signaling pathways. This activation is achieved by mutations in genes encoding key proteins. Other mechanisms of uncontrolled cellular proliferation are the loss of negative feedback loops. For example phosphatase and tensin homologue deleted from chromosome 10 (PTEN) negatively regulates PI3kinase, PTEN loss is associated with a number of different cancers. Cancer cells can also continue to proliferate by avoiding senescence and apoptosis. For example, in cultured cells high levels of RAS, MYC and RAF expression are associated with senescence however, in melanoma cells for example mechanisms leading to senescence and apoptosis are overcome.(29)

Key Characteristic 2 - evasion of growth suppression
Cancer cells avoid growth suppressing mechanisms within cells. TSG play a key role in preventing inappropriate cell proliferation. pRB transduces growth inhibitory signals that originate largely outside the cell. If cells have excessive genomic damage, or insufficient nucleotide, oxygen or glucose reserves, p53 can halt the cell cycle until conditions improve or if the situation is irretrievable, trigger apoptosis. Thus, inactivation of TSG such as p53 or pRb allows evasion of growth suppression. (23, 24) These genes and genes connected to them are commonly inactivated in cancers.

Key Characteristic 3 - Resisting Cell Death
Apoptosis or programmed cell death usually occurs when cells experience physiological stressors such as hypoxia or hypoglycaemia.(24) Thus, cancer cells must gain mechanisms to avoid apoptosis. Apoptosis is controlled by two main systems: (i) internal stresses such as DNA damage are monitored by p53 protein (24), the so called ‘intrinsic system’ (ii) external stressors are monitored by the Fas ligand and receptor, this is the ‘extrinsic system’. Better understanding of the cells apoptotic machinery and how cancer evades these mechanisms has
been achieved.\(^\text{(24)}\) This hallmark is believed to be an important factor in cancer development.

Another mechanism by which cancer cells grow is by inhibiting autophagy, a process by which cellular organelles are broken down and recycled. Autophagy occurs in times of cellular stress such as nutrient deficiency. Autophagy allows organelles to be broken down and their catabolites to be used by other cells in nutrient deficient environments. Thus, by being relatively resistant to autophagy, cancer cells can benefit from the catabolites produced by neighbouring healthy cells.\(^\text{(24)}\)

**Key Characteristic 4 - Enabling Replicative Immortality**

Most cells in the body are only able to undergo a certain number of replication cycles. However, cancer cells have an unlimited replication potential achieved by loss of senescence and by altering telomeric length. In normal cells, telomeres are tandem hexanucleotide repeats at the end of chromosomes, each time the cells divide they become shorter. Eventually they are so short they cannot protect the ends of DNA, and the chromosomes join up, and unstable chromosomes lead to the cell becoming non-viable. In cancer cells, there is activation of the telomerase enzyme which adds telomeres to the ends of DNA, thus the number of cell divisions is not limited. These mechanisms work together to promote uncontrolled cellular growth, e.g. early in cancer development, cells experience a telomere loss crisis allowing breakage-fusion-break cycles to occur. This allows deletions and amplifications of the genome to occur. However, apoptosis does not occur as TP53 mediated surveillance of genome integrity is impaired, subsequent telomerase activity allows defective cells to proliferate despite acquired mutations.\(^\text{(24)}\)
The hallmarks of cancer as described by Hanahan and Weinberg. (24)

<table>
<thead>
<tr>
<th>Enabling Characteristic</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genome Instability and Mutation</td>
<td>Successive alterations give cancer cells a survival advantage. This requires the caretakers of the genome which include genes that detect DNA damage, repair damage or those genes which inactivate DNA damage (e.g. p53) to not function properly.</td>
</tr>
<tr>
<td>Tumour Promoting Inflammation</td>
<td>Leucocytic infiltration of tumours promotes tumour growth by producing bioactive factors and growth factors aiding processes such as epithelial-mesothelial transition, proliferation and angiogenesis.</td>
</tr>
<tr>
<td>Reprogramming Energy Metabolism (Warburg effect)</td>
<td>Anaerobic glycolysis occurs in normoxic conditions. This is less efficient than aerobic respiration, therefore cancer cells utilize more glucose. Glycolysis produces intermediates that are substrates required for active cell proliferation. Tumours may contain two populations of cells; one producing glucose aerobically and the other anaerobically, thus balancing energy and substrate production.</td>
</tr>
</tbody>
</table>

Table 1.2.2 The Enabling Characteristics of Cancer. (taken from Hanahan and Weinberg) (24)
Key Characteristic 5- Induction of Angiogenesis
Tumours need a blood supply to gain nutrients and eliminate waste. In adults, angiogenesis is quiescent and ‘switched on’ only transiently e.g. in wound healing or within the female reproductive tract. Cancers are dependent on angiogenesis as they require new vessel formation in order to provide nutrients and eliminate waste so that they can continue to survive and grow. Thus, in cancers, the ‘angiogenic switch’ is permanently switched on. Angiogenesis is usually under finely balanced control achieved by counterbalanced growth factors. For example, vascular endothelial growth factor (VEGF) binds to one of three receptor tyrosine kinases (VEGFR1-3), and its downstream signaling is regulated at many levels reflecting its important and complex function. VEGF signaling can be upregulated by hypoxia but is abnormally activated in tumours.(24) The abnormal angiogenesis seen in cancer also allows tumour cells to gain nutrients and leave their environment and metastasize. Aberrant angiogenesis is a target for many novel cancer therapies including in renal cell carcinoma (RCC). (24, 30)

Key Characteristic 6- Activation of invasion and metastasis
Cancers cause clinical morbidity due to their ability to invade and metastasize. In order to invade and metastasize cancer cells need to detach from other cells and the extracellular matrix and alter shape. This process is known as the ‘invasion-metastasis cascade’ Simply, these are successive changes including: invasion of local tissue, intravasation of cancer cells into the blood and the lymphatic tissues, escape of cancer cells into the distant tissues, formation of micrometastases and finally development of macroscopic tumours i.e. ‘colonialisation’. (24) This cascade is controlled by the epithelial-mesenchymal transition (EMT). This is a process whereby transcription factors achieve a change in the cell biology by facilitating the following changes; loss of adherens junctions, change of cell shape to a
spindle shape, expression of matrix degrading enzymes, increased motility and increased resistance to apoptosis. One mechanism by which this is achieved is through loss of E-cadherin protein which is important in cell-to-cell contact. (24)

1.2.2.2 Other important enabling characteristics of cancer and stress factors

**Enabling Characteristics**

In addition to the key characteristics of cancer, there are a number of enabling characteristics that allow tumorigenesis to occur. These include: (i) genome instability, (ii) reprogramming metabolism, (iii) tumour promoting inflammation and are summarized in figure 1.2.2.

**Genomic instability:** Mutant clones gain a selective advantage over the subclones. This is achieved by gaining successive alterations which increased the chance of the subclone to dominate the other cells. Genetic changes can occur due to epigenetic changes and mutation. Cells are normally resistant to mutation, this accumulation of mutations is achieved by; sensitivity to mutagenic agents and breakdown of genomic surveillance mechanisms. These are achieved by alteration in p53 and other ‘caretaker’ genes. Caretaker genes include those involved in: detecting DNA damage, activating DNA repair machinery, inactivating and intercepting mutagenic agents before they damage DNA. Furthermore, impaired telomerase activity causes karyotypic instability by causing chromosomal amplification and breakdown. (24)

**Leucocytic infiltration:** Tumours are associated with leucocytic infiltration similar to that noted in inflammation. Leucocytes aid tumorigenesis by: providing growth factors, degrading extracellular matrices, aiding angiogenesis, secreting survival factors and facilitating EMT. (24) Inflammatory cells also produce free radicals such as reactive oxygen species which are mutagenic promoting genetic instability.
**Anaerobic glycolysis:** Cancer cells utilize glycolysis instead of oxidative phosphorylation to meet their energy requirements, even in normoxic conditions. Glycolysis is eighteen times less efficient than oxidative phosphorylation and requires increased glucose utilization hence the up-regulation of glucose transporters like GLUT-1. The glycolytic switch is mediated by oncogenes such as RAS which can upregulate transcription factors such as HIF that increase glycolysis. The glycolytic pathway may confer an advantage on cells as it produces macromolecules which can be used to produce organelles required in rapidly proliferating cells. Within a tumour, there are some cells that utilize aerobic respiration, these cells utilize the lactate produced by cells using glycolysis. This enables a symbiotic relationship minimizing waste. (24)

**Immune evasion:** Tumour cells often express atypical proteins however they evade immune detection and eradication. This enables micrometastases to spread and tumour invasion to take place. The reasons why tumour cells evade normal immunological responses are incompletely characterized, however may be due to secretion of factors such as TGF-β which suppress immunity. (24)

**1.2.2.3 The stress phenotype of cancer**
Cancer cells typically display distinct stressors such as DNA damage, proteotoxic, mitotic, metabolic and oxidative stress. These occur in non-cancer cells but represent a potential vulnerability of cancer cells to cell death (25) therefore these maybe targets for cancer therapies.

**DNA damage and replication stress:**
Genomic instability causes cancer cells to have numerous genetic alterations including; mutations, deletions, chromosomal rearrangements and aneuploidy. (25) This instability
occurs due to endogenous DNA damage, combined with activation of a DNA damage stress response. The mechanisms have been described previously and can lead to translocations and amplification. These can cause oncogene activation and genomic instability which can be a target for cancer therapies.\(^{(25)}\)

**Proteotoxic Stress:** Aneuploidy and gene copy number differences can shift the cell towards expressing proteins associated with cell proliferation. There is also a difference in protein subunit production that can cause problems in protein stoichiometry, resulting in increases in quantities of toxic unfolded aggregates in the cell. The presence of unfolded protein aggregates produce proteotoxic stress which also can be exploited by targeted treatments.\(^{(25)}\)

**Mitotic Stress:** Chromosome mis-segregation causes a chromosome instability phenotype, this results in a shifting chromosome distribution allowing cells to evolve rapidly. This phenotype is present due to alterations in genes associated with mitosis and mitotic pathways. Furthermore, abnormal chromosome segregation can occur due to aforementioned problems in oncogenes, double strand breaks and loss of TSG.\(^{(25)}\)

**Metabolic Stress:** This glycolytic switch called the Warburg effect leads to increased use of glucose by the cancer cell. The glycolytic switch provides cancer cells with a survival advantage as they are able to thrive in low oxygen conditions. This promotes tumour invasion and immune evasion.\(^{(25)}\) Some researchers are exploiting the cancer cells dependence on glycolysis by targeting glucose uptake as a potential cancer treatment.\(^{(31)}\)

**Oxidative Stress:** Cancer cells produce more reactive oxygen species than normal cells, this occurs due to mitochondrial dysfunction and oncogenic signaling. Reactive oxygen species are highly reactive and contribute to increased levels of endogenous DNA damage.\(^{(25)}\) Reactive oxygen species may also represent a vulnerability of the cancer cell.
1.2.3 Oncogenes and Tumour Suppressor Genes

The fundamental genetic changes in cancer are in two sets of genes (i) oncogenes in which mutations lead to a dominant gain in function and (ii) tumour suppressor genes in which mutations have recessive loss of function (figure 1.2.3).

1.2.3.1 Oncogenes

The archetypical oncogene is RAS, which was shown to have a transforming function in the late 1970s (32). As more genes were found to be altered in cancer, it became apparent that the progression from the normal to malignant phenotype occurred due to the gain of alterations that enabled cells to evade normal homeostatic mechanisms(32). RAS is mutated in many human cancers including colorectal, melanoma and lung cancers. There are three RAS genes which encode four proteins (33). RAS promotes tumour formation by activating a number of mechanisms required for cancer formation. RAS proteins are transducers that enable cell surface receptors to be coupled to intracellular signaling pathways. Genetic alterations of RAS cause incessant activation of a large number of downstream effector pathways: mutated RAS allows cells in G0 to enter the cell cycle in the absence of growth factors, increases transcription of growth factors, growth factor receptors and other transcription factors required for cell cycle progression. The consequence of these genetic changes is rapid, less controlled cell growth. Examples of oncogenes and their functions are described in table 1.2.3.1.

Oncogenes are derived from proto-oncogenes by a number of different processes (Figure 1.2.3(B). These include activation by increased copy number (i.e. amplification) seen in HER2 positive breast cancers, translocation e.g. BCR/ABL in chronic myeloid leukaemia and point mutation commonly seen in B-RAF e.g. V600E in melanoma. The end result of these genetic changes are constitutive activation of the gene i.e. uncontrolled positive stimuli.
Cancer cells can become dependent on the products of these genes to continue to survive and this process is called oncogene addiction. Oncogene addiction is believed to be achieved by oncogenes producing strong pro-survival signals. This is perceived by some to be the ‘Achilles Heel’ of cancer cells and maybe utilized as a therapeutic strategy. The potential of this strategy is discussed in chapter 5 of this thesis.

<table>
<thead>
<tr>
<th>Oncogene</th>
<th>Associated cancer</th>
<th>Mechanism of action as per Hanahan and Weinberg</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Myc</em></td>
<td>Haematological malignancy, sarcoma and some epithelial cancers</td>
<td>Sustained signalling – transcription factor influencing 10-15% of cellular genes.</td>
</tr>
<tr>
<td><em>BRAF</em></td>
<td>Colon, melanoma, thyroid, ovary</td>
<td>Sustained signalling – Ser/Thr kinase</td>
</tr>
<tr>
<td><em>EGFR</em></td>
<td>Lung, upper gastric cancers</td>
<td>Sustained signalling – EGF receptor activation</td>
</tr>
<tr>
<td><em>ABL</em></td>
<td>Chronic myeloid leukaemia</td>
<td>Enhanced tyrosine kinase activity</td>
</tr>
<tr>
<td><em>Bcl-2</em></td>
<td>Diffuse B cell lymphoma</td>
<td>Resistance to cell death - anti-apoptotic protein</td>
</tr>
<tr>
<td><em>MDM-2</em></td>
<td>Liposarcoma</td>
<td>Resistance to cell death- complexes with p53</td>
</tr>
<tr>
<td><em>ALL1</em></td>
<td>Acute myeloid leukaemia</td>
<td>Sustained signalling, resistance to cell death – involved in chromatin remodelling</td>
</tr>
</tbody>
</table>

Table 1.2.3.1 Some oncogenes, their clinical associations and mechanisms of action (35, 36)

1.2.3.2 Tumour Suppressor Genes (TSG).
Knudson first described TSG in the context of retinoblastoma, a paediatric retinal tumour. Sporadic cases had a later onset than those inherited in an autosomal dominant manner. Knudson hypothesized retinoblastoma occurred due to molecular inactivation of both alleles of the retinoblastoma gene (Rb). Inherited retinoblastoma presents earlier, because individuals have one functional Rb gene and therefore only one allele needs to be inactivated.
Whereas in sporadic retinoblastoma inactivation of both alleles is required therefore, presentation occurs at a later stage. This is known as the ‘Knudson two hit hypothesis’ and is described in figure 1.2.3. Inactivation of a gene can occur due to: non-sense, frameshift, splice site and truncating mutations, deletions or epigenetic silencing. pRB is mutated in a wide range of cancers indicating its important role in cell cycle control (pRb has been shown to stop the expression of genes required for progression into the S phase of the cell cycle).(26) TSG mutations lead to loss of function, thus both alleles need to be inactivated to cause an effect, examples of common TSG are in table 1.2.3.2.

<table>
<thead>
<tr>
<th>TSG</th>
<th>Associated cancer</th>
<th>Mechanism of action as per Hanahan and Weinberg(24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF2</td>
<td>CNS malignancy e.g. schwannoma, ependymoma,</td>
<td>Sustained signalling – usually inhibits several key signalling pathways including mTOR and PI3 kinase.</td>
</tr>
<tr>
<td>P53</td>
<td>Many cancer subtypes</td>
<td>Resisting cell death – usually induces apoptosis</td>
</tr>
<tr>
<td>ARH1</td>
<td>Ovarian</td>
<td>Sustained signalling and resistance to cell death – usually inhibits PI3 kinase and other kinases, is associated with autophagy</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Breast, ovarian, prostate</td>
<td>Resisting cell death- involved in DNA repair</td>
</tr>
</tbody>
</table>

Table 1.2.3.2. Common TSG, their cancer associations and mechanisms of action (24, 37-39)

1.2.3.3 Mechanisms of genetic alteration in proto-oncogenes and TSG

In order for oncogenes and TSG to influence cancer development a genetic or epigenetic change is required. The common mechanisms of gene activation and silencing are described in figure 1.2.3. These include: point mutations, deletions, duplications, inversions, translocations, frame-shift, pre-mature stop, splice site disruption, and epigenetic silencing(40). Chromosome gain and loss can also lead to cancer associated genetic changes, aneuploidy has long been associated with tumour cells. In a typical cancer cell, 10% of the cancer genome is associated with gains and losses.(41) Data from whole genome sequencing
has also led to the identification of two processes associated with genetic alteration; chromothripsis and kataegis.

Chromothripsis occurs when usually one of two chromosomes is shattered and then scrambled fragments are misjoined on repair.\(^{(42)}\) It occurs in 2-3% of human cancers and is particularly prevalent in bone and paediatric cancers.\(^{(41)}\) It is believed to occur due to abnormal chromosome separation in mitosis, chromosomes are entrapped within micronuclei which prematurely condense and can then be pulverised. The process of chromothripsis can generate many oncogenes and TSG in one event.\(^{(43)}\)

Kataegis is localised areas of multiple point mutations that occur in \textit{cis} near rearrangement breakpoints.\(^{(41, 42)}\) This is believed to occur by activation-induced deaminase and apolipoprotein B mRNA editing enzyme catalytic polypeptide like protein families.\(^{(41)}\) Kataegis is much more common than chromothripsis occurring in one in forty cancer genomes.\(^{(43)}\) As with chromothripsis this process enables multiple mutations to occur at one time rather than in a step-wise fashion. Potentially, these mechanisms can lead to increased rapidity in oncogenesis.

\textbf{1.2.3.4 Driver and passenger mutations}

Mutations in oncogenes and TSG represent key changes in cancer. Cancers develop in a process where successive genetic changes allow cells to gain growth advantages which leads to the progressive conversion of normal cells into cancer cells.\(^{(23)}\) With the clonal evolution of cancers, an accumulation of genetic changes occurs, resulting in the presence of numerous mutations some of which are thought to be key to driving the process of oncogenesis (‘driver mutations’) whereas other mutations are so called ‘passenger’ mutations. Driver mutations are therefore defined as mutations which provide a growth advantage to tumour cells, whereas passenger mutations occur at the same time as driver mutations but do not influence cell
growth (44). In a somatic cancer cell there can be around sixty gene alterations which alter protein function (44). Determining which of these alterations are ‘driver mutations’ is a challenge facing cancer genomics. This is explored in chapter 4 of this thesis.

1.3 Developments in cancer genomics
The first genome was sequenced at a cost of $3 billion. The current cost of genome sequencing is approximately $5000.(45) The technological advancements in genomics have led to there being a great deal of information available about the cancer genome. Much of this information has been achieved by large multinational collaborations such as the Cancer Genome Atlas (TCGA) and International Cancer Genome Consortium (ICGC).(46) In fact, there has been a lag between improvements in the understanding and interpretation of genomic data compared to its production. The average solid tumour contains around sixty mutations that will alter protein function (47). Most of these substitutions are single nucleotide variations (95%), the remainder are usually single nucleotide insertions and deletions. Somatic cancers have more translocations than normal cells but these are not usually driver translocations and occur in non-coding regions, so called ‘gene deserts’.(47) Translocations are better tolerated in cancer cells compared to healthy cells as the normal DNA damage sensors within the cell (e.g. p53) are not functioning properly.(47) Different tumour types contain different numbers of mutations, lung cancer and melanoma have over a hundred mutations as they are associated with potent mutagens (cigarette smoking and ultraviolet light exposure) (48, 49). Colorectal cancer associated with mismatch repair can be associated with even more mutations. Thus, it can be very difficult to determine the significance of these mutations.
A. Mechanism of carcinogenesis achieved by (i) an inherited TSG mutation and (ii) a non-inherited TSG mutation. In order for tumourigenesis to occur both alleles need to be inactivated i.e. a double hit is required e.g. by point mutation, deletion, splice site mutation or epigenetic silencing.

B. The conversion of a protooncogene into an oncogene. Gene activation must take place, this can occur by a number of different mechanisms (e.g. gene translocation, amplification or activating mutation). The consequence of this is uncontrolled transcription of the gene. Typically these genes influence an aspect of cancer growth e.g. angiogenesis or DNA replication.
Identifying ‘driver mutations’ is further complicated by the fact that mutations occur more frequently over a period of time. The ‘gatekeeper’ mutation enables a normal epithelial cell to grow more than other cells to produce a sub-clone of cells. (47). Subsequently, further mutations occur, each driver mutation provides a further growth advantage. The older the tissue the more mutations are likely to be present i.e. the same tumour in an older person will have more mutations than the equivalent tumour in a younger person. Furthermore, tissues that undergo frequent self-renewal such as gastrointestinal and urogenital cancers have more mutations but these tend to occur in the ‘pre-cancerous’ stage and do not confer a growth advantage. (47). These are passenger mutations. (47)

Given the number of different mutations present in any given sample, the task of differentiating driver and passenger mutations is difficult. When data-sets were small this often involved in vitro analysis of potential mutations with assays such as colony formation assays and soft-agar assays to indicate anchorage dependent growth. (50, 51) These techniques are time consuming and not easily translated to the evaluation of a large number of miss-sense variants. Therefore, in silico methods of evaluation of genes have been adopted. One such method involves labelling somatic cancer predisposition genes (CPG) as potential TSG if in ≥20% of cases there is inactivating mutation, similarly an oncogene could be defined as a gene in which ≥20% of cases there are recurrent missense mutations (the 20/20 rule). This has been validated in some tumours, however can lead to some rarer driver mutations being missed. (47). The number of driver mutations present in each cancer varies in each tumour type however there are believed to be between three and six in most solid tumours. (47)
1.4 The application of cancer genomics to patient care

1.4.1 Examples of personalized cancer treatment

Information regarding cancer development that has been determined from cancer genomics has already been translated into routine clinical practice. One of the first targeted treatments derived from genomic studies is trastuzumab which is a monoclonal antibody directed towards HER-2(52) used in breast cancer. HER-2 amplification is determined by immunohistochemistry which if equivocal is further evaluated by fluorescence in-situ hybridization (FISH). Trastuzumab is standard of care for individuals with HER-2 positive breast cancer which has been radically treated (i.e. in the adjuvant setting)(53, 54) and those with metastatic disease(55).

Currently, clinicians treating non-small cell lung cancer routinely provide personalized medicine. The first of the new drugs used in ‘personalised’ non-small cell lung cancer care was established after retrospective analysis of the tumours of patients who responded to EGFR (epidermal growth factor receptor) inhibitors (56). 21% of adenocarcinomas have activating mutations of EGFR (57, 58). Now all lung adenocarcinoma are screened for activating mutations in EGFR. Individuals with these mutations receive orally available EGFR inhibitors such as erlotonib (59) and gefitinib.(60) Response rates to oral agents in these patients are much higher than with conventional chemotherapy (74% c.f. 31%) as is progression free survival (PFS) (10.8 months c.f. 5.4 months).(60) Crizotinib is an orally available ALK inhibitor, this however was designed to treat the 5% of patients with non-small cell lung cancer with an ALK re-arrangement determined by FISH (61, 62). It was associated with a 57% response rate in the first line treatment of advanced ALK positive disease(62). In comparison to chemotherapy, crizotinib had a better response rate (65% versus 20%) and
PFS (7.7 versus 3.0 months) and had better responses than chemotherapy in the second line setting.(61)

The most commonly cited example of personalized cancer care is the use of vemurafenib in melanoma.(63) The V600E mutation of \textit{BRAF} was identified in 59\% of melanoma cell lines (64), \textit{BRAF} activates the MAPK-ERK signaling pathway contributing to cell growth and proliferation.(65) Therefore, \textit{BRAF} was thought to be an important target in melanoma. In individuals with a \textit{BRAF} mutation determined by real-time PCR, vemurafenib was associated with a 48\% response rate versus 5\% with chemotherapy and 84\% six month survival compared with 64\% in the chemotherapy group.(63) Further study of individuals on vemurafenib revealed that 50\% of patients develop resistance to drug. Resistance is multifactorial, however re-activation of MAPK signaling is a significant contributory factor. Translational laboratory work revealed that complete MAPK inhibition would be required to cause cell death in V600E melanoma. Therefore, a MAPK inhibitor (MEK) was combined with \textit{BRAF} inhibition \textit{in vitro} and demonstrated to stop MAPK signaling and induce death. This combination was evaluated in patients and shown to improve PFS (4.8 months compared with 1.5 months).(66) The model of personalized care exemplified by \textit{BRAF} and ALK inhibitors is what is hoped can be achieved by genetic analysis of cancer cells, so the most appropriate agent can be given to patients dependent on their ‘cancer genotype’. This approach maximizes efficacy, reduces toxicity and potentially decreases cost. Therefore, genomics has been incorporated in to early phase trial design in order to facilitate focusing of drugs to the appropriate patients. However, the examples of vemurafenib and crizotinib exemplify that genomic medicine is not a panacea for cancer care. Despite these drugs targeting key driver genes, because of the inherent genetic plasticity of cancer cells, resistance occurs. Thus, work on cancer treatments must follow the model of translational oncology by
being iterative and going from the patient to the ‘bench’ and back again. The genomic spectrum of patients’ tumours may also need to be sampled multiple times as resistance develops.

In inherited cancers, genome analysis is becoming increasingly used and will be discussed in chapter 3. Genome and exome analysis of individuals with family histories of cancer have enabled the detection of new cancer related genes such as *PALB2* associated with breast and pancreatic cancer.(67) Potentially, genomic assessment of patients with inherited syndromes may allow assessment of the risk of the development of cancer, facilitate risk reducing strategies including surgery and chemo-prevention in addition to production of pharmacogenomics markers.(67)

### 1.4.2 Application of genomics to diagnosis and prognosis of cancer

As described previously, in most cases treatment for cancer remains empirical based on the organ in which the cancer is derived and histopathological features. Individual gene mutations are routinely evaluated in some conditions in order to guide targeted treatment choices. These include: *KRAS* analysis in colorectal cancer as mutations in *KRAS* are associated with resistance to EGFR inhibitors (68, 69), and *ALK* and *EGFR* analysis in non-small cell lung cancer (see section 1.4.1). Although there are some direct to consumer whole genome sequencing companies in the US (70), genome sequencing at present remains predominantly a research tool used in clinical trials. However, there have been some attempts to bring genomic technologies into clinical care. Clinically, genomics have been used in prognostication and stratification. Women with early stage breast cancer can receive adjuvant chemotherapy with concordant toxicities with relatively low absolute survival benefits if selected using clinical criteria alone (often an absolute benefit of -5%). Gene-expression profiles such as 70 gene Mammaprint profile approved in the US by the FDA and the 21 gene
recurrence score (Oncotype DX) which has been recommended by the American Society of Clinical Oncology(2) have been developed to estimate the risk of relapse. Patients identified at high risk of relapse can be offered chemotherapy whereas those at lower risk can be managed expectantly. Other gene expression arrays have been developed in colon cancer to determine who may benefit from adjuvant treatment.(2) These arrays however are still in the process of prospective evaluation in the form of randomized controlled trials.(2, 71)

1.5 The scope of the research presented in this thesis
Given the considerable progress made in cancer genomics, the aim of this research is to improve the understanding of molecular pathophysiology of cancer. This includes improved identification of individuals with genetic predisposition to cancer, identification of potentially new cancer associated genes (actionable targets) and using molecular aberrations to identify possible new ‘druggable’ targets. Actionable targets represent cancer drivers which alter the cancer’s growth and development but may not be druggable. Whereas druggable targets represent genes that produce a protein that can be bound by a small molecule at a required binding affinity and affect its function.(72) For example, HER2 overexpression is a poor prognostic sign in breast cancer hence is an actionable target. As there is anti-HER2 treatment it is also a druggable target.

In order to facilitate the study of cancer genomics, two exemplars of cancer genetics are evaluated. Phaeochromocytoma is evaluated in order to establish genetic testing protocols as although it is a relatively rare cancer it is associated with some seminal findings in cancer genetics. Historically, it was thought to be only associated with a 10% genetic risk (73) but is now believed to be one of the most important genetic cancers with over 40% of individuals
harbouring a mutation causing the condition(74). Furthermore, laboratory investigations into the function of mutations in SDHx have revealed the novel role of Kreb’s cycle intermediates such as succinate and fumarate in pseudohypoxia and the hypermethylator phenotype(74, 75).

The second cancer studied in this thesis is familial RCC, although familial RCC is relatively rare (representing 3% of all renal cancers)(76), studies of the condition have led to key findings in the oncogenesis of renal cancer. Notably, the identification of the von Hippel Lindau gene (VHL) in families with the disease(77) was later found to be inactivated in almost 90% of all clear cell renal cancers(78). Functional evaluation of VHL led to agents being used therapeutically which were up-regulated by loss of VHL e.g. anti-VEGF agents. Therefore, familial RCC represents a good model for the evaluation of CPG and potentially new TSG. The advantage of studying familial kidney cancer is also that there are fewer mutations present therefore, potentially fewer passenger mutations improving the efficiency of evaluating variants. Thus, in this thesis research into novel familial RCC genes and new therapeutic targets in RCC will be described.
CHAPTER 2 METHODS

2.1 Materials

2.1.1 Companies materials obtained from

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<td>Agilent Biotechnology</td>
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<td>Bioline</td>
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<td>Biorad</td>
<td>Hemel Hemstead, UK</td>
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<td>Stratech Scientific Limited</td>
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</tr>
<tr>
<td>Web Scientific</td>
<td>Crewe, UK</td>
</tr>
</tbody>
</table>

2.1.2 General chemicals

General laboratory chemicals were obtained from Sigma-Aldrich unless otherwise specified. Methanol, ethanol and isopropanol were supplied by Fisher Scientific. Agarose, DNA and protein ladders were obtained from Fermentas. Tissue culture flasks, plates and dishes were obtained from Corning and Sarstadt. Pipettes were purchased from Appleton Woods. PCR plates and tubes were obtained from WebScientific UK.
2.2 DNA samples
All patients gave written informed consent for genetic studies and ethical approval was provided by the South Birmingham Research Ethics Committee (Molecular Pathology of Human Genetic Disease Study).

2.2.1 Germline familial RCC samples
Germline DNA from patients with a family history of RCC were obtained from the West Midlands Familial RCC Registry. DNA was extracted using standard protocols by the West Midlands Regional Genetics Service (WMRGS) (79). The registry was analysed in order to identify DNA samples from individuals with no known mutations in RCC associated genes, notably \textit{VHL}, \textit{FLCN}, \textit{MET}, \textit{SDHB}. DNA was chosen from individuals who had been reviewed by a consultant cancer geneticist and believed to have an inherited renal cancer, have a personal history of RCC, and either a strong family history of RCC or multiple RCC.

2.2.2 Sporadic RCC DNA samples
Paired normal kidney and RCC samples were provided to Professor’s Maher’s laboratory as a gift from Professor Noel Clarke, University of Manchester. DNA had been extracted from these samples by Mr Dean Gentle. RCC samples were derived from patients without a family history and with a single tumour.

2.2.3 Familial phaeochromocytoma germline samples
Germline DNA from patients with familial phaeochromocytoma were obtained from the West Midlands Familial Phaeochromocytoma Registry. DNA was extracted using standard protocols by the WMRGS as described previously (79). DNA studied was chosen from patients with no known mutations in the common phaeochromocytoma predisposing genes: \textit{VHL}, \textit{SDHB}, \textit{SDHD}, \textit{RET} and \textit{NF1}.
2.2.4 Sporadic phaeochromocytoma DNA samples
DNA from sporadic phaeochromocytoma samples were provided to Professor Latif as a gift from Department of Urology; Yokohama City University School of Medicine, Japan.

2.3 DNA extraction from tissues
DNA was extracted from cell lines in order to perform mutational analysis. The Roche DNA isolation kit for cells and tissues was used. DNA was extracted from cell pellets. Cells were cultured as described in section 2.11. The media was removed and the cells washed in sterile phosphate buffered saline (PBS) (appendix 1). Cells were trypsinised using 1ml of trypsin (Invitrogen) and resuspended in 10 ml of media. The cells were then centrifuged at 280 x g for three minutes. The supernatant was removed and the pellet was resuspended in 10ml of ice cold PBS. This was then re-centrifuged and the supernatant removed. The pellet was resuspended in PBS and centrifuged again. The supernatant was removed and the pellet snap frozen in liquid nitrogen.

In order to extract DNA, 1ml of cellular lysis buffer was added to each cell pellet and mixed by gentle flicking. 1.5µl of the supplied Roche proteinase K solution was added and the sample vortexed for 2-3 seconds to ensure adequate mixing. The sample was placed on a pre-heated heat block at 65° for one hour. 100µl of RNase solution at a concentration of 10mg/ml was then added and vortexed for a further 2-3 seconds to ensure the RNase solution was well mixed. The sample was then incubated at 37° for 15 minutes and divided into different 500µl aliquots. 1ml of the Roche supplied precipitation solution was added to each aliquot. Each sample was mixed by vortexing for 5-10 seconds. The samples were then placed on ice for 5 minutes. The samples were centrifuged at 26,900 x g for 30 minutes. Whilst the samples are being centrifuged, double the number of eppendorfs was labeled. After centrifugation, the supernatant was carefully pipetted into the aforementioned labeled eppendorfs. To each
sample 560µl of isopropanol was added (0.7 x sample volume) and then gently mixed. The samples were centrifuged at 1370 x g for 10 minutes. The supernatant was gently pipetted off and discarded. 500µl of cold 70% ethanol was added to the pellet. This was flicked gently to ensure the pellet was washed with ethanol. The pellet was then centrifuged at 1370 x g for 5 minutes, the supernatant was gently pipetted off and the sample allowed to air dry. 10µl of nuclease free water was then added to each sample and then the same samples were pooled. The concentration of the DNA was estimated using the Nanodrop® Spectrophotometer ND 1000. The A_{260/280} value for the DNA was between 1.7 and 1.9 suggesting adequate quality.

2.4 Whole Exome Sequencing (WES)
DNA from eight individuals with RCC who had been seen by a consultant cancer geneticist and identified as having inherited RCC was sent for WES. These individuals also had either a strong family history of familial RCC or multiple tumours. WES was performed by the group of Dr Michael Simpson, Division of Medical and Molecular Genetics, Kings College London, Guy’s Hospital exome sequencing and bioinformatic analysis service. The process is summarized below.

WES was performed on DNA extracted from peripheral blood lymphocytes using standard techniques. 5µg of DNA with an OD of 1.8-2.0 was required for WES. DNA was sheared using adaptive focused acoustics from Covaris (Woburn, USA). This method enables DNA to be fragmented at controlled temperatures in a sealed tube, reducing the risk of contamination and damage to the stability of the DNA. The protocol was carried out on a bench top centrifuge. After shearing, the DNA was attached to Solid Phase Reversible Immobilisation Beads (SPRI) beads. (Beckman Coulter, High Wycombe, UK). The beads are paramagnetic and made of polystyrene covered with a magnetite layer and then coated in
carboxylate. In the presence of a mixture of polyethylene glycol (PEG) and sodium chloride, DNA binds to the carboxylate. The beads are able to bind vast amounts of DNA, the binding of DNA depends on the concentration of salt in the solution and the ratio of DNA to beads. Once the DNA has been bound to the beads, the beads were washed remove excess unbound DNA. Double SPRI was used to select DNA, the first binding removes base pairs >250bp as they are bound to beads and those less than 250bp are in the supernatant. The beads were then discarded and the supernatant re-incubated with the SPRI beads and concentrations so that all DNA between 150 and 200bp is bound. The supernatant was discarded and the DNA was eluted off the beads and the DNA recovered. Overall DNA recovery with this technique is between 80-90%.

After the DNA was cleaned, the 5’ ends of DNA were phosphorylated and a poly-adenosine tail was added to the 3’ end. The fragmented DNA was ligated to adapter sequences. These adapter sequences correspond to sequences on the Illumina flow cell. After the DNA was attached to adapter sequences, PCR enrichment took place and excessive reagents removed. After enrichment, exon capture was performed with Sure Select Target Enrichment system (Agilent Technologies UK Ltd, Berkshire). The DNA was then hybridized with 120mer biotinylated RNA library baits. Post hybridization, the DNA was mixed with streptavidin coated magnetic beads. DNA which had been adequately hybridized with RNA baits bound to the streptavidin and then these beads were removed using magnetic fields. The unbound fraction (i.e. that without RNA baits) was discarded. The beads were then washed so that the DNA was removed and the RNA digested. The DNA with appropriate adapter sequences formed a library which was ready for next generation sequencing (NGS).

Sequencing was performed on the Illumina Analyser IIX with 76 base paired ends and has been described previously by Dr Simpson’s group (80, 81) The paired end reads for each
exome were in two FASTQ files. Initial checks on the quality of the data were performed using the FASTQC programme (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The Phred score of the sequence should be ≥15 and the minimum coverage 25x. The depth and breadth of sequence coverage was calculated using the BedTools software package (82). Similarly, sequence alignment, identification of single nucleotide variants (SNV), small deletions were identified by the SAMTools software package, this package also filtered the SNV for quality (variant frequency >20%, coverage ≥5%)(83). The sequence was aligned to the reference human genome sequence (hg 18 NCBI build 36). Low read scores were excluded as were reads going to multiple alignments. The Annovar software package (84) was used to annotate variants to genes and transcripts. The data was then filtered and variants identified in NCBI dbSNP build 129 and common SNPs in the 1000 genomes data (85) were removed. Furthermore data was compared to 250 control exomes sequenced by the same method.

2.5 Whole genome amplification
In order to perform and aid mutational analysis germline DNA was amplified using the Qiagen REPLI-g Minikit. Filter tips were used for the transfer of all DNA and buffers. These were obtained from Corning Ltd. 42 samples were amplified in each experiment. Stock DNA at a concentration of (>100ng/µl) was diluted 1 in 10 with TE Buffer (see appendix 1). 1µl of the diluted DNA was aliquoted into a clean, dry PCR plate. A further 1.5µl of TE buffer was added to each sample. 500µl of nuclease free water was added to buffer DLB. REPLI-g mini DNA polymerase was then thawed on ice. Buffers D1 and N1 were prepared for these samples. Buffer D1 consisted of 25.2µl of reconstituted buffer DLB and 118.9µl if nuclease free water. Buffer N1 consisted of 34.8µl of stop solution and 197.2µl of nuclease free water. 2.5µl of buffer D1 was added to each sample and mixed by
pipetting. The samples were incubated at room temperature for 3 minutes. Whilst the samples were incubating, a master-mix was prepared. This consisted of 42 µl of nuclease free water, 1218µl of REPLI-g mini reaction buffer, 42µl of REPLI-g mini DNA polymerase. 5µl of buffer N1 was added to each sample and then 30µl of master-mix was added and mixed by gentle pipetting. The samples were then placed in a PCR machine with a heated lid and incubated at 30° for 10-16 hours. The samples were then heat inactivated at 65° for 3 minutes. The PCR samples were then diluted 1 in 20 with TE buffer and 3µl of DNA were used for subsequent PCR reactions. If a variant was found, the stock DNA was analysed in order to confirm the mutation.

2.6 Polymerase Chain Reaction
Unless stated otherwise all PCR reagents were obtained from Roche (Fast Start Taq DNA Polymerase) stored at -20°. Working concentrations (10mM) of dNTP were produced by adding 12.5µl of 100mM stock deoxyadenosinetriphosphate (dATP), deoxyctosinetriphosphate (dCTP), deoxyguaninetriphosphate (dGTP) and deoxythymidine triphosphate (dTTP) in 450µl ddH2O. dNTP were stored at -20°. Stock dNTP were obtained from Roche. Primers were obtained from AltaBiosciences or Invitrogen.

2.7 Sequencing genes
2.7.1 Primer design
The reference sequence of the gene was obtained from the UCSC genome browser (86), intronic regions were in lower case and exonic regions in uppercase. The cDNA sequence of the gene was then obtained from Ensembl (87). The Primer 3 programme (88) was used to design appropriate primers. A nucleotide blast search (89) was performed in order to ensure specific binding to the gene in question. Ensembl was also used to confirm the primers were not placed in areas with SNP(87).
2.7.2 Polymerase chain reaction (PCR) for sequencing
All PCR reactions were performed in a BioRad Tetrad 2 Peltier thermocycler unless stated otherwise. Table 2.7.2.1 demonstrates the standard PCR reagent concentrations used in each reaction. A negative control was also performed in each reaction to ensure no contamination. Thermal cycling conditions varied depending on the primer combination (table 2.7.2.2 in appendix 2). Those for CDKN2B exons 1 and 2 are described in table 2.7.2.3 in appendix 3. 5µl of sample was mixed with 2µl of loading dye (appendix 1). The samples were electrophoresed on a 1.5% volume/volume (%) (v/v) agarose gel produced by warming 3g of Agarose (Sigma) in 200ml of 1x TBE (10x TBE obtained from GeneFlow Ltd (89mM Tris Borate pH 8.3, 2mM Na$_2$EDTA) in the microwave for 2 minutes. The liquid was allowed to cool by running under a cold water tap and once able to be held comfortably, 2µl of ethidium bromide (10ng/ml) was added to the liquid. The gel was then poured and allowed to set. 100bp ladder (Fermentas) was also added to the gel to allow comparison between samples. Gels were visualised using UV visualised (Ingenius Syngene Bioimaging Machine) and photographed using Gene Snap v 7.04, Synoptics Limited.
Table 2.7.2.1. Concentrations of reagents used in a PCR reaction. * if whole genome amplified DNA was used 3µl of DNA was used and thus the ddH₂O decreased to 5.8µl. This correlated with 10-200ng of genomic DNA, 0.1-1ng of plasmid DNA

2.7.3 Cleaning the PCR product

Prior to carrying out the sequencing reaction, the amplified DNA was cleaned using a combination of exonuclease I (New England Biolabs) and alkaline phosphatase (Fermentas) known as the ExoSAP reaction. Exonuclease I removes excess primer and alkaline phosphatase removes excess dNTP. The reaction concentrations for the ExoSap and conditions for the PCR reaction are described in table 2.7.3.

(A) Reagents required for ExoSAP cleaning of one PCR reaction and (B) The cycling conditions for the ExoSAP reaction.

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</tbody>
</table>

Table 2.7.3. Reagents and conditions required for the ExoSAP reaction. (A) Reagents required for ExoSAP cleaning of one PCR reaction and (B) The cycling conditions for the ExoSAP reaction.
2.7.4 Gel extraction
DNA from PCR reactions for amplification of tumour/normal DNA pairs was extracted prior to sequencing instead of carrying out Exosap reactions. This was because the sequence was not clear when Exosap reactions were performed. Gel extraction was performed using the Qiagen gel extraction kit. PCR product was run out on a 2% v/v agarose gel, the product was then visualised using a UV light and excised using a clean scalpel. The scalpel was cleaned with ethanol before each band was excised. The cut band was placed in a clean dry Eppendorf container, 300µl of buffer QG was added to each eppendorf and incubated at 50º for 10 minutes until all the gel was dissolved. The eppendorf was vortexed every 2-3 minutes. Once dissolved 100µl of isopropanol was added to the sample and mixed. Qiaquick spin columns were labelled appropriately and the sample added to the column. The column was centrifuged at 17900 x g for 1 minute to bind the DNA. The follow through was discarded and 500µl of buffer QG added to the column, the column was spun again at 17900 x g for 1 minute. The wash through was discarded and 750µl of buffer PE added to the spin column, the column was incubated at room temperature for 5 minutes. The column was spun again at 17900 x g for 1 minute. The follow through was discarded and then the column was spun again at 17900 x g to ensure that the excess buffer had been removed. The column was then placed into a clean dry eppendorf and 30µl of double distilled water (ddH₂O) added to the column. The column was incubated for 5 minutes at room temperature and subsequently spun at 17900 x g for 1 minute to elute DNA.

2.7.5 The sequencing reaction
A sequencing reaction was carried out on clean DNA using the reagents as described in the table below. Big Dye terminator version 3.1 sequencer kit (Applied Biosystems). The sequencing reaction was carried out as described in table 2.7.5 below.
Table 2.7.5 Reactions and conditions for the sequencing reaction. (a) Reagents required (b) thermocycling conditions.

2.7.6 Ethanol precipitation
Sequencing reactions were typically 10µl and DNA was precipitated as follows. 1µl of precipitation buffer (appendix 1) was added to each well followed by 25µl of 100% ethanol. The plate was then placed in an Eppendorf 580R centrifuge at 2254 x g for 30 minutes. The plate was then removed and excess ethanol gently removed by tapping on some filter paper and the plate was spun upside down for 10s at 23 x g. 50µl of 70% ethanol was added to each well and the plate spun at 2254 x g for 20 minutes. The excess ethanol was then removed as described previously and another 50µl of 70% ethanol added to the plate. This was centrifuged for a second time at 2254 x g for 20 minutes and the excess ethanol removed. The ethanol was then removed from the sample by heating the PCR plate to 65° for 15 minutes and then re-suspending the DNA in 10µl of Hi-Di (Applied Biosystems). The DNA was then heated at 95° for 5 minutes and placed on a 3730 ABI DNA Analyser. Analysis for mutations was performed using either Sequencing Analysis 5.2 (Applied Biosystems) or Mutation Surveyor (Soft Genetics).
2.7.7 Determination of predicted functional significance of genetic alterations using *in silico* analysis

The possible functional significance of genetic alterations was determined using *in silico* tools. Polyphen-2 (Polymorphism phenotyping version 2) (90) was used to predict whether alterations would have a damaging effect on the protein structure and also consider the evolutionary conservation of the particular amino acid. The SIFT programme (91) was used to model the influence of amino acid changes to protein function. In order to determine whether missense alterations influenced splice sites, the splice site prediction programme (NNSplice 0.9)(92) was used to predict the splice sites of the gene investigated.

2.8 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

This was performed to establish the presence of RNA corresponding to specific genes. The first step in this process is RNA extraction.

2.8.1 RNA extraction

RNA extraction was performed using the Qiagen RNeasy kit, prior to commencing the procedure, 10µl of β mercaptoethanol were added to the provided RLT buffer. Cells from which RNA was to be extracted were harvested. This consisted of aspirating off media, washing cells with 2ml of ice cold PBS and then adding 350µl of buffer RLT. Cells were removed using a cell scraper and then the cells were placed in a clean eppendorf. The eppendorf was centrifuged at 300 x g for 3 minutes and the supernatant taken off and placed in a fresh eppendorf. 350µl of ice cold ethanol was added to the supernatant and mixed by gentle pipetting. This mixture was placed on an RNeasy mini-spin column and centrifuged for 15s at 17900 x g. The follow-through was discarded and 700µl of RW1 was added to the column and this was centrifuged at 17900 x g for 15s. The follow through was discarded and then 500µl of buffer RPE was added to the spin column and centrifuged again at 300 x g for 15s. The follow through was discarded and a further 500µl of buffer RPE was added to the
spin column and the column was centrifuged again for 300 x g for 2 minutes. After the follow through was discarded, the column was centrifuged again to remove excess buffer. The column was then transferred to a new eppendorf tube and 30µl RNase free water was added to the column and centrifuged at 300 x g for 1 minute. The RNA was quantified using a spectrophotometer, the quality was assessed by checking the ratio of the absorbance at 260nm and 280nm and ensuring the ratio was between 1.9 and 2.1.

2.8.2 Conversion of RNA to DNA
1µg of RNA was converted to DNA using the superscript II system (Invitrogen). Briefly this consisted of diluting 1 µg of RNA in 10µl of nuclease free water and adding 50ng of random hexamers and 1 µl of 10mM dNTP mixture to an eppendorf. The eppendorf was mixed well and centrifuged briefly and heated to 65° for 5 minutes and then quick chilled on ice. 4µl of single strand buffer, 1µl of 0.1M dithiothreitol, 40 units of RNaseOUT inhibitor and 200 units of the superscript II reverse transcriptase were added to the eppendorf. The mixture was mixed gently and incubated at the following temperatures: 25° for 5 minutes, 55° for 60 minutes, 70° for 15 minutes. DNA quantity is then determined using a nanodrop as described in section 2.3.

2.9 Evaluating sporadic samples for copy number changes using Multiplex Ligation Dependent Probe Amplification (MLPA)
This was carried out using the ME024-B1 9p21 CDKN2A/B region kit produced by MRC Holland. This kit detects copy number changes in genes located at 9p21.3. Each individual MLPA reaction evaluates using 33 probes evaluating the genes of interest and compares them to 12 reference probes. The peak size of individual probes is compared to normal samples (a total of four was included in each experiment) and the intra-experimental reference probes. Each MLPA run contained a total of 20 samples including normal controls and one TE buffer control (see appendix 1). The MLPA reaction was performed using the manufacturer’s
protocol (MSP-003 updated 23-01-12) which was split into a number of stages: (i) DNA denaturation, (ii) hybridisation (iii) ligation (iv) PCR reaction.

2.9.1 Sample preparation and DNA denaturation
Initially, three sets of 0.2µl thin walled PCR tubes were labelled 1-20 in three different colours on three different racks. A table was made with the following headings: sample number, tumour (T) or normal (N), Label (1-20) and dilution required to make 250ng. The table was completed and the DNA diluted to a concentration of 250ng using TE buffer in 0.2µl PCR tubes. 1µl of diluted DNA was added to 4µl of TE in another labelled tube, to the tube labelled 20 only 1 µl of TE was added. A drop of mineral oil (Sigma) was added to each sample, lids closed and then the tubes were placed in a tetrad thermocycler ensuring that there is random distribution of the controls within the sample. The DNA was then denatured for 5 minutes at 98 ° and then cooled to 25° for 30 minutes.

2.9.2 Hybridisation
The provided Salsa probe mix was mixed by gently flicking and pipetting and the hybridisation master mix prepared for 22 samples as described in the table 2.8.2 below. 3 µl of the hybridisation mixture was added to each tube underneath the mineral oil, mixed by gentle pipetting and the lids closed firmly. The tubes were then placed in the thermocycler at 95° for one minute and then 16 hrs at 60°

<table>
<thead>
<tr>
<th>Hybridisation Mix (µl)</th>
<th>X22</th>
</tr>
</thead>
<tbody>
<tr>
<td>SALSA Probe</td>
<td>1.5</td>
</tr>
<tr>
<td>MLPA Buffer</td>
<td>1.5</td>
</tr>
<tr>
<td>Total</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 2.9.2 Master Mix preparation for hybridisation

2.9.3 Ligation reaction
These were performed following the overnight hybridisation reaction. Initially the master-mixes for the reactions were made as described in the table 2.9.3, mixed by gentle pipetting
and kept on ice. The thermocycler was then set to 20° forever and the tubes removed from the cycler. 13μl of ligase-65 buffer A was added to each tube and mixed by pipetting, this was the ‘dosage mix’. 10μl of the above dosage mix was added to the unused and pre-labelled tubes and placed in another rack. The cycler was incubated at 48° forever and the tubes were added to the cycler. 10μl of the dosage mix was added to the tubes, mixed by pipetting, the tubes were then closed and incubated at 48° for 30 mins, 98° for 5 mins and then 20° forever. Then the PCR step was performed.

(A)

<table>
<thead>
<tr>
<th>Ligase-65 Buffer A (μl)</th>
<th>X22</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>66</td>
</tr>
<tr>
<td>Water (μl)</td>
<td>10</td>
</tr>
<tr>
<td>Total (μl)</td>
<td>13</td>
</tr>
</tbody>
</table>

(B)

<table>
<thead>
<tr>
<th>Ligase - 65 Mix FOR DOSAGE ONLY</th>
</tr>
</thead>
<tbody>
<tr>
<td>X1</td>
</tr>
<tr>
<td>Ligase-65 Buffer B (white cap)</td>
</tr>
<tr>
<td>1.5</td>
</tr>
<tr>
<td>Ligase-65 (μl) (green cap)</td>
</tr>
<tr>
<td>0.25</td>
</tr>
<tr>
<td>Water (μl)</td>
</tr>
<tr>
<td>8.25</td>
</tr>
<tr>
<td>Total (μl)</td>
</tr>
<tr>
<td>10</td>
</tr>
</tbody>
</table>

Table 2.9.3 Ligase Master-mixes for copy number assays;

(A) Master-mix for Ligase A (B) Master-mix for Ligase B

2.9.4 PCR reaction

Salsa polymerase was warmed in the hand to reduce viscosity and a master-mix of Salsa PCR primers and Salsa Polymerase was prepared (see table 2.9.4). This was mixed by gentle pipetting and stored on ice. At room temperature, 5μl of the polymerase mix was added to each tube and mixed by gentle pipetting. The tubes were then placed in a thermocycler and the following programme initiated: 35 cycles of: 30 seconds 95°, 30 seconds 60°, 60 seconds
72° followed by 20 minutes at 72°. After completion of the task the tubes were opened in another room and the product transferred into another set of clean labelled tubes. These were stored at 4° for up to a week or for longer periods at -15° to -25° wrapped in foil.

<table>
<thead>
<tr>
<th></th>
<th>SALSA PCR</th>
<th>x44</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salsa PCR PRIMERS (brown cap)(µl)</td>
<td>1</td>
<td>44</td>
</tr>
<tr>
<td>Salsa Polymerase (orange cap)(µl)</td>
<td>0.25</td>
<td>11</td>
</tr>
<tr>
<td>Water(µl)</td>
<td>3.75</td>
<td>165</td>
</tr>
<tr>
<td>Total(µl)</td>
<td>5</td>
<td>220</td>
</tr>
</tbody>
</table>

Table 2.9.4 Reaction components for PCR reaction

2.9.5 Fragment separation by electrophoresis
0.7µl of the above PCR reaction was aliquotted into a PCR plate. To this 9µl of formamide (Life technologies) and 0.2µl of GeneScan LIZ-500 size standard (Life Technologies) were added. The plate was sealed, incubated for 2 minutes at 80° and cooled rapidly. The reaction was then run on an AB3730 sequencer equipped with fragment analysis software.

2.9.6 Relative quantification of amplification products.
Data was analysed using the Genemarker software (edition 1.71), Softgenetics LLC, USA.

The TE control was analysed to ensure that there was no contamination. The internal quality controls were then assessed. A CDKN2B panel was devised using the probe sizes given in the MRC Holland Protocol. Genes were labelled A-I on the basis of the position on the chromosome (described in table 2 of the MRC Holland Protocol), and probes marked according to size (described in table 1 of the protocol) with a letter _ name of the gene, and a _ followed by the appropriate number (e.g. A_DOCK8, G_CDKN2A.1). Control and reference probes were labelled with the prefix and then the size for reference probes. The software compared the peak heights in the samples to the mean of the controls to produce a ratio. Deletions were denoted by a loss <0.75 and gain by a ratio of 1.3.
2.10 Experiments performed by transfecting cells with a vector containing **CDKN2B**

Prior to commencing cloning experiments LB broth, LB agar and Kanamycin were prepared as per appendix 1. LB broth containing kanamycin was produced at a final concentration of 50ng/ml by adding 50µl to 500ml of LB broth. Kanamycin plates were produced by allowing LB agar to cool to room temperature and adding 25µl of kanamycin to every 50ml of LB agar. The agar was poured gently into 10cm dish and left to cool. The plates have their lids placed on them, were labelled and stored upside at 4°C.

2.10.1 **CDKN2B clone**

The **CDKN2B** clone was obtained from Cambridge Bioscience (True ORF Gold RC204895). This was streaked onto a kanamycin plate and incubated at 37°C overnight. Six individual colonies were picked and placed in 2ml of sterile LB broth containing kanamycin. This was incubated overnight at 37°C and agitated at 250RPM in a shaking incubator. The clone was then purified using the QIagen miniprep spin kit as described below as per manufacturer’s instructions(93).

2.10.1.1 Purification of the high copy number plasmid

The bacterial broth was purified using the QIagen mini-spin kit as per manufacturer’s instructions(93). Briefly, 1.5ml of bacterial broth was placed in an eppendorf and spun at 17,000 x g for 10 minutes. The supernatant was then discarded and the pellet resuspended in 250µl of buffer P1. 250µl if buffer P2 containing LyseBlue was added to the eppendorf and mixed by inverting. The solution then became colourless. 350 µl of Buffer N3 was added to the sample and immediately mixed by inversion until the solution was colourless. The eppendorf was centrifuged at 17,000 x g for 10 minutes. During centrifugation, QIAprep spin columns were labeled using a permanent marker. The supernatant from the centrifugation was then pipetted onto the appropriately labeled spin column. The spin column was centrifuged at 17,000 x g for 60s and the follow through discarded. 500µl of buffer PB
was added to the spin column and then centrifuged for 60 seconds at the same speed. The
follow through was discarded and 750µl of buffer PE added to the spin column. This was
centrifuged for a further 60s, and the follow through discarded. The column was then
centrifuged at 17,000 x g for a further minute to remove residual buffer. Then the column
was added to a clean microcentrifuge tube and 30µl of nuclease free water added to the
column. The column was left for one minute and then centrifuged at 17,000 x g for a further
minute. The concentration of the plasmid was determined by spectrophotometry.

2.10.1.2 Confirmation of sequence of the clone
The clone was sequenced as described previously using the following primers:

VP1.5 (Forward Primer) 5’to3’ GGACTTTCCAAAATGTCG, XL39 (Reverse Primer) 5’ to
3’ ATTAGACAAGGCTGGTGGG.

The sequencing reaction contained the following concentrations: 4µl of purified vector, 0.5 µl
of big dye, 2 µl of 10x buffer, 1 µl of primer, 2.5 µl of ddH₂O

2.10.2 Purification of large quantities of plasmid
2.10.2.1 Transformation of bacterial cells with CDKN2B
Once the plasmid was confirmed to contain the correct CDKN2B sequence, alpha-select
competent silver competent cells (Bioline) were transformed. The following technique was
utilized: Silver competent cells were gently thawed on ice. 30µl of silver competent cells
were aliquotted into a clean sterile eppendorf, 5µl of plasmid was placed in the cells and
stirred gently using a pipette tip. The mixture was incubated on ice for 20 minutes. The
eppendorf was then placed into a heat block at 42° for 45s. The cells were then incubated on
ice for 2 minutes. 500µl of SOC medium (Invitrogen) was added to each eppendorf which
was then snapped shut and wrapped with parafilm (Sigma Aldrich). The eppendorf was then
placed in a horizontal position and secured to prongs in an orbital incubator (Gallencamp Orbital Incubator) and incubated at 37\(^{\circ}\)C for 1 hour. 100µl of cells were then plated out on kanamycin plates for >15hrs. A colony was then picked the following day using a sterile pipette and placed in 100ml of sterile kanamycin containing media overnight (12-16 hours) in a conical flask.

2.10.2.2 Purification of plasmid DNA
The Qiagen Endotoxin Free Maxiprep kit (94) was used to purify DNA from the overnight culture prepared above. The culture media was placed into two 50ml falcon tubes and centrifuged for 15 minutes at 4\(^{\circ}\)C at 6000 x g. Whilst the culture was being centrifuged, one vial of RNase was added to buffer P1 and one vial of LyseBlue added to buffer P1. After centrifugation, 10ml of buffer P1 was added to the first pellet and this resuspended. Then this was removed and placed in the second falcon and the second pellet was also resuspended by gentle pipetting. 10ml of buffer P2 was added to the falcon tube and mixed by inverting 6 times, at this point the liquid became blue. The mixture was then incubated for 5 minutes. A further 10ml of chilled buffer P3 was then added to the mixture. The falcon tube was inverted 6 times until there was a colourless liquid. The liquid was placed in a Qiagen filter making sure not to pipette any of the suspended flocculent material. The remainder of the flocculent material was then filtered and added to the Qiagen filter cartilage and the lysate incubated at room temperature for 10 minutes. The cap was removed from the Qiagen filter cartilage and the plunger gently inserted the filtrate was collected into a new labeled falcon tube. 2.5ml of buffer ER was added to the filtered lysate and mixed by inversion ten times. The mixture was incubated on ice for 30 minutes. In the interim, a Qiagen tip-500 was equilibrated by adding 10ml of buffer QBT and allowing it to pass through the tip by gravity. The buffer was discarded and the filtrate from the maxi-cartridge was applied to the Qiagen tip and allowed to
pass through the tip by gravity. The tip was washed twice using 30ml of buffer QC. The DNA was eluted into a fresh falcon tube using 15ml of buffer QN. The DNA was precipitated by adding 10.5ml of isopropanol and centrifuged at 14000 x g at 4\(^\circ\) for 30min. The pellet was re-suspended in 1.5ml of 70% ethanol and converted into a clean eppendorf. This was centrifuged at 26,900 x g for 10 minutes. The supernatant was removed and the pellet was air dried for 10 minutes. The pellet as then re-suspended in 500µl of TE buffer. The concentration of the DNA was then estimated using a nanodrop and the DNA diluted to an end concentration of 1µg/µl. DNA was stored at -20\(^\circ\).

2.10.3 Site directed mutagenesis
Site directed mutagenesis was performed on the CDKN2B clone obtained from Cambridge Biosciences (True ORF Gold, Expression Validated cDNA clones, RC204895). The clone had been sequenced to ensure that it contained the correct sequence. Site directed mutagenesis was performed using the Quik Change II Site Directed Mutagenesis Kit (Stratagene, obtained from Agilent Technologies).

2.10.3.1 Primer design
Primers were designed using guidelines recommended by the company (95). The mutation was placed at the centre of the primer with 15 further bases extended in both directions. The Tm was \(\geq 78\)\(^\circ\) and determined using this equation:

\[
Tm=81.5+0.41(\%GC)-((675/N)-\%\text{ mismatch})).
\]

The GC content of the primer was at least 40% and the primer should terminate in one or more C or G bases. Primers were not 5’ phosphorylated, purified by polyacrylamide gel electrophoresis and obtained from Invitrogen (see table 2.10.3.2 (appendix 4))

2.10.3.2 Mutant strand synthesis reaction
PCR reactions were performed for each mutation in addition to one control reaction.
The control reaction was set up as follows: 2.5µl of 10x reaction buffer, 1 µl of pWhitescript 4.5kb control plasmid, 0.625 µl of oligonucleotide control primer number 1, 0.625 µl of oligonucleotide control primer number 2, 0.5 µl of dNTP mix, 19.25 µl of ddH2O. Then 0.5 µl of PfuUltra DNA polymerase (2.5U/µl) was added.

The sample reactions were set up as follows: 50ng of CDKN2B gene expression plasmid, 2.5µl of 10x reaction buffer, 0.5 µl of dNTP mix, 1 µl of forward primer (1µl of 1:10 dilution of 100µM stock), 1 µl of reverse primer (1µl of 1:10 dilution of 100µM stock), 19.25 µl of ddH2O. Then 0.5 µl of Pfu Ultra DNA polymerase (2.5U/µl) was added.

These reactions were completed as per table 2.10.3 in a thermocycler. After the PCR reaction is completed the samples were placed on ice for 2 minutes to ensure the samples were cooled to ≤37 °.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>95</td>
<td>30</td>
</tr>
<tr>
<td>3</td>
<td>55</td>
<td>60</td>
</tr>
<tr>
<td>4</td>
<td>68</td>
<td>180</td>
</tr>
<tr>
<td>5</td>
<td>Cycle to step 2 for 12 cycles</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.10.3.3 Thermocycling conditions for Mutant Strand Synthesis

2.10.3.3 Digestion of amplification products
1µl of the DpnI restriction enzyme (10U/µl) was added to the previously described amplification reaction. This was mixed by gentle pipetting and incubated at 37° for one hour. This step is to ensure digestion of the non-mutated parental DNA.

2.10.3.4 Transformation of XL-1 Blue Supercompetent Cells
XL-1 Blue supercompetent cells were gently thawed on ice. 25µl were aliquotted into pre-chilled eppendorfs. 1µl of the aforementioned digested amplified PCR product was added to
the cells and mixed by stirring. The mixture was incubated on ice for 30 minutes. The mixture was then heat pulsed for 45 seconds at 42°C using a heat block and then placed on ice for 2 minutes. 500µl of pre-warmed SOC media (Sigma) was added to the eppendorf and the transformation reaction was incubated at 37°C for 1 hour shaking at 250RPM in the orbital incubator. 125µl of the transformation mixture was placed on two kanamycin plates. These were incubated at 37°C for greater than 16 hours.

2.10.3.5 Confirming accurate site directed mutagenesis.
10 colonies were picked using a clean sterile pipette, each colony was incubated in 3ml of LB broth containing kanamycin. These were incubated at 37°C in a shaking incubator. 1.5ml of the culture was transferred into an eppendorf and DNA extracted using a Qiagen minispin kit as described in section 2.10.1.1. A sequencing reaction was then carried out using 4µl of DNA, 1 µl of primer, 0.5 µl of Big Dye, 2 µl of 10x buffer and 2.5 µl of ddH2O. Once the mutation was confirmed the DNA was amplified using the Qiagen endotoxin free maxiprep kit as described previously (see section 2.10.2.2).

2.11 Cell culture
All tissue culture experiments were carried out in class II tissue culture hoods. Prior to use the hoods were cleaned with 1% trigene solution (Starlabs).

2.11.1 Cell lines used in this thesis
The following RCC cell lines were utilised in this study: SKRC-39, KTCL-26, SKRC-47, 786-P, RCC4, ACHN. These were all previously used and characterised by Professor Maher’s research group (50, 96-98). The cells were maintained in complete medium described below. In addition to this, paired isogenic SKRC-39 cell lines were used in the kinase screening experiments. These cells were previously produced in our laboratory by transfecting SKRC-39 with PC DNA 3.1 vector alone or PC DNA vector 3.1 containing the VHL gene. These
cells were maintained in complete media containing 1% v/v G418 (PAA laboratories). All cell lines were maintained in a humidified incubator containing 5% CO$_2$ at 37$^\circ$.

2.11.2 Media for cell culture
Native cell lines were maintained in ‘complete medium’ which consisted of Dubecco’s Modified Essential Medium (Sigma) containing 10% fetal calf serum (PAA laboratories), 1% penicillin and streptomycin(Sigma) v/v, 1% L-glutamine(Sigma, UK) and 1% v/v non-essential amino acids (Sigma). Cell lines selected for G418 sensitivity were maintained in the complete medium with 1% v/v G418 solution at 0.5mg/ml (PAA laboratories).

2.11.3 Maintaining cells
Cells were maintained in T75 flasks until they were required for experiments. PBS, media and trypsin were warmed to 37$^\circ$ prior to use using a Grant Sub Aqua 18 water-bath. Cells were maintained in the media described above and split every five days. Cells were visually inspected using a Nikon TMS light microscope for viability and absence of infection. Media was removed from the cells and discarded. The cells were washed using 10ml sterile PBS and 1ml of trypsin (Sigma) was added after the PBS was discarded. The flask was gently agitated to ensure the bottom of the flask was evenly covered with trypsin and incubated at 37$^\circ$ for 10 minutes. The flask was agitated and light microscopy was used to ensure that there were no further adherent cells. 10ml of fresh media was added to the flask. The cells were re-suspended, and 9ml of cells discarded. A further 9 ml of fresh media was added to the flask. Every three months, mycoplasma testing was performed.

20.11.4 Mycoplasma testing
Cells were tested for possible mycoplasma infection three monthly basis or if suspected using the EZ-PCR mycoplasma test kit (Geneflow). 10ml of cells were centrifuged at 200 x g for 10 minutes using a Labnet International benchtop spectrafuge 24D centrifuge. The supernatant was pipetted out and placed in a new clean centrifuge. The supernatant was then
centrifuged at 16300 x g for 10 minutes. The supernatant was discarded and the pellet re-
suspended in 50µl of the buffer mix. The tube was heated at 95° for 3 minutes. Three
reactions were prepared in separate 200µl PCR tubes using 17.5µl of water, 5µl of reaction
mix, and either 2.5µl of test sample, 2.5 µl of water (negative control) and 1 µl of positive
control supplied. The tubes were covered with mineral oil and the following PCR reaction
carried out: 94° for 30s, then 35 cycles of 94° for 30s, 60° for 120s, 72° for 60s, then 94° for
30s, 60 ° for 120s and 72 ° for 5 minutes. The PCR products were loaded on a 2% agarose gel
without any loading dye. A 270bp fragment would indicate the presence of mycoplasma.

2.11.5 Counting cells for experiments
Cells were trypsinised and resuspended in 10ml of media as described above. 1ml of cells
were aliquotted into a container, 20µl of cells were placed on an improved Neubauer
haemocytometer. The haemocytometer was visualised using a light microscope at x10
magnification, the number of cells in the four outer sections of the haemocytometer were
counted. Cells touching the outer and upper borders were not counted. The total number of
cells were divided by four to give the number of cells x 10^4/ml. Cells were then diluted as
appropriate.

2.11.6 Cryopreservation of cells
Cells contained in a T75 flask were trypsinised and re-suspended in 10ml of medium. These
cells were centrifuged at 280 x g for 5 minutes. The media was decanted and discarded.
Subsequently, the pellet was suspended in 3mls of 10% FCS containing 1% v/v of DMSO.
The resuspended cells were aliquotted into three different cryovials (Sarstedt). These were
then placed in a Nalgene Mr Frosty freezing device containing isopropanol, this was placed in
the -70° freezer for 48 hours prior to transfer of the cryovials to liquid nitrogen.
2.11.7 Resurrecting cells from storage.  
On removal from liquid nitrogen cell lines were placed in an icebox and transported to the tissue culture facility. The cryovials were placed into a waterbath maintained at 37°. Once the cells were defrosted they were placed in to a new sterile 75ml tissue culture flask.  
Warmed media was then added drop wise to the cells and maintained as described previously.

2.11.8 Colony formation assays
Colony formation assays were performed using the native CDKN2B plasmid and the mutated forms of the plasmid described in section 2.10.3. The plasmids were amplified using the Qiagen endotoxin free maxi-prep kit and diluted to a concentration of 1µg/µl using nuclease free water. Colony formation assays were performed in VHL inactivated KTCL-26 cell lines which are known to contain Ser68X mutation in exon 1 of VHL. This line was chosen as in most clear cell RCC VHL is inactivated. The first transformation was carried out using the native CDKN2B plasmid and a vector without the gene (empty vector EV). 6 x 10^4 cells/ml were plated out in 6 well plates on day 0. These cells were suspended in complete medium.  
On day 1, transfection was carried out in triplicate. For one transformation, 2µl of plasmid was diluted in 200 µl of optiMem solution (Invitrogen) and 6µl of Fugene HD (Promega) was added directly to the optiMem taking care not to touch the sides of the eppendorf. The eppendorf was vortexed and incubated at room temperature for 15 minutes. The transfection reaction was added dropwise to the media. On day 4, the cells were washed in PBS and trypsinsed. Cells were resuspended in 10ml of complete media containing G418. For the first experiment, these cells were further diluted at the following concentrations 0, 1:5, 1:10, 1:20. Plates were incubated in a humidified incubator at 37°. They were inspected twice weekly and media replaced after 5 days and then as required. After three weeks the plates were stained to allow counting. This was performed as follows: media was aspirated off the plates, each plate was washed with 10ml of PBS. Then to each plate a few drops of crystal
violet solution (250ml ddH₂O, 250ml methanol and 0.5% v/v crystal violet) was added. The plate was gently tipped to ensure even covering and then washed off gently using ddH₂O. The plate was washed three times and then placed upside down on tissue paper. Once the plates had dried the total number of colonies counted.

The best results came from performing a 1 in 5 dilution, i.e. diluting the 2ml of cells in the 6 well plate in 10ml of G418 containing media. Thus the experiment was repeated three times, the mean number of colonies were determined. Unpaired t tests were used to determine the significance of any difference between means. One in five dilutions were also used for subsequent experiments using mutant plasmids compared to empty vectors. In order to confirm transfection efficiency, immunoblotting was carried using lysates from the transfected cells (see section 2.15).

2.12 Investigating possible synthetic lethal interactions

2.12.1 Screening for synthetic lethal interactions using a siRNA screening library. The Silencer® Select Human Kinase siRNA Library v4 (Ambion) Kinase library contains 2130 unique siRNA targeting a total of 710 human kinase genes. Within this experimental system there is post-transcriptional silencing of genes with double stranded RNA in the form of siRNA with sequence homology to the target sequences of interest(99). Initially the working parental plates were diluted to produce working plates for use in the experiment. This was done by diluting the parental plate 1:10 using sterile nuclease free water (Ambion). 2µl of stock siRNA was added to a PCR plate containing 20µl of sterile nuclease free water. This plate was labelled working plate. There were three oligonucleotides for each gene, thus for each experiment three plates were made labelled ABC1-4. These and the parental plates were stored at -70°.
Prior to setting up the experiment media, trypsin, optiMem (Gibco) and INTERFIRin (Polyplus transfection, Autogen Bioclear Ltd) were warmed at room temperature. OptiMem was placed into a reservoir and then 22.5μl added to a second clean PCR plate labelled master mix plate ‘A/B/C 1_3’. To this 6μl of siRNA from the working plate was added to the corresponding well on the PCR plate. In column 12 of the master mix plate 6μl of siRNA directed against luciferase was added to wells 12A-D, these wells represented the controls. This mixture was homogenised by placing on a Grant-bio PMS-100 plate shaker. To a second clean PCR plate, 60μl of INTERFIRin was added to each well in column 1, this column was the master column. To each column in each master mix plate add 1.5μl of INTERFIRin using a multichannel pipette. The master mix plate was covered with a thermolid and left on a plate shaker for 10 minutes.

Whilst the plate was mixing, SKRC-39ev and SKRC-39wt cells were trypsinised and diluted to a concentration of 6 x 10⁴ cells/ml. After the cells had been diluted, 10μl of the siRNA INTERFERin mixture was transferred from the mastermix plates into the corresponding black plates (Costar, Black bottomed 96 well Assay plates, Corning Inc) which were labelled A/B/C, 1/2/3_1/2/3 EV or WT. Each diluted cell line was placed in a separate sterile reservoir and 90μl of cells was pipetted into wells in the corresponding black assay plate using a multichannel pipette. Each well was mixed by gentle pipetting. To the last 2 wells in column 12 of each black assay plate (12E-H) 10μl of 100mM mitramycin was added as a negative control, 2 wells were left empty i.e. contained cells only. The cells were maintained at 37° in a humidified 5% CO₂ atmosphere using standard techniques for 76 hours.
2.12.2 Assessment of cell viability using cell titre blue assay
After incubation for 76 hours, 20µl of cell titre blue reagent (Promega) was added to each well containing transfected cells (black plates). Plates were agitated for 10 seconds and incubated at 37°C for 4 hours. Fluorescence recorded at 560/590nm using the Perkin Elmer Victor X3 plate reader (PerkinElmer). Data was analysed by comparing the normalised luminescence values of SKRC39wt cells to SKRC39ev cells this represents the change in cell viability of VHL expressing cells compared to those lacking VHL.

2.12.3 Assessment of cell apoptosis using the caspase-glo assay
Depletion of genes was carried out as described in 2.12.1 however cells were plated into white bottomed 96 well assay plates (Costar, Corning). Apoptosis induced by kinase gene knock down was determined using the Caspase-Glo 3/7 Assay (Promega). Caspase-Glo 3/7 reagent was prepared as per the manufacturer's recommendations. It was allowed to equilibrate at room temperature. Under sterile conditions 30µl of media was removed from each well and 50µl of caspase-glo reagent was added to each well. The plates were then incubated at room temperature for an hour, luminescent light was measured in a Victor X3 Plate Reader (Perkin Elmer).

2.13 Protocol for transfection of siRNA using Dharmacon SmartPool siRNA
Dharmacon ON-TARGETplus SMARTpool siRNA was purchased from ThermoScientific directed towards STK10, PLK1 and CDKN2B. siRNA tubes were centrifuged to ensure the siRNA pellet was at the bottom of the tube. 100µM stock was prepared by adding 50µl of RNAase free water to the tube. siRNA was diluted in RNAase free water in sterile conditions using filter tips. The stock tube was mixed by gentle pipetting and then placed on an orbital shaker for 90 minutes at room temperature. The tube was then aliquotted into smaller tubes and then stored at -70°C. The experimental conditions investigated were as follows:
1. Untreated cells in triplicate: SKRC39EV, SKRC39WT, 786-0, KTCL-26, RCC4
2. Cells treated with mithramycin: SKRC39EV, SKRC39WT, 786-0, KTCL-26, RCC4
3. Cells treated with luciferase: SKRC39EV, SKRC39WT, 786-0, KTCL-26, RCC4
4. Cells treated with siRNA to PLK1: SKRC39EV, SKRC39WT, 786-0, KTCL-26, RCC4
5. Cells treated with siRNA to STK10 siRNA: SKRC39EV, SKRC39WT, 786-0, KTCL-26, RCC4
6. Cells treated with siRNA to STK10 siRNA and PLK1: SKRC39EV, SKRC39WT, 786-0, KTCL-26, RCC4
7. Cells treated with siRNA to STK10 siRNA and PLK1: SKRC39EV, SKRC39WT, 786-0, KTCL-26, RCC4

The cells were plated out as below in figure 2.13 in triplicate on black assay plates.

2.13.2 Reverse transfection methodology
A serial dilution of the 100µM stock was performed in sterile RNase free tubes to produce a 1µM working solution. In a separate RNase free tube, DharmaFECT1 was diluted by adding 1.8µl Dharmafect 1 in 373.2 µl of buffer per sample. As there were 30 samples, 54 µl was diluted in 11.196 ml. For each transfection, 600µl of Dharmafect 1 was added to a clean sterile tube, to this 9µl of siRNA was added i.e. 9µl siRNA directed against STK in one tube and to a second tube 9µl siRNA directed against PLK was added to Dharmafect 1. For ‘50%’ dose, 4.5µl of siRNA was used. 25 µl of the siRNA/Dharmafect mixture was pipetted into black bottomed assay plates as shown above and incubated at room temperature. Cells were trypsinized and diluted at a concentration of 5x 10⁴ cells/ml in complete media without antibiotics. 100µl of cells to each well as per the experimental plan (figure 2.13). The plate
was incubated at 37° in 5% CO₂ for 72 hours and cell viability was assessed using cell titre blue as described previously (2.12.2).

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<td>RCC-4</td>
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<td>SKRC39-WT</td>
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<td>STK10+PLK1</td>
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<td>KTCL-26</td>
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<td>STK10+PLK1 at 50%</td>
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<td></td>
<td></td>
<td></td>
<td>STK-10 25nM</td>
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Figure 2.13 Experimental design for knockdown of STK-10 and PLK1. The layout of Black Assay Plates used to analyze the effect of depletion of STK-10, PLK and both on RCC cell lines.

2.14 **Forward transfection using Dharmacon smartpool siRNA**
SKRC39ev and SKRC39wt cells were plated out in antibiotic free media on day one at a concentration of 2.5x10⁵/ml in 6 well plates. On day two, transfection was performed as per manufacturer’s instructions. siRNA was diluted to a working concentration of 5µM in 1 x siRNA buffer (i.e. 1 in 20 dilution of 100µM stock). 3 eppendorfs were labelled tube 1 and also control, STK10, PLK1. To each eppendorf was added 190µl of OptiMEM and 10 µl of
siRNA. These were mixed by gentle pipetting. A further three eppendorfs were labelled tube 2 Control, STK10 and PLK1. To each tube two 1µl of Dharmafect 1 transfection reagent and 199µl of OptiMEM were added. These were mixed by gentle pipetting and the tubes were incubated at room temperature for 5 minutes. Subsequently, the contents of eppendorfs labelled 1 were added to the corresponding tube 2. After gentle mixing, the tube was incubated at room temperature for 20 minutes. In the meantime, the media covering the previously plated out cells was replaced with 1600µl of media without antibiotics. After, the incubation had completed 400µl of the appropriate transfection mixture was added dropwise to SKRC39ev and SKRC39wt cells. Each experiment was carried out in triplicate. After 72 hours the cells were harvested by washing the cells in ice cold PBS and adding trypsin. The cells were then scraped off using a cell scraper. Subsequently, they were centrifuged in ice cold PBS twice at 13000 x g for 5 mins and the pellet snap frozen in liquid nitrogen.

2.15 Immunoblotting to detect changes in protein expression

2.15.1 Preparing protein lysates

Protein from cell lines was extracted by initially trypsinising cells and resuspending the cells in media. The cells were centrifuged at 13000 x g for 10 minutes. The supernatant was removed and the pellet resuspended in 10ml of sterile PBS. This was centrifuged again at 13000 x g for 10 minutes. PBS was removed and the pellet snap frozen in liquid nitrogen immediately or 1.5x the pellet volume of RIPA buffer (appendix 1) was added to the pellet and incubated on ice for 10 minutes. After incubation the mixture was transferred into an eppendorf and centrifuged at 13000 x g for 10 minutes. The supernatant was then removed and placed in a clean eppendorf and stored at -70° in a clean labelled eppendorf.

Protein was extracted from cells treated in the 6 well setting by placing the cells on ice and removing the media of the cells and washing them in 2ml of sterile ice cold PBS. The PBS
was removed and 100µl of RIPA buffer was added to each well. The cells were then removed using a cell scraper (Corning Ltd) and transferred to a clean eppendorf. After incubation for 10 minutes with more RIPA, the cells were centrifuged at 13000 x g for 10 minutes. The supernatant was stored.

2.15.2 Protein estimation
The Biorad DC Protein Assay kit (Biorad laboratories) was used to estimate the concentration of proteins. The assay was carried out in a 96 well flat bottomed assay plate. Reagent A* was prepared by adding 20µl of reagent S to reagent A. This was stored up to a week at room temperature. Standard albumin concentrations were then prepared using BSA 2µg/µl (Sigma). The following concentrations were produced as follows. BSA (µg/µl): 0.4, 0.8, 1.2, 1.6, 2.0 (produced as described in figure 2.15.2). These were plated out as below in a 96 well plate. 5µl of each test sample was pipetted in duplicate in columns 3-4 position A and B onwards. Then 25µl of mixture A* were added to each sample and 200µl of reagent B added and mixed by gentle pipetting. The plate was left at room temperature for 15 minutes and then the absorbance measured using the WorkOut 2.0 programme (Dazdaq Ltd) on a Perkin Elmer Victor 3 Plate Reader which compared absorbance to a standard curve produced using the standards.
Figure 2.1.5.2. Protein concentration estimation

A) A table showing how to dilute the bovine serum albumin (BSA) into the required concentrations.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>BSA (2µg/µl)</th>
<th>Lysis Buffer (RIPA)</th>
</tr>
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<tbody>
<tr>
<td>0.4</td>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td>0.8</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>1.2</td>
<td>12</td>
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<td>1.6</td>
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<td>4</td>
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<tr>
<td>2.0</td>
<td>20</td>
<td>0</td>
</tr>
</tbody>
</table>

B) The layout of the protein estimation plate. Each sample is placed in duplicate, columns 1 and 2 allow calibration of protein using albumin dilutions. Proteins to be estimated (P) are placed in columns 3 onwards in duplicate. (Key LB=lysis buffer, BSA=bovine serum albumin)
2.15.3 Preparation of sodium dodecyl sulphate polyacrylamide gels

Proteins were separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using equipment provided by BioRad Ltd. Different concentrations of gel were used to resolve different sized proteins. The constituents required to produce 12% gels are described in figure 2.14.3 below. Ammonium persulphate (APS) was prepared by dissolving 0.1g of APS in 1ml of ddH2O. 1.5mm plates and combs were initially washed using soapy water and then cleaned with 70% ethanol. The plates were then gently placed together using the above equipment. The resolving gel was added to the plate using a 1ml pipette ensuring no bubbles were present until the specified position on the plate (leaving approximately 1.5cm of the plate free from gel). The gel was overlaid with ddH2O, the remainder of the resolving gel was retained in a universal container once this was set the water poured off the gel and plate dried using filter paper. The stacking gel was then placed on top of the resolving gel and the comb placed into the gel. Once set the gel was removed for use.

<table>
<thead>
<tr>
<th></th>
<th>Resolving (12%) Gel</th>
<th>Stacking gel</th>
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</thead>
<tbody>
<tr>
<td>ddH2O (ml)</td>
<td>6.6</td>
<td>6.8</td>
</tr>
<tr>
<td>Acrylamide (ml)</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Tris Buffer (ml)</td>
<td>5 (1.5M Tris pH 8.8)</td>
<td>3 (0.5M Tris pH 6.8)</td>
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<tr>
<td>10% SDS (μl)</td>
<td>200</td>
<td>100</td>
</tr>
<tr>
<td>APS (μl)</td>
<td>150</td>
<td>100</td>
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<tr>
<td>TEMED (μl)</td>
<td>10</td>
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Table 2.15.3 Concentrations of reagents required to make two 1.5mm SDS-PAGE gels

2.15.4 Sample preparation

Typically 20μl of sample were loaded onto 1.5mm thick gels. After estimation of protein concentrations between 20 and 40μg of protein were placed in an eppendorf, together with the appropriate quantity of 5x loading buffer (appendix 1). The sample was heated to 95° for 5 minutes on a heat block. The gel tank was set up and plates placed in the equipment. 1x running buffer was made by diluting 10x stock (Ultrapure Tris/Glycine/SDS (Geneflow)) in ddH2O. Running buffer was placed between the plates and in the tank. The sample was
subsequently loaded onto the gel, with 5μl of protein ladder in an adjacent well (Pageruler Plus, Fermentas). The sample was electrophoresed at 130V for 90 minutes or until the loading dye had run off the gel.

2.15.5 **Transfer of proteins onto a nitrocellulose membrane by electrophoresis**

Transfer was performed using the mini trans-blot Biorad equipment. Transfer buffer was prepared in advance by diluting 100ml of 10x stock (Ultrapure Tris/Glycine (Geneflow) with 200ml of methanol and 700ml of ddH$_2$O and stored at 4°C. 4 pieces of Whatmann filter paper and nitrocellulose membrane (Amersham Hybond M (GE Healthcare)) were cut to the size of the front plate. Filter papers and fibre pads were placed in in a tray containing cooled transfer buffer in order to ensure the system was adequately moistened. Nitrocellulose was activated by placing in methanol. The plates were also placed in to the tray and prised apart using the special wedge shaped tool. The stacking gel was removed and discarded. The gel was removed carefully from the plates. The Biorad cassette was placed in the tray with the black side was facing down, then a fibre pad, followed by two pieces of filter paper and the gel were placed. Then the membrane was placed on the gel and gently rolled to ensure no air bubbles were present. Two further pieces of filter paper and fibre pad were placed over the membrane and the cassette was closed. The cassette was placed with the black side (cathode) facing the cathode. A magnetic stirrer was placed in the equipment as was a container containing ice in order to aid the transfer process. The stirrer was switched on and the transfer reaction performed by using electrophoresis at a voltage of 100V for 1 hour (see figure 2.15.5). After one hour, the apparatus was switched off and the cassette opened, the membrane had its top left corner removed to allow orientation and was removed using forceps.
Figure 2.15.5. The principle of electrophoretic transfer of protein. The nitrocellulose membrane contains pores between 0.45\(\mu\)m and 0.2\(\mu\)m, proteins are transferred from the gel to the membrane due to the flow of current (voltage/resistance). Resistance is due to the buffer, fibre pads and filter pads. Resistance can also be increased by increasing temperature and thus the transfer equipment is cooled.

2.15.6 Blocking the membrane and probing the membrane.
The membrane was blocked by incubation with 5% non-fat milk (Marvel) in PBS for one hour. The primary antibody was then diluted in 5% milk and 0.6% BSA (for antibodies used in this thesis see figure 2.15.6 in appendix 6). The membrane was placed in 50ml falcon containing 5ml of the antibody/milk/albumin mixture. The membrane was washed by agitation in PBS-Tween for 10 minutes four times (see appendix 1 for recipe). The membrane was incubated with secondary antibody for 1 hour. It was washed using PBS Tween four times for 10 minutes. The secondary antibody was conjugated with horseradish peroxidase and thus chemo-luminescence was used to detect the protein. After washing, excess PBS-Tween was removed by dabbing the corner of the membrane with paper towel. Then the ECL plus reagent was made by adding 25\(\mu\)l of reagent 1 to 1ml of reagent 2 (ECL, ECL plus and
ECL Prime were purchased from GE Healthcare. The membrane was incubated with the reagent for 5 minutes and then the protein visualized using Biomax XAL film (Kodak). The CC developer was used to visualize the proteins. For some proteins such as tubulin, ECL was used to visualize proteins (1 ml of reagent 1 and 1 ml of reagent 2 were mixed, placed on to the membrane and incubated for 10 minutes prior to developing), ECL prime was also used for other proteins (1 ml of reagent 1 was added to 1 ml of reagent 2 and incubated for 5 minutes prior to developing). Differences in expression were quantified where appropriate.

A photograph of the immunoblot was taken using the Ingenius Syngene Bioimaging System. The blot was on a white plate and white light was used. The image was stored using Gene Snap v 7.04 (Synoptics Ltd), this image was then opened using GeneTools vs 4 (Synoptics Ltd). The absorbance of each band was determined manually and the corresponding background was also determined. The absorbance of the background was subtracted from the absorbance of the study band. This was then performed for the corresponding band from the housekeeping gene. Then a ratio was determined of the normalized absorbance of the gene of interest compared to the normalized housekeeping gene. This was done for three experiments and the mean result used.

2.15.7 Reprobing of the membrane
In order to check loading or look for other proteins the membrane was stripped and reprobed. This was achieved by boiling the membrane in ddH2O for 5 minutes. The membrane was blocked in 5% milk for an hour and then incubated in primary antibody. This was then washed as described previously and incubated with the secondary antibody. Detection was performed as described in section 2.15.6. Tubulin was used as a loading control as it is ubiquitously present in all cells.
2.16 Real time evaluation of depletion of STK-10 using siRNA
SKRC39ev and SKRC39wt were incubated with either 5nM or 10nM siRNA directed against 
STK-10 and LUC (Ambion) as described above. RNA was extracted from cell pellets using 
the Qiagen RNeasy kit and assessed by running 2 μl of product on a 1.5% agarose gel. RNA 
was quantified using a nanodrop, subsequently, 1μg was converted into cDNA using the 
Superscript II cDNA synthesis kit (Invitrogen) (as described in 2.8.2). The cDNA 
concentrations were measured using a nanodrop and a working concentration of 100ng/μl 
made by diluting the DNA with water. In order to determine the standard curve of CT values 
at different concentrations of cDNA were achieved by diluting the SKRC-39ev DNA treated 
with siRNA against 5nM LUC and STK-10 to achieve concentrations of 0, 1, 5, 50, 100 and 
500ng of cDNA/reaction. The total volume of cDNA required for each reaction is 9μl and 
thus further water was added to make up the final volume. In each reaction, 1μl of gene 
expression assay mix (Taqman, Applied Biosystems) and 10 μl of TaqMan Universal PCR 
master mix (2x) was added. In order to determine the background rates, SKRC39ev cDNA 
derived from cells treated with 5nM LUC was used. The two gene expression assays 
completed were for STK-10 and GAPDH. For the background data each sample condition 
was replicated. This provided the background data for experiments to determine the relative 
knockdown achieved for each gene in SKRC39ev and SKRC39wt cells. Prior to analysis, the 
plate was sealed with a plastic seal and centrifuged at 400 x g for 1 minute. Real time PCR 
was carried out using a Biorad IQ5 Multicolour Real Time-PCR Detection System under the 
following conditions: step 1 incubation at 95° for 3 mins, step 2 incubation at 95° for 10s 
followed by step 3 incubation at 55° for 30s, with steps 2 and three repeated 40 times. cDNA 
at a concentration of 100ng/reaction from SKRCEv and SKRCwt cells treated with 5 and 
10nM LUC and STK-10 were analysed for expression of STK-10 and GAPDH using the real 
time PCR protocol described above. The CT values for a mean of three wells calculated using
the REST programme, this compared the CT values to the background CT values and then allowed comparison between LUC controls and treated wells. Each experiment was repeated three times and mean results presented.

2.17 Drug testing using a growth inhibition assay
Erlostonib was purchased from Stratech Scientific Limited (Suffolk, UK), the stock powder was diluted in 11.63ml DMSO to make 20mM stock and stored at -70°. SKRC-39ev and SKRC-39wt cells were seeded on day 0 at a concentration of 2 x10^4 cells/ml (see figure 2.17). The first and last rows and columns contained media alone in order to reduce the influence of evaporation on cells. Cells were plated out in two 96 well plates, one plate was to be used for drug treatment as shown below, the control contained only cells. The control plate was harvested on day one by addition of 20µl of cell titre blue to each well and incubating for 4 hours. Luminescence was measured using a Perkin Elmer Plate reader. On day one, the experimental plate was set up. 100x drug concentrations of erlotonib were produced in DMSO (five concentrations 1µM to 10mM). These concentrations were further diluted 1:10 in media in a clean sterile eppendorfs, the dilution was performed by adding 108µl of media to each eppendorf and then adding 12µl to the first eppendorf (to make a concentration of 100µM) and then performing serial dilutions in further eppendorfs. 10µl of each drug concentration was added to each well, with each row representing a final concentration. The plate was incubated at 37° for 72 hours. The plate was harvested by adding cell titre blue and measuring the luminescence. Data was exported into a Microsoft Excel spreadsheet and the luminescence reading on Day 1 (control) subtracted from Day 3 (experimental) for cell line at a given concentration. The mean and standard deviation for each concentration was determined and this was then analysed using the Prism programme (GraphPad Software Ltd), and the GI50 dose determined. The GI value is the concentration of a test drug where 100x
\[(T-100)/(C-T_0)\]. Where \(T\) is the optical density of the test well at 72 hours, \(C\) is the control optical density and \(T_0\) is the optical density on day 1. It is a measure of the ability of a drug to inhibit cell growth. The mean GI50 dose for three experiments was compared using a T test for SKRC39ev and SKRC39wt cells to determine any selective difference.

Figure 2.17 Experimental layout determining the effect of erlotonib in RCC. The layout of 96 well plates used to analyse the influence of different doses of erlotonib on SKRC39ev and SRC39wt cells.

2.18 Use of the Kinexus microarray to evaluate changes in protein expression

In order to determine the effect of PLK1 and STK10 depletion on global protein expression the Kinexus Microarray service was utilised. This produced an unbiased screen of the differential expression of dye labelled proteins from cell lysates. The array contains 518 pan-specific antibodies, 359 phosphospecific antibodies, 234 kinase specific antibodies and 44 antibodies directed towards protein phosphatases. The proteins which the antibodies were directed towards included those involved in: cell proliferation, stress and apoptosis. The
antibodies in the array are arranged in two identical fields. This enables two experimental conditions to be directly compared. Within each field there are sub-grids with 11 x10 spots containing antibody.

Protein was sent to the company in the form of a cell pellet, shipped on dry ice. This pellet was achieved by transfecting 2 million SKRC39ev and SKRC39wt cells with STK-10, PLK1, and control siRNA Dharmacon smartpool DNA as described in 2.14. These cells were washed in ice cold PBS and detached using trypsin. The cells were centrifuged at 500 x g and washed in ice-cold PBS. The pellet was then snap frozen in liquid nitrogen and placed in dry ice. Cell pellets were sent in order to compare protein expression in control cells and STK10 and PLK1 depleted cells in SKRC39ev and SKRC39wt cells.

The company lysed the cell pellets and then labelled the lysate with a proprietary fluorescent dye. Free dye molecules were removed. The array was then ‘blocked’ to reduce non-specific binding, then the array was incubated with labelled lysate. Excess lysate was washed away and the array imaged using a Perkin Elmer Scan Array Reader laser array reader.

Kinexus also processed the data from the array, this involved signal quantification using pre-determined algorithms and determining z-ratios. The background-corrected raw intensity data was transformed to the logbase 2. A z score was then determined by subtracting the overall intensity of all the spots within a sample from the raw intensity for each spot and dividing it by the standard deviations (SD) of all measured intensities within each sample. Z ratios were determined by taking the differences between the average Z scores and dividing them by the SD of all the differences for that particular comparison. A Z ratio of +/- 1.2-1.5 was considered significant. The manufacture provided a short-list of proteins which represented those which had demonstrated a significant change in expression.
Array technology is subject to false negative and false positive results as non-denatured proteins are used and also there is differential labelling of proteins with dye. The manufacturer’s information states that only 30-45% of results are validated (100). Thus 18 proteins were chosen as described in section 5.8.9.2 to validate the results of the array using the Kinetworks custom KCPS 1.0 screen which uses immunoblotting to confirm the array results.

2.19 Statistical considerations
Most statistics performed in this thesis utilised the IBM SPSS Statistics Programme version 21, 2012. Logistic regression was used to assess different models of genetic testing in phaeochromocytoma and these models were described as receiver operator characteristics using SPSS. Pearson’s correlation was performed in SPSS to determine any correlation in z-ratios of SKRC39ev and SKRC39wt cells treated with siRNA directed towards STK10 or PLK1. Significance was determined if the p value was <0.05. Student’s t tests were used in GraphPad Prism 5, all tests were two tailed and p<0.05 was considered as significant.

GraphPad Prism 5 was also used to determine the GI50 values described in section 2.17.
CHAPTER 3 THE MOLECULAR DIAGNOSIS OF CANCER – PHAEOCHROMOCYTOMA

Preface

Work presented in this chapter has been published in a review article by Jafri and Maher(73) and a paper by Jafri et al (101).

3.1 The rationale for genetic testing in cancer
In inherited disease, one of the most important promises of cancer genomics is to enable clinicians to prevent cancer or detect early disease in those with a genetic predisposition (102). Identification of a germline variant in those with inherited predispositions to cancer allows doctors to screen for early disease and remove disease prior to it becoming clinically significant. In addition, risk reduction strategies can be performed such as prophylactic mastectomy in the case of the breast cancer predisposition gene BRCA1 and BRCA2(103). These interventions could result in a significant decrease in the illness burden experienced by those affected by genetic mutations and also reduce costs to health services. Furthermore, family members can be tested and if negative, costly and demanding screening programmes can be ceased. Conversely, affected members can be screened appropriately and family planning is facilitated.

In order for a genetic test to be utilized it must fulfill four criteria described by Korf and Rehm(103). These criteria are the test should be: (i) valid, (ii) able to diagnose a condition (iii) guide clinical management, (iv) not cause discrimination(103). Some cancers are inherited, defined as arising from an alteration in the germline DNA either inherited or
occurring due to mutation in an egg or sperm cell. (103) Thus, individuals can be tested for a cancer predisposing gene (CPG) to determine their risk of the condition. Some cancer conditions have numerous associated CPG and therefore identifying the gene responsible for the cancer can be time consuming and costly, termed by some as a ‘diagnostic odyssey’. Therefore, it is important to determine whom to test for inherited predispositions and which genes to test.

Phaeochromocytoma is the archetypal genetic disease. It was initially believed that 10% of phaeochromocytoma were inherited (104), however many genetic studies of phaeochromocytoma have established that around forty percent of tumours have a genetic cause (105). This means that phaeochromocytoma may be the most inherited cancer. The intensive investigation into the genetics of phaeochromocytoma has led to novel insights into tumour biology. Phaeochromocytoma is a condition for which numerous genes are potentially responsible and therefore the aim of this chapter is to determine the characteristics of individuals who test positive for mutations in common phaeochromocytoma genes and characteristics that are associated with specific gene mutations in order to determine whom and which genes to test. Throughout the time of this doctoral thesis, genetic technology has been associated with new developments in phaeochromocytoma, therefore new methods of gene testing and new phaeochromocytoma associated genes will also be discussed.
3.2 The genetics of phaeochromocytoma

3.2.1 An overview of phaeochromocytoma

Phaeochromocytoma is a relatively rare tumour derived from neural crest cells with an incidence of two to eight cases per million per year. The nomenclature of phaeochromocytoma can be confusing, the World Health Organisation tumour classification defines phaeochromocytoma as chromaffin tumours arising from the adrenal medulla, and extra-adrenal tumours as paraganglioma (75). However, in the published literature the term paraganglioma may be used to refer to tumours derived from sympathetic nervous system or parasympathetic tissue (sympathetic tissue is located within the adrenal medulla, prevertebral and paravertebral thoraco-abdominal and pelvic paraganglia or in the reproductive organs, prostate, bladder, liver and organ of Zuckerkandl and parasympathetic paraganglia are located in the vicinity of the major arteries and nerves, e.g. carotid body, jugular, vagal, tympanic, pulmonary and aortic paraganglioma) (73). In this chapter, phaeochromocytoma refers to both chromaffin tumours that arise from the adrenal medulla (adrenal phaeochromocytoma (aPCA)) and those extra-adrenal tumours derived from sympathetic ganglia (extra-adrenal functional paraganglioma (eFPGL)). Phaeochromocytomas are usually secretory and typically present with features of catecholamine excess. In contrast, paraganglioma arising from the parasympathetic nervous system (usually within the head and neck and herein referred as HNPGL) are predominantly endocrinologically inactive.

3.2.2 The genetics of ‘syndromic’ phaeochromocytoma

Amongst individuals with phaeochromocytoma around ten percent may have a family or personal history of either phaeochromocytoma or another cancer (106). Such individuals may have the phaeochromocytoma as part of a recognized syndrome affecting multiple organs. There are three main syndromes associated with phaeochromocytoma, (i) von Hippel Lindau (VHL) disease associated with mutations in the \( VHL \) gene (107), (ii) neurofibromatosis
associated with mutations in the \textit{NF1} gene \cite{108} and (iii) multiple endocrine neoplasia associated with alterations in the \textit{RET} gene \cite{107}. However, the penetrance of these genes can be variable as can the phenotype of individuals affected by a particular gene mutation, for example some individuals with VHL only have a phaeochromocytoma\cite{109}.

\subsection*{3.2.2.1 VHL associated phaeochromocytoma}
Twenty percent of individuals with suspected VHL disease present with a phaeochromocytoma\cite{110}. VHL is an autosomal dominantly inherited disease with an incidence of 1 in 36,000.\cite{111} It is associated with numerous abnormalities including: retinal angioma, haemangioblastoma, endolymphatic sac tumours, serous cyst adenomas,\cite{112} clear cell RCC (average lifetime risk >70\% by age 60 years) \cite{113} and pancreatic neuroendocrine tumours \cite{114}. VHL disease is categorized into type 1 which is not associated with phaeochromocytoma and type 2 which is associated with phaeochromocytoma. Type 2 VHL is further subdivided in to 2A which is associated with haemangioblastoma, phaeochromocytoma and rarely RCC, type 2B which is associated with haemangioblastoma, phaeochromocytoma and RCC, type 2C phaeochromocytoma only\cite{109}. Interestingly missense mutations in \textit{VHL} are more likely to be associated with phaeochromocytoma compared to truncating mutation \cite{110}. In particular, phaeochromocytoma is associated with mutations that lead to an alteration in an amino acid at the surface of the protein (pVHL) compared to those causing a truncated protein, exon deletion or a missense mutation at an amino acid at the core of a protein \cite{110}. Probably, patients with \textit{VHL} gene deletions are unlikely to develop PPGL as some basal VHL protein expression is required for phaeochromocytoma development \cite{115}.
Early detection and treatment of retinal angiomas and renal tumours associated with VHL disease reduces morbidity and mortality. Thus, the detection of a germline \textit{VHL} mutation in phaeochromocytoma can lead to the initiation of tumour surveillance protocols in patients and their families. aPCA/eFPGL in VHL disease is characterised by a younger age at diagnosis (mean 28 years) and increased frequency of bilateral or multiple tumours although the risks of malignancy are not elevated (4). As many VHL-associated aPCA/eFPGL are detected during routine imaging surveillance, they are more frequently asymptomatic at diagnosis than sporadic aPCA/eFPGL.

Alterations in the \textit{VHL} gene is believed to cause phaeochromocytoma due to stimulation of the pseudohypoxic pathway (see section 4.2.1 for a detailed explanation). Briefly, there is decreased proteosomal degradation of HIF1α and HIF2α leading to HIF accumulation and downstream activation of multiple target genes. However, \textit{VHL} mutations can be tumorigenic without activation of HIF as seen in type 2C disease where there is HIF independent phaeochromocytoma formation(116).

### 3.2.2 RET associated phaeochromocytoma

The \textit{RET} gene encodes a tyrosine receptor kinase that when activated promotes growth and differentiation of neural crest tissue(117). Gain of function mutations in \textit{RET} affecting the specificity of the receptor to the substrate or constitutive activation of the gene lead to MEN2 and loss of function mutations lead to Hirschsprung’s disease (117, 118). Interestingly, a case of familial phaeochromocytoma described in 1886 was shown to result from a germline \textit{RET} mutation 121 years later (119). MEN2 is inherited in an autosomal dominant manner and is divided into three clinical subtypes, two of which (MEN2A and MEN2B) are characterised by the development of medullary thyroid cancer (MTC) and aPCA (and hyperparathyroidism in
MEN2A and a marfanoid habitus and mucosal neuromas in MEN2B). The third subtype familial medullary thyroid cancer is believed to be a variant of MEN2A without PPGL (120).

Mutations in RET are an example of cancer phenomics, where different mutations are associated with different clinical pictures. Eighty-five percent of cases of MEN2A are associated with five mutations in cysteine (Cys609, Cys611, Cys618, Cys620 in exon 10 and Cys634 in exon 11) which mean that intramolecular disulphide bonding within RET cannot take place (117). Therefore, the unpaired cysteines from two mutant RET molecules can bind causing dimerisation and constitutive activation of the receptor tyrosine kinase. These proteins have less transforming activity than missense mutations in the intracellular tyrosine kinase domain(117). The p.Met918Thr mutation is associated with 95% of MEN2B. This amino acid is at the catalytic core of RET is associated with a strong transforming activity. This may be responsible for MTC and aPCA having an earlier age at onset in MEN2B than MEN2A. Individuals with MEN2A have about a 50% risk of developing aPCA but the mean age at diagnosis of MTC is earlier than that of aPCA (~40 years) and therefore are less likely to present with sporadic non-syndromic phaeochromocytoma. Nevertheless, a germline RET mutation can be detected in about 5% of such cases (104). Individuals with MEN2A often develop aPCA (which maybe synchronous or metachronous) but eFPGL and malignancy are rare (121).

3.2.2.3 Neurofibromatosis 1 (NF1) associated phaeochromocytoma

Germline mutations in NF1 cause neurofibromatosis, a multi-system disorder involving both cutaneous tumours (localised and plexiform neurofibromas, neurofibrosarcoma) and internal
tumours such as aPCA/eFPGL, central nervous system tumours (glioma, astrocytoma and optic gliomas), carcinoid and leukaemia(122). Although, NF1 is relatively common (incidence 1 in 3000 persons), the prevalence of aPCA/eFPA in NF1 is below 1% (108). Therefore, NF1 is not a common diagnosis in aPCA/eFPGL patients (123). The median age at diagnosis of aPCA/eFPGL in NF1 is relatively late (~41 years) and so other features of the disease (e.g. cutaneous cafe-au-lait spots, axillary freckling, neurofibromas, Lisch nodules, etc) are usually present (making the clinical diagnosis of NF1 straightforward)(124, 125). The NF1 gene is a large gene (57 exons) and NF1 is associated with a wide variety of, frequently de novo, inactivating mutations making genetic analysis difficult. Therefore, although molecular genetic analysis is becoming more available, it is usually not indicated for diagnosis because of the ease of clinical diagnosis (123, 126). Somatic NF1 mutations have recently been found to be frequent in sporadic phaeochromocytoma, with somatic inactivating mutations being identified in twenty five out of sixty-one sporadic tumours in one cohort(127) and 21% of another cohort(108). Inactivation of NF1 is associated with stimulation of the Ras/raf/mek/erk signalling pathway which is key to coupling responses from the extracellular membrane to transcription factors, controlling important processes such as cell cycle progression, apoptosis and differentiation(128).

3.2.3. Genetic causes of non-syndromic phaeochromocytoma

3.2.3.1 Succinate Dehydrogenase (SDH) subunit mutations
Succinate dehydrogenase (SDH) is a mitochondrial protein involved in the tricarboxylic acid cycle and oxidative phosphorylation.(129) SDH catalyses the oxidation of succinate into fumarate in the tricarboxylic acid cycle and acts as an electron donor to complex III in the electron transport chain via ubiquinone or co-enzyme Q(129). SDH is composed of four subunits SDHA, SDHB, SDHC, SDHD. The main catalytic subunit is SDHA which converts
succinate to fumarate via the reduction of a flavin adenine dinucleotide. Electrons pass three iron sulphate centres in SDHB which transfer them to ubiquinone. Two smaller subunits, SDHC and SDHD anchor the protein complex to the inner mitochondrial membrane. (129) Succinate dehydrogenase complex assembly factor 2 (SDHAF2) is a highly conserved co-factor of flavin adenine dinucleonucleotide, SDHAF flavinates SDHA which is essential for the structure and function of the SDH complex (130). Mutations that lead to loss of function of any of the four SDH subunits and SDHAF are associated with development of PPGL. These will be described below and vary in terms of their associated phenotype and mutation frequency. Mutations in SDHx lead to decreased activity and stability of the SDH enzyme causing increased proteasomal degradation. This means that the oxidation of succinate to fumarate is impaired causing succinate accumulation. (131)

SDHB associated PPGL
SDHB mutations are an important cause of HNPGL and aPCA/eFPGL (79, 104, 132). In addition, SDHB mutations predispose to renal tumours and may present with familial RCC (133). Important clinical features of SDHB aPCA/eFPGL are a high frequency of extra-adrenal location and malignancy. Approximately 20% of mutation carriers will develop malignant disease and up to 50% of patients with a malignant eFPGL harbour a germline SDHB mutation (79, 104, 134, 135). Compared to all patients with SDH mutations, the overall risk of HNPGL (e.g. carotid body tumours) is higher with SDHD mutations whereas the risk of aPCA/eFPGL is higher with SDHB mutations (104, 133). Initial reports had suggested a high clinical penetrance for SDHB mutations, however, as testing of apparently sporadic cases has become more widespread and asymptomatic relatives of mutation carriers undergo genetic testing, the observed penetrance of SDHB mutations has fallen (e.g. 25-40% in non-probands).
(136). In addition to renal tumours, SDHB mutations may predispose to thyroid tumours (though the absolute risk is small). SDHB and SDHC mutations have been found in 12% of individuals with gastrointestinal stromal tumours without PDGFRA receptor mutations (137, 138).

SDHB related tumours occur at a younger age than sporadic tumours with a mean age of presentation of 30 years of age, with some patients presenting even earlier (139). As individuals with a SDHB mutation have a nineteen fold higher risk of malignancy (74) and have a worse prognosis than other tumours, screening in these carriers of SDHB mutations should occur from the age of ten. SDHB mutations may cause malignancy as a result of intracellular metabolic changes that allow hypoxia inducible factor (HIF) accumulation and epigenetic alterations. High levels of intracellular succinate cause SDH inactivation and inhibit HIF prolyl hydroxylases (PHD). Hydroxylation of HIF is required for binding of HIF to VHL, and subsequent ubiquitinisation and degradation. Thus, inhibition of PHD leads to accumulation of HIF and expression of HIF associated genes such as those involved in angiogenesis, cell proliferation and cell survival. This hypothesis has been supported by gene expression array data in which tumours with SDHx mutations overexpressed HIF1α and HIF2α target genes. Another potential mechanism of tumourigenesis achieved by SDH mutations is production of reactive oxygen species due to a defect in the function of the electron transport chain complex II. SDH alterations in vitro can be associated with oxidative stress, and genomic instability.

Succinate accumulation can also lead to the inhibition of α-ketoglutarate dependent dioxygenases including histone demethylases. Typically, CpG clustered together in the form of CpG islands are demethylated in normal tissue and methylated in cancer cells, and CpG that are outside islands are highly methylated in normal tissue and demethylated in
cancer. Letouze and colleagues used a methylation array to evaluate phaeochromocytoma tumours and found that tumours fell within three clusters. They classified phaeochromocytoma depending on their methylation pattern as being in cluster M1, M2 or M3. M1 tumours were SDH deficient and demonstrated hypermethylation of both CpG in islands and outside islands ‘the hypermethylator phenotype’. M2 tumours were VHL deficient and had no hypermethylation in CpG islands but were hypomethylated if not in islands. M3 tumours included NF1 and RET deficient tumours together with some VHL deficient tumours and demonstrated hypomethylation of CpG both within islands and outside islands. Patients with M1 tumours were younger and had a worse prognosis than those with M2 and M3 tumours. A mouse model of SDHB deficiency confirmed succinate accumulation in the presence of SDHB mutations, and its association with a hypermethylator phenotype. Integrative analysis of gene expression data and methylation data revealed hypermethylation was associated with down regulation of genes with key oncogenic functions. For example, down-regulated genes included those associated with neuroendocrine differentiation (RBP1), catecholamine metabolism (e.g. PMNT which encodes phenyl-ethanolamine N-methyltransferase which catalyses the conversion of noradrenaline to adrenaline), and EMT (KRT19). Thus, hypermethylation secondary to succinate accumulation can explain the malignant potential of SDH associated tumours as differentiation and invasiveness are altered by methylation.

SDHD associated PPGL
SDHD mutations were initially associated with HNPGL and subsequently with aPCA/eFPGL. Although early studies suggested that SDHD mutations were more strongly associated with HNPGL than aPCA/eFPGL, a recent report suggests that
this is mainly attributable to a very low risk of aPCA/eFPGL with a common SDHD missense mutation (p.Pro81Leu)(79). Missense mutations or in-frame deletions have been suggested to be less deleterious to protein stability compared to absent or unstable SDHD protein (79, 139). Multifocal tumours are more common with SDHD mutations.(144). PPGL associated with SDHD are usually benign with an estimated prevalence of malignancy between 0-10%(139). SDHD associated PPGL have a parent-of-origin pattern of penetrance such that disease occurs (though there are very rare exceptions) after paternal transmission of the mutation. Thus if a male inherits a SDHD mutation from their mother then they will be unaffected but any of their children that they transmit the mutation to will be at risk of HNPGL/PPGL.(145) A similar inheritance pattern is seen with SDHAF2 and MAX mutations (see later).

SDHC associated PPGL
SDHC mutations may be present in ~4% of HNPGL patients and though aPCA/eFPGL can occur it is rare (146-150). In the French paraganglioma cohort, fourteen of the sixteen patients with SDHC mutations had HNPGL and two had a thoracic eFPGL (150). Malignancy is rare in SDHC associated tumours, given the rarity of these mutations, they are not routinely investigated.(139)SDHA and SDHAF2 Associated PPGLSDHA mutations were initially described in a biallelic state (homozygous or compound heterozygous) in children with an autosomal recessively inherited juvenile encephalopathy (Leigh syndrome) (151, 152). Mutations in SDHA were initially thought to be absent from patients with HNPGL or aPCA/eFPGL. However, a recent report has described heterozygous germline SDHA mutations in a small subset of patients with HNPGL and aPCA/eFPGL(153, 154). Furthermore, SDHA mutations have also been identified in patients with gastrointestinal stromal cell tumours (155).

SDHAF2 encodes a protein required for the encorporation of FAD cofactor in the subunit A of the succinate dehydrogenase complex, flavination is necessary for the function of SDH and
thus SDHAF2 is highly conserved (139). SDHAF2 mutations in have been described in association with HNPGL but, to date, not with phaeochromocytoma (130, 156, 157). The SDHAF2 gene maps to chromosome 11q13 and, as with SDHD (11q23), there are parent-of-origin effects on expression such that tumour development only occurs after paternal inheritance (130, 156).

3.2.3.2 MAX associated phaeochromocytoma
Exome sequencing of three unrelated individuals with a similar transcriptome profile identified germline inactivating mutations in MAX (158). Loss of wild type MAX was also noted in tumour DNA and immunostaining revealed loss of MAX expression consistent with a tumour suppressor function (158). MAX is a key component of the MYC-MAX-MXD1 network that regulates cell proliferation and differentiation and there is crosstalk between this network and the mTOR pathway (to which TMEM127 has been linked (see 3.2.3.3)). Interestingly, evidence was found that paternal transmission of the gene was necessary for tumour development (as with SDHD and SDHAF2). Most cases of MAX associated phaeochromocytoma were bilateral confirming the association between genetic abnormalities and multiple tumours (159). A proportion of MAX associated PPGL are malignant (160). Overall, however MAX mutations were very infrequent occurring in between less than 1% (159) and 1.12% (160) of PPGL. As with other phaeochromocytoma susceptibility genes, MAX has also been identified in 1.65% of somatic PPGL (160). Individuals with MAX mutations were shown to have three times more urinary normetanephrine than metanephrine associated with intermediate levels of tissue PMNT. Thus this clinical finding could be used to identify those with MAX mutations (160).
3.2.3.3 TMEM-127 mutation associated PPGL
Following genetic linkage studies that mapped a novel locus for familial phaeochromocytoma to chromosome 2q11, germline mutations were identified in TMEM127 (161). The presence of loss of function mutations and allele loss in TMEM127 phaeochromocytoma was consistent with the TMEM127 acting as a TSG. TMEM127 encodes a three spanner transmembrane protein which is highly conserved and, although the function of TMEM127 is not well defined, it has been linked to mTOR signalling (161). In an extensive cohort analysis of 990 individuals with phaeochromocytoma or HNPGL the frequency of TMEM127 mutations was ~2% (162). In contrast to VHL and SDHB mutations, patients with mutations in TMEM127 do not have an early age at diagnosis (median age of 41.5 years). Typically patients have adrenal phaeochromocytomas (often bilateral) and malignancy is infrequent (161, 162). The role of genetic testing for TMEM127 mutations in clinical practice is not well defined as, though mutations are uncommon (0.9% frequency in a series of 642 French patients (163), they may be present in patient groups that are frequently excluded from genetic testing (162). Furthermore, the recent description of HNPGL and eFPGL in association with TMEM127 mutations demonstrates how the phenotype associated with inherited phaeochromocytoma can continue to evolve (164). A summary of the phenotype and inheritance of different germline phaeochromocytoma mutations is described in table 3.2.3.

3.2.3.4 Other phaeochromocytoma associated genes
Two apparently very rare causes of phaeochromocytoma are mutations in KIF1B beta and PHD2. Mutations in these genes have been described in only a few or single families respectively (165-167) and neither gene is routinely analysed in clinical practice. IDH1 has been observed in one sporadic paraganglioma and is also not believed to be a significant cause of PPGL (168).
<table>
<thead>
<tr>
<th>Gene</th>
<th>Phenotype</th>
<th>Mechanism of action</th>
<th>Approximate Frequency (%)</th>
<th>Inheritance</th>
</tr>
</thead>
<tbody>
<tr>
<td>VHL</td>
<td>aPCA, retinal angioma, haemangioblastoma, endolymphatic sac tumours, serous cyst adenomas</td>
<td>Pseudohypoxia and in some cases HIF independent</td>
<td>11</td>
<td>Autosomal dominant</td>
</tr>
<tr>
<td>RET</td>
<td>aPCA, medullary thyroid cancer,</td>
<td>Activation of tyrosine kinase</td>
<td>5</td>
<td>Autosomal dominant</td>
</tr>
<tr>
<td>NF1</td>
<td>sPCA/eFPGL, neurofibroma, central nervous system tumours</td>
<td>Ras/Raf/Mek/Erk pathway</td>
<td>~1</td>
<td>Autosomal dominant</td>
</tr>
<tr>
<td>SDHB</td>
<td>HNPGL and sPCA/eFPGL</td>
<td>Pseudohypoxia and hypermethylation</td>
<td>4</td>
<td>Autosomal dominant</td>
</tr>
<tr>
<td>SDHD</td>
<td>HNPGL and sPCA/eFPGL</td>
<td></td>
<td>4</td>
<td>Paternal</td>
</tr>
<tr>
<td>SDHC</td>
<td>HNPGL rarely sPCA/eFPGL</td>
<td></td>
<td>~1</td>
<td>Autosomal dominant</td>
</tr>
<tr>
<td>SDHAF2</td>
<td>HNPGL</td>
<td></td>
<td></td>
<td>Paternal</td>
</tr>
<tr>
<td>MAX</td>
<td>Often bilateral phaeochromocytoma</td>
<td>mTOR signalling,</td>
<td>~1</td>
<td>Paternal</td>
</tr>
<tr>
<td>TMEM127</td>
<td>Often bilateral phaeochromocytoma usually benign</td>
<td>mTOR signalling</td>
<td>~1</td>
<td>Autosomal dominant</td>
</tr>
</tbody>
</table>

Table 3.2.3. A summary of common genetic changes in aPCA/eFPGL and HNPGL. Clinical phenotypes, frequency and inheritance are described. (104, 112) (161)

3.3. Current testing strategies in phaeochromocytoma
The identification of inherited phaeochromocytoma genes provides the opportunity to define the frequency of specific gene mutations in both familial cases and in apparently non-syndromic sporadic cases. Unexpectedly, mutation analysis of RET, SDHB, SDHD and VHL
revealed germline mutations in up to 25% of sporadic cases suggesting that at least a third of patients with phaeochromocytoma had inherited disease (104). Although it has been suggested that all patients with phaeochromocytoma should be offered genetic testing, such a policy is (with conventional mutation detection methodologies) expensive (e.g. a recent estimate for testing SDHB, SDHC, SDHD, VHL and RET by conventional Sanger sequencing was $4100 (169)). Thus more targeted screening has been advocated. Clinical features that influence the odds of detecting a mutation in an individual case will include whether there is (a) a personal or family history to indicate a syndromic cause, (e.g. MTC, HNPGL, haemangioblastoma etc.), (b) a positive family history for phaeochromocytoma, (c) multiple primary phaeochromocytomas, (d) malignancy, (e) extra-adrenal location and (f) age at diagnosis (generally inherited tumours occur at an earlier age than non-inherited). In the seminal study by Neumann et al (13), 70% of individuals with phaeochromocytoma aged less than 10 years had a detectable mutation (in SDHB, SDHD, RET or VHL) compared to 8% of those who presented after age 40 years.

If a targeted approach to mutation analysis is to be pursued then it is generally agreed that those with a personal or family history of a specific syndromic cause and, in apparently non-syndromic cases, those with a positive family history for phaeochromocytoma, or multiple or bilateral tumours should be analysed. It is usual to test individual genes sequentially and the order of testing can be individualised according to the likelihood of a syndromic cause (e.g. RET if there is a family history/personal history of medullary thyroid cancer, SDHB/D if a HNPGL etc.) or, in apparently non syndromic cases with familial or multicentric phaeochromocytoma, the presence of extra-adrenal or malignant tumours would favour starting with SDHB analysis (but VHL if an adrenal phaeochromocytoma). There are no generally agreed criteria regarding which patients with apparently non-syndromic sporadic
phaeochromocytoma should be offered genetic testing for the most frequently mutated genes (SDHB, SDHD, VHL and RET). Clearly, the more targeted mutation analysis is designed to be, the more cost effective (in terms of expenditure per mutation detected) but less sensitive it will be. It is generally suggested that all patients with malignant phaeochromocytoma should be offered testing for SDHB, VHL and SDHD mutations as about 40-50% of such patients will have a detectable mutation (~35%, 5% and 1% respectively). For patients with sporadic non-syndromic extra-adrenal phaeochromocytomas the mutation detection rate is also high and thus it is suggested that SDHB, VHL and SDHD should be tested in such cases. For patients with a single benign adrenal phaeochromocytoma it is generally agreed that the age at diagnosis be used to prioritise testing – though the precise age cut-off has varied between investigators. In the largest study yet reported, Erlic et al (169) suggested that an age cut-off of 45 years would appreciably reduce the costs of genetic testing at the expense of only missing a relatively small number of mutation positive cases (<5%). As testing for TMEM127 and MAX becomes more widely available then the indications for including testing for these genes may need to be incorporated into genetic testing protocols.

3.4 Aims
This work aims to determine the characteristics of individuals with non-syndromic PPGL, in particular the frequency of mutations in SDHx and VHL genes. As no clear guidelines are currently available regarding whom to test and in which sequence to test genes, characteristics of mutation carriers will be determined in order to inform clinicians regarding optimum testing strategies.

3.5 Study methodology
3.5.1 Patients included in the study
The study population comprised probands with non-syndromic presentation of PPGL and HNPGL entered prospectively into the West Midlands PPGL/HNPGL database between 01
September 2001 and 01 September 2011. This database included all individuals referred to the WMRGS for genetic testing for SDHB, SDHD or VHL. If more than one member of a family was referred, only the proband was included in this study. Patients were referred from clinical genetics departments and endocrinologists from across the UK. RET was not analysed although it is believed to contribute to only a small proportion of non-syndromic patients and NF1 was not tested as it is usually diagnosed clinically (123, 126). Testing of other genes such as TMEM127 and MAX was not performed as they had only been recently identified and therefore were not been routinely analysed.

3.5.2 Data collated in this study
Referring clinicians were contacted regarding demographic and clinical features of cases studied. Data was collated on a standardised proforma and entered on a specified database. Demographic and clinical data including the following: gender, age at presentation, method of presentation (sporadic versus familial versus multicentric), location of tumour, presence of bilateral disease and evidence of malignancy were collected. Malignancy was defined as the presence of distant or local/regional metastasis. Information on the clinical and genetic features of tested relatives was collated in order to determine the penetrance of SDHB mutations.

3.5.3 Molecular genetic analysis
The WMRGS received 10ml of EDTA or ACD anticoagulant blood for each individual included on the study. Genomic DNA was extracted from peripheral blood leukocytes according to standard protocols described previously by our group (79). Briefly, all DNA samples were analysed in the same genetic laboratory (WMRGS). Mutation analysis was performed on the exonic and intronic flanking regions including splice sites for all exons of VHL (NM_000551.2), SDHB (NM_003000.2) and SDHD (NM_003002.2). In addition to
PCR based mutation scanning, multiplex ligation dependent probe amplification (MLPA) was performed for SDHB, SDHD, and VHL (MRC Holland). All genetic alterations were reviewed by consultant clinical geneticists and compared with available mutation data. (79, 115, 170)

3.5.4 Statistical considerations
Data was analysed using the SPSS Statistical Package (v18). Logistic regression was used to determine the predictive power of models explored and ROC curves drawn to determine the accuracy of models using different age cut offs. Student’s t tests were used to compare the ages in different subgroups.

3.5.5 Ethical considerations
All patients gave written informed consent for genetic testing and evaluation of the genetic testing service was approved by Birmingham Women’s Hospital NHS Trust Research and Development Department.

3.6 Results
3.6.1 General demographic data for the cohort
Five hundred and one patients were identified as having either a PPGL or HNPGL. Four hundred and thirteen individuals had a PPGL and eighty eight individuals had a HNPGL. The median age of PPGL patients was 36 years (range 5-94 years), the median age of HNPGL patients was 39 (range 8-74). The male to female ratio was 0.84 for PPGL and 1.02 for HNPGL patients.

3.6.2 Clinical and mutational data for individuals undergoing susceptibility testing with phaeochromocytoma/paraganglioma (PPGL)
Of the PPGL cohort (n=413); 99 (24%) patients had a family history of either phaeochromocytoma or HNPGL and 314 had a sporadic presentation. 286 of the 314 patients (69% of total PPGL cases) with a sporadic presentation had a single tumour. 40 had bilateral
phaeochromocytoma and 30 patients had multiple PPGL (at least one paraganglioma (PPGL)) at the time of referral. 57 patients (14% of the total PPGL referrals) had evidence of malignancy. 131 of the PPGL probands had a PGL (36 of 99 familial cases, 86 of 286 sporadic cases, 9 of 30 multicentric cases, and 29 of 35 malignant cases). Comparison of the age distribution of UK sporadic cases referred for genetic testing with those in the population-based cohort described by Neumann et al (6) revealed a slight enrichment of younger cases in our series (see Figure 3.6.2).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No of patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive family history</td>
<td>99 (24)</td>
</tr>
<tr>
<td>Sporadic presentation</td>
<td>314 (76)</td>
</tr>
<tr>
<td>Bilateral disease</td>
<td>40 (10)</td>
</tr>
<tr>
<td>Multiple tumours</td>
<td>30 (7)</td>
</tr>
<tr>
<td>Malignant</td>
<td>57 (14)</td>
</tr>
</tbody>
</table>

Table 3.6.2. Clinical characteristics of patients with PPGL.

3.6.3 SDHB, SDHD and VHL mutation data

Overall, 129 of 413 (31%) probands with PPGL had a detectable mutation in either SDHB, SDHD or VHL. Some mutations had previously been described by Ricketts et al (11) however additional novel mutations are listed in figure 3.6.3. Overall, SDHB mutations were detected in 97 probands (accounting for 75% of mutations detected), SDHD in 13 probands (10%) and VHL in 19 probands (15%). 11 of 97 probands with a pathogenic SDHB mutation had a deletion of one or more exons, 10 had a nonsense mutation, 13 had a frameshift mutation, 19 had a splice site mutation and 43 had a missense mutation. There was one insertion. SDHD mutations were detected in 13 probands (missense =5, nonsense = 4, frameshift =2, splice site mutation=1 and a deletion were each seen in one case). Pathogenic
VHL mutations in 19 cases consisted of missense mutations (n=17), deletions of one or more exons (n=1) and frameshift mutations (n=1).

3.6.4 Characteristics of mutation positive and mutation negative probands

3.6.4.1 Family history
Sixty one of ninety nine (62%) probands with PPGL and a positive family history of PPGL or HNPGL had a detectable mutation in SDHB (n=47), SDHD (n=6) or VHL (n=8). The median age of probands with a positive family history was 26 years (range 7-69). Of ninety-nine, twenty-two had multi-centric tumours, thirty one had a PGL and eighteen had a malignant PPGL. The age of those with a family history and mutation was significantly less (t=2.9, p=0.004) than those without a mutation (mean 26 versus 36 years).

Figure 3.6.2. Age distribution of sporadic phaeochromocytoma. The age distribution of our cases included in this study (labeled UK) compared to those presented in the population based series described by Neumann and colleagues (labeled EAPR).(104)
Table 3.6.3. Mutations in SDHB and SDHD. This figure describes mutations in this cohort which have not previously noted in a published UK cohort.(79)

3.6.4.2 Multiple tumours

Individuals with SDHx mutations can also develop tumours other than PPGL, these tend to be rare and include RCC(133), pituitary adenoma (171, 172), papillary thyroid carcinoma and gastrointestinal stromal tumours.(173) In this cohort, ten of thirty probands with multiple tumours and no family history of PPGL or HNPGL had a detectable mutation in SDHB (n=7), SDHD (n=2) or VHL (n=1). The median age of these probands with multiple tumours was 44 years (range 12-83). Three had malignant tumours and ten had one or more extra-adrenal tumour. The mean age at diagnosis for a proband with multi-centric tumours without a positive family history with a mutation and those without a mutation were 39.5 and 51 years respectively (t=3.09, p=0.003).
3.6.4.3 Malignant PPGL
Thirty of fifty seven (53%) probands with a malignant tumour had a detectable mutation in SDHB (n=26), SDHD (n=3) or VHL (n=1). Excluding those with a positive family history and/or multiple tumours, 16 of 35 (46%) probands with a sporadic malignant tumour had a detectable mutation in SDHB (n=14), SDHD (n=1) or VHL (n=1). The mean age at diagnosis in this latter group for those with a mutation and those without a mutation was 29 and 50 years respectively and there were no differences in the frequency of extra-adrenal phaeochromocytoma between the two groups.

3.6.4.5 Location of PGL
Overall fifty-eight of one hundred and thirty one (44%) probands with an extra-adrenal paraganglioma (PGL) had a detectable mutation in SDHB (n=40), SDHD (n=6) or VHL (n=12). For patients with a PGL, the mean age of those with a mutation was 28 years compared to 41 years in those without a detectable mutation (t=4.13, p<0.001). For individuals with a solitary, benign, adrenal phaeochromocytoma 29% (71/242) had a detectable mutation in SDHB (n=55), SDHD (n=6) or VHL (n=10). Sporadic phaeochromocytoma patients with a mutation were significantly younger than those without a mutation (mean age 29 versus 40 years respectively (t= 4.4, p<0.001). The frequency of mutations in patients with a single sporadic phaeochromocytoma in different age groups is shown in figure 3.6.4A. Mutations were detected in all age groups. The proportions of mutations detected in patients with a single sporadic phaeochromocytoma at different age-at-diagnosis cut-offs (<40, <50, <60 years and >60 years) are shown in figure 3.6.4 B. As the age cut-off is increased the proportion of mutations detected increases at the expense of less specificity.
3.6.4.6 Presence of additional tumour types
In addition to PPGL and HNPGL, a variety of other tumour types have been reported to be associated, or potentially associated, with SDHB or SDHD mutations (133, 138, 172, 174, 175). Therefore, the influence of the presence of another different tumour on the likelihood of mutation detection in individuals with PPGL was evaluated. In this cohort, there were three individuals with PPGL and breast cancer and none of these patients had a detectable mutation. Two individuals with PPGL also had had thyroid carcinoma and neither had a mutation. Three individuals with PPGL and renal tumours were tested and one individual (reported previously (176) had a mutation. Neither of two patients with PPGL and a second endocrine tumour (prolactinoma or adrenal adenoma) had a mutation. This data suggests that there are other yet to be identified genetic causes of these multiple cancers.

3.6.5 Characteristics of mutation positive cases by specific genes
3.6.5.1 SDHB mutation positive probands
Ninety seven probands harboured a SDHB mutation. The median age of SDHB mutation positive probands was 27 years. 49% of SDHB mutation positive probands had a family history and 7% had multi-centric tumours at presentation. 43% of individuals with an SDHB mutation had a sporadic presentation. 22% percent of those with a bilateral phaeochromocytoma had an SDHB mutation. Twenty seven individuals with SDHB mutations had a malignant tumour (26%), thirty one of those with SDHB disease had extra-adrenal tumours (32%). This indicates that SDHB mutations are more likely to be associated with malignancy and extra-adrenal presentation.
Figure 3.6.4 Solitary adrenal phaeochromocytoma. (A) The proportion of individuals with a solitary adrenal phaeochromocytoma with a detectable SDHB, SDHD or VHL mutation according to age at diagnosis. (B) A comparison of the proportion of mutation positive patients detected and the proportion of cases tested with different age cut-offs for offering testing.
3.6.5.2 SDHD mutation positive probands
The median age of the thirteen patients with an SDHD mutation was 23 years and only one patient aged over fifty had a SDHD mutation. Forty six percent of those with a SDHD mutation (6/13) had a positive family history, 15% (2/13) had multicentric disease and 38% (5/13) had a sporadic presentation. The proportion of those with multicentric disease included those with HNPGL which are strongly correlated with SDHD mutations. 23% of individuals with SDHD mutations had bilateral tumours, 15% had malignant tumours and 31% of individuals had extra-adrenal tumours.

3.6.5.3 VHL mutation positive probands
Ten probands harboured a VHL mutation, their median age was 20. There were no patients aged ≥46 years. 38% had a positive family history of PPGL, 5% had multi-centric tumours and 57% had sporadic presentation. Nineteen percent had extra-adrenal tumours and 32% of individuals had bilateral disease. 10% had a malignant tumour.

3.6.6 Clinical characteristics and SDHB, SDHD or VHL mutation prediction
This data suggests that the likelihood of detecting a SDHB, SDHD or VHL mutation was increased by the presence of a positive family history of PPGL or HNPGL, multiple tumours, extra—adrenal location and younger age at onset. This was consistent with previous reports (see Jafri and Maher(73) and references within). However, whilst there is a consensus about the risk factors for the presence of a mutation, there is little consensus about the age up to which patients with sporadic phaeochromocytoma should be offered genetic testing.

Therefore different selection models were applied to this data to investigate the sensitivity and efficiency of different age cut-offs. Thus in model A, all patients with a positive family history, multiple tumours, extra-adrenal location and/or malignant PPGL plus those with sporadic adrenal phaeochromocytomas aged ≤45 years would be tested. Applying these criteria to this data and undertaking logistic regression analysis revealed that application of
model A would have detected 69% of all mutation positive cases (97% of SDHD mutations, 95% of VHL mutations and 76% of SDHB mutations). The effects of altering the age cut-off for sporadic phaeochromocytoma cases to 50 years (Model B) and 60 years (Model C) on the proportions of mutation positive cases detected were studied. Increasing the age cut off to 50 years would increase the number of mutation positive cases up to 69.1% and increasing the age cut off to 60 years would increase the number of mutation positive cases up to 76.4% respectively. The ROCs for each model are shown in figure 3.6.6. Although increasing the age cut-off improves the mutation detection rate, there is an increase in the number of tests undertaken. Thus, assuming an indicative rate of £1500 ($2300) for mutation analysis of SDHB, SDHD and VHL the cost per mutation detected for analyzing all PPGL cases is £4802 (approximately $7360). Using model A, the cost is £4170 (approximately $6380), £4245 for model B (approximately $6490) and £4500 for model C (approximately $6885).
Figure 3.6.6 Receiver Operating Characteristic (ROC) Curves for Phaeochromocytoma.

ROC curves if testing occurs in all individuals with family history, extra-adrenal, multiple and malignant phaeochromocytoma and aged ≤45, ≤50, ≤60 years old (models A, B and C, labelled less_45, less_50 and Less_60 respectively).
3.6.7 Clinical and mutational data for individuals undergoing susceptibility testing with head and neck paraganglioma (HNPGL)

Eighty eight probands presented with HNPGL and their median age at diagnosis was 40 years (range 8-75 years). 41% of the cohort had a positive family history of either HNPGL or PPGL. Eight of fifty-two patients without a family history presented with multiple tumours. Median age at presentation was 38 years in those with a positive family history (n=36), 36 years in those with sporadic multiple HNPGL (n=43) and 36 years in those with a single sporadic HNPGL (n=40).

3.6.7.1 SDHB and SDHD Mutation Data in HNPGL

Overall, 63% (n=55) of probands with HNPGL had a detectable mutation in SDHB (27%, n=24), or SDHD (35%, n=31). Pathogenic mutations in SDHB consisted of deletions of one or more exons (n=2), nonsense mutations (n=3), frameshift mutations (n=1), splice site mutations (n=3) and missense mutations (n=15). Pathogenic mutations in SDHD consisted of deletions of one or more exons (n=4), nonsense mutations (n=2), frameshift mutations (n=7), splice site mutations (n=0) and missense mutations (n=18).

3.6.8 Characteristics of mutation positive and negative HNPGL probands

3.6.8.1 Family history

33 of 36 (92%) probands with a positive family history of PPGL or HNPGL had a detectable mutation in SDHB (n=15) or SDHD (n=18). Two patients had multi-centric tumours and two had malignant tumours.

3.6.8.2 Multiple tumours

Eight of nine (89%) probands with multiple tumours and no family history of PPGL/HNPGL had a detectable mutation in SDHB (n=2), SDHD (n=6). The median age at diagnosis for those with multiple tumours and a mutation was 38.5 years compared with 62 years for those without a mutation.
3.6.8.3 Malignant HNPGL
Only three patients (2 of whom had a positive family history) were diagnosed with a malignant HNPGL. Two had truncating mutations (one SDHB nonsense mutation (c268C>T, p.Arg 90X) and one a SDHD frameshift mutation (c.94_95del TC, p Ala33 Ile fs*35). No mutation in SDHD, SDHB, or VHL was detected in the third case.

3.6.8.4 Single sporadic HNPGL
Sixteen of forty four probands of individuals with a sporadic presentation had a detectable mutation in SDHB (n=7) and SDHD (n=9).

3.6.9 Characteristics for mutation positive HNPGL cases for specific genes

3.6.9.1 SDHB mutation carriers
The median age of SDHB mutation carriers was 46 years. 63% (15/24) of SDHB mutation carriers had a family history and 8% (2/24) had multicentric tumours at presentation. 29% (7/24) of individuals with an SDHB mutation had a sporadic presentation.

3.6.9.2 SDHD mutation carriers
The median age of individuals with SDHD mutations was 38.5 years. 58% (18/31) had a positive family history, 19% (6/31) had multicentric disease and 29% (9/31) had a sporadic presentation. Two patients had both a positive family history and multiple tumours.

3.7. Discussion

3.7.1 Recommendations from this study in light of the current literature
At the time of performing this study, mutations in 10 genes (SDHB, SDHC, SDHD, VHL, NF-1, RET, TMEM127, SDHAF, SDHA, MAX), were accepted to cause inherited PPGL and/or HNPGL (see Jafri and Maher (73) and references within). A further two possible inherited PPGL genes had been reported in single reports (PHD2/EGLN1 and KIF1β (165, 166)). These potential inherited phaeochromocytoma genes were not routinely evaluated due to their relative rarity. At the time the contribution to inherited PPGL/HNPGL from mutations in
\textit{MAX, TMEM127, SDHAF, SDHA} and \textit{SDHC} appeared to be relatively small and/or not well defined (130, 153, 156, 160, 163, 177). Among non-syndromic PPGL cases \textit{SDHB, SDHD} and \textit{VHL} account for most cases and, though \textit{VHL} mutations may occasionally be associated with HNPGL, \textit{SDHD} and \textit{SDHB} are the major HNPGL genes (104, 135, 144, 150, 178-181). The frequency of \textit{VHL, SDHB} and \textit{SDHD} mutations among PPGL/HNPGL is variable because of the presence of founder mutations in some populations. This study was the first extensive study describing mutation analysis of PPGL and HNPGL cohorts from the United Kingdom and was similar in size to large studies from the French COMETE Group (150) and Spain (179). In addition, in contrast to earlier studies(104), all patients in the current study were tested for germline exonic deletions/duplications in \textit{SDHB, SDHD} and \textit{VHL}.

Two limitations of the current study were that \textit{RET} germline mutation analysis was not performed as part of the genetic testing service and that the cohort represents a referral-based series and not a population-based series. \textit{RET} gene mutations are less common in non-syndromic populations however can occur in 5% of non-syndromic patients.(104) Use of a referral based population potentially contributes to ascertainment bias as possibly more patients with a high risk phenotype were included in the study. Nevertheless, many of the findings of this study are consistent with those of large population and referral-based studies from outside the UK in that a positive family history, multiple tumours, malignant disease, extra-adrenal location and younger age at diagnosis were all indicators of an increased mutation detection rate.

This cohort had a larger representation of malignant PPGL than the cohort described by Erlic \textit{et al} (169) (12.5% compared with 4%). Furthermore, the frequency of germline \textit{VHL} gene
mutations detected was less than that reported by Neumann et al (104). This latter finding might reflect more intensive investigation to detect subclinical features of VHL disease before genetic testing in the UK or some referral bias. However, the age distribution of sporadic cases in our series described in figure 3.6.2 was not markedly dissimilar to that reported in population-based series (6).

HNPGL are mostly associated with mutations of SDHB and SDHD (169), though SDHC and rarely TMEM127, SDHAF and VHL mutations may be detected (130, 153, 161, 182). A minority of patients had a malignant HNPGL (3%), this proportion was similar to that seen by Boedeker et al who identified 3.6% of 195 individuals tested for HNPGL had evidence of distant metastases. (183) All malignant HNPGL in Boedeker and colleagues study had SDHB mutations. In this cohort, one patient had a SDHB mutation, one patient had a SDHD mutation and one patient had no detectable mutation in SDHB, VHL, or SDHD.

The high mutation detection rate in this study compared to population-based series (104) may reflect a combination of ascertainment bias and MLPA analysis to detect SDHB and SDHD (and SDHC) exonic deletions. As universal genetic testing of all PPGL patients using conventional sequencing technology is expensive (even for just the major inherited PPGL genes; see below) a number of groups have suggested clinical stratification strategies to increase mutation detection efficiency and cut costs. Thus, Erlic et al (180) analysed mutation detection predictors in 989 cases of non-syndromic phaeochromocytoma and suggested that germline mutation testing for RET, SDHB, SDHD and VHL could be most efficiently focused on patients with one or more of the following: family history of PPGL/HNPGL, age <45 years, multiple tumours, extra-adrenal location and previous HNPGL. Although there is a consensus that patients with a positive family history or multiple tumours should be offered genetic testing and most would suggest at least SDHB and SDHD mutation testing in patients...
with extra-adrenal or malignant tumours, there is less agreement regarding age cut-offs for targeting genetic testing of patients with a single benign phaeochromocytoma and no family history. Application of the Erlic et al (180) testing criteria to our cohort would detect most *SDHB, SDHD* and *VHL* mutations in the cohort. However, a significant minority of cases would not be detected. Increasing the age cut-off to 60 years would have increased the proportion of mutations detected but increased genetic testing costs (e.g. for sporadic benign phaeochromocytoma patients and age cut-offs of 40 years, 50 and 60 years would result in the detection of 52%, 72% and 93% respectively of all mutations, but would have involved testing 52%, 69% and 89% respectively of all sporadic benign cases). An age cut-off of 45 or 50 years might appear to offer the best balance between mutation detection and genetic testing costs. The majority of mutations “missed” by an age cut-off of 45/50 years were in the *SDHB* gene. Thus, there is a clear rationale for suggesting that if an age cut-off of 45/50 years is instigated then this could be combined with *SDHB* immunostaining (79, 154, 184) to identify those older patients who could benefit from mutation analysis. Although clinical and immunohistochemical criteria can be used to prioritize patients for genetic testing, it seems likely that, as the number of inherited PPGL genes increase (and the proportion of cases with a genetic basis), the demand for more extensive genetic testing will increase.

The cost of *VHL, RET, SDHB* and *SDHD* by conventional (Sanger) sequencing technology has been estimated to be approximately £1800 ($2700) (181), but the advent of next generation sequencing (NGS) offers the potential for faster and less expensive testing of multiple genes simultaneously (see section 3.7.3). Hence, in the near future comprehensive mutation analysis of all proven PPGL and HNPGL genes (excepting *NF1*) in all patients may emerge as the standard testing strategy and therefore patients that do not satisfy current local
criteria for genetic testing should have DNA stored for future analysis. A major advantage of a comprehensive NGS testing strategy is that all genes are tested simultaneously rather than serially and so the time to mutation detection is reduced.

However, it remains important to evaluate the results of genetic testing in service-based settings so that the recommendations for genetic testing are kept under review. This will enable genetic testing recommendations to reflect the characteristics of their associated population. For example, in this series, a significant proportion of patients did not have a detectable mutation whereas series of HNPGL and PPGL from the Netherlands have revealed that six founder mutations accounted for 88.8% of all mutations(177). Patients included in research studies are not necessarily representative of those in the general population and the phenotype associated with a particular gene may emerge over time. For example, TMEM127 mutations were initially thought to be confined to adrenal phaeochromocytoma cases, often aged >40 years, but were subsequently reported in patients with paraganglioma and HNPGL (164).

### 3.7.2 New developments in the genetic basis of phaeochromocytoma

#### 3.7.2.1 Recently identified genes in phaeochromocytoma

NGS techniques have recently facilitated the identification of novel phaeochromocytoma associated genes. As these have only recently been characterized the extent they influence phaeochromocytoma susceptibility remains to be ascertained. However, the genes identified to fit the current paradigm regarding the pathophysiology of phaeochromocytoma.

**3.7.2.1.1 Fumarate Hydratase (FH) as a PPGL associated gene**

FH is a known TSG previously confirmed to be the cause of Hereditary Leiomyomatosis RCC (see section 4.2.3 for more detail)(185). FH has been demonstrated to be associated with familial PPGL in 4 out of 598 cases (74, 186), Clark et al have also independently
identified FH mutations in phaeochromocytoma(187). Individuals with FH associated PPGL are more likely to have multiple tumours and these tumours are more likely to be malignant(186). Loss of heterozygosity of FH has been demonstrated in tumours and clinically these tumours have similarities to those seen in SDHB deficient individuals such as having elevated normetanephrine levels(186). FH deficiency causes fumarate accumulation which acts as an oncometabolite in much the same way as succinate. In SDHB deficient tumours, succinate inhibits PHD enzymes and TET enzymes leading to increased pseudohypoxic signalling and epigenetic abnormalities. Fumarate accumulation causes TET inhibition which can be demonstrated using immunohistochemistry showing low levels of staining for 5-hydroxymethylcytosine. Immunohistochemical staining of SDHB and FH deficient tumours revealed low levels of 5-hydroxymethylcytosine compared to NF1 and RET deficient tumours. Similarly tumours from individuals with FH mutations demonstrated protein succination due to the interaction of fumarate with sulphydryl groups on proteins. Immunohistochemical staining of tumours could therefore be used as an adjunct to the identification of the most appropriate mutation. It is hypothesized that the association of malignancy with SDHB and FH mutations is due to increased pseudohypoxic signalling and inhibition of demethylation leading to increased vascularization and increasing the probability of metastasis and EMT.

3.7.2.1.2 HIF2A as a PPGL associated gene
HIF2A mutations were initially identified in patients with polycythemia and phaeochromocytoma(188-190). Most mutations found in HIF2A have been found in sporadic tumours, and more recently have been identified in the absence of erythrocytosis.(191) Mutations in HIF2A caused stabilization of HIF2α by affecting the residue involved in prolyl
hydroxylase binding. This alteration leads to failure of HIF-2α ubiquitinisation and degradation. A recent study revealed mutations of HIF2A occurred in tumour DNA from sporadic PPGL (2.4% of the cohort) but no inherited PPGL. One specific mutation Pro531Thr was associated with decreased VHL binding and caused tumours when transfected cells were injected into mice. Furthermore, HIF2A mutations were associated with increased expression of hypoxia associated genes, and a dedifferentiated phenotype.\(^{(192)}\) HIF2A associated PPGL can be multi-centric tumours which are usually thought to be inherited through families. The variable phenotype associated with HIF2A mutations may be caused by genetic mosaicism, with the mutation occurring as a \textit{de novo} event in early embryogenesis in the post-zygotic phase.\(^{(193)}\) Thus individuals with a HIF2A mutation should undergo lifelong surveillance and their offspring should be screened for the mutation.

\textbf{3.7.2.1.3 HRAS as a PPGL associated gene}

Unlike the other PPGL associated genes, HRAS mutations have only been described as somatic events in sporadic PPGL.\(^{(194, 195)}\) Four of fifty eight patients in one series\(^{(195)}\) had missense mutations in HRAS present in tumour DNA which were absent in the germline DNA. The mutation was associated with a benign tumour with a low proliferative index and male sex. There was evidence of elevated RAS signalling in the tumours and patients had increased metanephrines. Germline HRAS mutations are associated with Costello syndrome which is not known to be associated with PPGL. A second European study\(^{(194)}\) also found that there was a ten percent rate of HRAS mutations in sporadic PPGL however, did not find any clinical correlates associated with mutation positivity.

\textbf{3.7.2.2 Clustering of phaeochromocytoma according to transcriptomic and genomic data}

Rather than phaeochromocytoma being subdivided based on whether they present as part of a syndrome or not, phaeochromocytoma can be clustered according to their genotype and
corresponding cell biological abnormalities. This categorisation has been greatly aided by gene expression arrays and other genomic techniques. Analysis of PPGL on the basis of their gene expression has also facilitated the discovery of new phaeochromocytoma associated genes, for example the identification of \textit{MAX} and \textit{FH} was facilitated by analysing gene expression data.

\textbf{Cluster I Pseudohypoxia associated PPGL (VHL, SDHx, FH, HIF2A associated tumours)}

Transcriptional profiling studies have demonstrated that \textit{VHL} and \textit{SDHB} associated tumours display an increased expression of genes related to pseudohypoxia and angiogenesis.(141) In particular there was increased expression of hypoxia inducible factor 2\(\alpha\) (HIF-2\(\alpha\)), NOX4 (required for HIF-2\(\alpha\) transcriptional activity) and VEGF(141). HIF-2\(\alpha\) overexpression is the key differentiator of cluster I and II PPGL.(196). Transient expression of HIF-2\(\alpha\) is believed to occur in the chromaffin cell progenitor cells early in embryogenesis, thus overexpression of HIF-2\(\alpha\) in PPGL is a reflection of their tissue of origin and describes the susceptibility of the chromaffin progenitors to mutations that lead to stabilisation of HIF(196). PPGL with a \textit{HIF2A} mutation also form part of cluster I displaying upregulation of hypoxia induced genes(191). Cluster I PPGL are associated with secretion of higher quantities of immature catecholamines. This is due to decreased expression of key components of the catecholamine biosynthetic and secretory pathways. In particular, the enzyme PNMT which converts norepinephrine to epinephrine is absent resulting in increased secretion of norepinephrine. This is not seen in cluster II PPGL (see below) which tend to secrete epinephrine.(196)

Within the ‘pseudo-hypoxia cluster’, transcriptomics enables differentiation of \textit{VHL} and \textit{SDHB} associated tumours. \textit{VHL} associated tumours are associated with activation of the TNF
pathway and HIF-1α. The latter is associated with increased Egln3 transcription. HIF1-α is important in the Warburg effect (discussed in section 1.2.2.2), it has several functions including: switching on glycolytic enzymes, inducing glucose transporters, inducing lactate dehydrogenase and mediating the expression of pyruvate dehydrogenase kinase 1. (197) Pyruvate dehydrogenase kinase 1 inhibits the conversion of pyruvate to acetyl coA reducing mitochondrial function and oxidative phosphorylation (197). VHL associated PPGL also have no SDHB expression (197). Glycolysis is stimulated preferentially in VHL associated phaeochromocytoma compared to SDHB associated PPGL indicating a HIF independent mechanism. It was found that VHL activates p53 which in turn regulates a glycolysis regulator. (197) SDHB associated tumours have increased expression of cell migration and adhesion genes which may be associated with their increased tendency for malignancy. (141) VHL associated tumours tend to be adrenal and benign whereas other cluster I tumours tend to be extra-adrenal and have an increased malignant potential. (131)

**Cluster II: PI3K/AKT/mTOR and Ras/Raf/Erk associated PPGL (NF1/RET/H-ras/MAX associated tumours)**

Tumours with either NF1, RET or HRAS mutations share a transcriptional profile. (108) Cluster II tumours represent 70% of sporadic tumours (115) and tend to be adrenal (131). These tumours except those associated with MAX tend to be benign. Alterations in these genes are all associated with increased intracellular signalling. Typically this involves increased signalling of the PI3K/akt/mTOR and Ras/Raf/Erk pathways. Other genes upregulated in this cluster include those genes associated with initiation of translation, protein synthesis, adrenergic metabolism (e.g. PNMT) and neuroendocrine differentiation. (115) There is no difference in the gene expression profile of tumours associated with RET and NF1 mutations indicating a common tumourigenesis pathway. (115)
MAX associated tumours are found within a subcluster of cluster II, they are characterised by intermediate PNMT expression (more than cluster I tumours and less than cluster II tumours) and intermediate HIF-2α expression (more than cluster II tumours and less than cluster I tumours). MAX inhibition in a cell line model was associated with decreased PNMT expression which suggested a causative role. HIF1α levels were higher in MAX associated tumours than in either cluster I or cluster II tumours (196).

3.7.3. Use of genetic testing to guide clinical management
Identification of germline mutations in aPCA/eFPGL and HNPGL has important clinical implications. Firstly, as the paternal mode of inheritance has been noted in MAX and SDHD mutations appropriate screening regimens can be applied. Individuals carrying mutations can be screened for other associated conditions such as kidney cancer in VHL associated disease and MTC in RET associated disease. In individuals found to have SDHB associated disease for example, the risk of malignant disease means a more cautious approach could be adopted. Finally, improved understanding of the pathophysiology associated with these mutations can lead to stratified medicine approaches in PPGL. For example, cluster I associated PPGL could be treated with angiogenesis inhibitors such as sunitinib (198, 199) and demethylating agents are being trialled (200). Cluster II tumours may be treated with key pathway inhibitors such as mTOR inhibitors (200). This model of using genetics to understand tumour biology and then inform treatment choice maybe an example for other cancers in the future.

3.7.4 Future directions for genetic testing in phaeochromocytoma and paraganglioma
Given the fast moving pace of improvements in sequencing technology, the costs of genetic testing are decreasing year on year. Furthermore, in PPGL new genes are being discovered in the condition on a yearly basis. This has led to a trend towards more generalized testing in phaeochromocytoma with some authors suggesting all patients with the condition be tested.
The advantage of this strategy is that the detection rate can increase as apparently only 30-50% of individuals have a ‘strong enough’ family history to warrant genetic testing (202). Furthermore, rather than testing single genes sequentially in individuals with phaeochromocytoma, multiple genes can be tested at the same time. This leads to a quicker result and an increased chance of a gene mutation being identified. This ‘blanket’ testing of certain cancers has already been introduced in ovarian cancer in patients in Canada and Australia where all patients with ovarian cancer are tested for \textit{BRCA1} and \textit{BRCA2} as half of all patients with mutations in these genes have no positive family history. Critically mutations in these genes are associated with improved platinum sensitivity and thus testing has clinical implications (203).

The advantage of early identification of genetic causes of phaeochromocytoma in those at risk of phaeochromocytoma or paraganglioma is that screening can be initiated earlier. Disease can be identified at an early stage and tumours removed. This has the advantage that surgery would be less extensive and the serious risks associated with the disease can be abrogated. This can in severe cases be sudden death due to sudden catecholamine release. Furthermore, related family members can be tested and then would be offered screening investigations if necessary or avoid screening if negative for the mutation (204). Dependent on the type of mutation identified different surveillance strategies may be instigated for example \textit{SDHB} mutation associated tumours are associated with a poor prognosis whereas \textit{HRAS} mutations are associated with a more benign course at present.

\textbf{3.7.5 Use of NGS in the diagnosis of phaeochromocytoma.}

NGS (detailed in section 4.7 of this thesis) has been developed as means of interrogating numerous genes simultaneously. This is both cheaper and quicker than the sequential approach utilized in this chapter. The advantages of using NGS should be considered with
regard to two possible negative aspects of the technique. Firstly, testing of larger number of genes by NGS can be associated with an increased risk of identifying a variant of unknown significance (over 30% in some estimates)\cite{202}. It is unclear how to manage such patients and having such a variant can be associated with anxiety and distress. Secondly, potentially an incidental finding of a mutation in another non-cancer associated gene could be identified. There are no clear guidelines regarding which alterations to report to patients in the UK.

Two main methods have been utilized to harness the potential power of NGS in the diagnosis of phaeochromocytoma associated genes (i) whole exome sequencing (WES) and (ii) multiplex gene testing using custom designed libraries for genes of interest\cite{204, 205}. These techniques require careful validation in addition to the bioinformatics needed in order to quality assure and filter available data.

WES does not cover the entire exome and the sequences captured are dependent on the probes used within the library. One sample presented in McInerley-Leo’s cohort\cite{204} had no discernible mutation using one exome sequencing library, however when the library was analysed in did not contain all the exons in \textit{SDHC}. Thus, NGS was performed using a different exon capture library and an SDHC mutation was identified. This group compared the different capture rates for the main phaeochromocytoma genes for different exome capture platforms. They identified that within the ‘real world’ laboratory setting, capture of exomic regions was not as efficient as the manufacturer supplied data, indicating validation of coverage should be performed. Furthermore, different capture libraries had different coverage methods for example the Nimblegen and Agilent systems tended to have some coverage of all genes whereas the Illumina Nextera system was more ‘all or nothing’.\cite{204} In addition, the 5’UTR was not included in the Nextera exon capture system thus some alterations could be overlooked. This group however argued that WES has two main advantages over multiplex
gene sequencing (i) it is useful for gene discovery as novel alterations in ‘new’ genes can be identified and (ii) the technique does not need to be updated every time a new gene is identified. A disadvantage of WES in a condition where there are many known genes is that potentially the technique may be more expensive and due to the amounts of data more time consuming. Interestingly, this group was able to perform the analysis relatively quickly (<1hr for analysis after initial bioinformatics (quality control, sequence alignment, removal of common SNPs and filtering for common phaeochromocytoma genes). The results were reported to clinicians within 5 weeks suggesting this is a feasible technique. A similar study in Sweden also employed WES in the genetic screening of paraganglioma and phaeochromocytoma. This group used the Agilent Sure Select exome capture kit and also focused on their analysis on the known phaeochromocytoma associated genes. Their data was filtered in house using standard protocols and after this had been achieved reporting the data required only 30 minutes. This group also felt that the ‘turn around’ time of NGS could be a week.

NGS in phaeochromocytoma or cancers in general is not without its complications. A recent survey of the use of a cancer susceptibility gene testing panel revealed 30% of all cancer panels performed contained a variant of unknown significance or ‘benign’ variant. There is at present little work being carried out in order to determine how to characterize these variants and thus individuals with such genetic variants will experience a degree of uncertainty which may adversely influence them for years to come. Furthermore, for some cancer susceptibility genes the penetrance and lifetime risk is not clearly defined. Therefore, counselling regarding the implications of carrying such a gene is yet to be clarified.
Multiplex sequencing using a panel of susceptibility genes has been successfully achieved recently (205, 208, 209). Rattenberry and colleagues (205) used multiplex sequencing on a Roche platform to evaluate nine PPGL associated genes (MAX, RET, SDHA, SDHB, SDHC, SDHD, SDHAF, TMEM127, VHL). They found the technique to have a sensitivity of 98.7%. However, exons with a GC content of greater than 66% produced poor results. There was also a considerable proportion of variants of unknown significance (61%). The technique was associated with a considerable reduction in costs (70%) and time taking less than 60 days compared to 160 days. The study demonstrated that multiplex testing was accurate and associated with substantial time and cost savings compared to Sanger sequencing. NGS sequencing of the same nine genes was also carried out on germline DNA from thirty-one patients with histologically proven PPGL using an Illumina platform at x50 coverage, MLPA was also performed to identify deletions (208). This universal approach was associated with a 31% rate of identification of mutations, the population tested was predominantly sporadic and interestingly two SDHAF mutations were identified which would otherwise have been missed. This group calls for universal testing as one patient with a TMEM127 mutation was 57 years old and would have been missed by most targeted testing protocols and one patient with a SDHAF2 mutation was 67 and would also have been missed. Family members of these individuals would therefore require testing and potentially screening furthermore the patient with a TMEM127 mutation would benefit from further surveillance as TMEM127 mutations are associated with bilateral disease (208). However, careful cost-benefit analyses are required before universal screening is performed.

In a Swedish study (209), tumour DNA from eighty-six patients were sequenced for mutations in ELGIN1(PHD2), KIF1ββ, MAX, MEN1, NF1, RET, SDHA, SDHB, SDHC,
SDH, SDHA, TMEM127 and VHL. Ninety seven percent of the exons were covered with problems in areas of GC rich nucleotides and the average depth of coverage being x 915. NGS confirmed known mutations i.e. in SDHB and subsequent analysis of germline DNA revealed 19% of sporadic tumours harboured a germline mutation. As this panel included genes less commonly investigated i.e. KIF1ββ, the study identified novel missense mutations and identified somatic KIF1ββ mutations. This cost of the analysis by this group was believed to be €189 per sample. As the group identified some novel mutations in sporadic tumours, the group suggested that a universal screening approach may be advantageous as some alterations would have been missed by conventional screening. However, the proportion of cancers that are associated with the ‘novel genes’ identified in PPGL is very small and thus others argue that routine testing of these genes may not be cost effective. (159)

3.8 Conclusions
There are no current UK based guidelines to guide clinicians regarding which individuals with PPGL or HNPGL should be offered genetic testing. Decisions regarding who to test rest with endocrinologists and clinical geneticists based on their personal experience, clinical interests and the published literature. Within the literature mutation frequency varies according to population studied: for example the founder mutations in the Black Forest region of Germany (210) and the Netherlands (177) lead to different mutations frequencies to those described in this study. The UK benefits from a heterogenous population and thus may well have different mutation frequencies and characteristics. Therefore, this study provides important information to guide clinicians in the UK population. This data corroborates the recommendations of other key publications in the field, notably suggesting that all individuals with bilateral tumours, familial tumours, malignant, and extra-adrenal tumours should be tested. Furthermore, individuals with HNPGL have a greater likelihood of harbouring a
mutation than those with a solitary adrenal phaeochromocytoma. With regards to the best age for testing individuals with solitary adrenal phaeochromocytoma susceptibility genes there is no clear age cut off which will lead to no cases being missed. The cases that are missed are predominantly $SDHB$ mutations and therefore perhaps testing could be combined with immunohistochemical tumour analysis in these older individuals. This immunohistochemical analysis (211) is not currently routinely performed. Thus, the age at which patients should be tested requires a judgement based on a combination of the currently available literature regarding mutation frequency and the costs of testing.
CHAPTER 4. INVESTIGATING THE MOLECULAR PATHOLOGY OF RENAL CELL CARCINOMA (RCC)

Preface
Some of the work in this chapter has been previously published Jafri *et al.*, (212)

Background

4.1 Understanding the challenges in the current management of RCC.

4.1.1 Background: histology and epidemiology
RCC is a cancer derived from the malignant proliferation of the renal tubular epithelium, it is the eighth most common cancer in the UK (213). There are several different distinct pathological subtypes of RCC. These subtypes are associated with a variety of clinical disease spectra. Different subtypes can be associated with a number of inherited causes of RCC which will be discussed in section 4.2. RCC is typically classified according to its histological subtype. The most common subtypes of RCC are clear cell carcinoma (75%) followed by papillary, chromophobe, sarcomatoid and oncocytoma (which are benign tumours) (214). With increased understanding of the molecular pathology of cancer, this classification may be superseded in the future. The most recent pathological consensus into the classification of RCC (215) has recommended additional histological subtypes based on genotypic and phenotypic data. These include the new subtypes of; tubulocystic RCC, acquired cystic disease-associated RCC, clear cell (tubulo) papillary RCC, the MiT family translocation RCCs (in particular t(6;11) RCC), and hereditary leiomyomatosis RCC syndrome-associated RCC. There were also two provisional potential subtypes of RCC which are based on the presence of a genetic alteration; *SDHB* deficiency-associated RCC; and *ALK* translocation RCC (215) It is likely that with better understanding of the molecular pathogenesis of malignant tumours this classification may be further complicated by the
presence and absence of genetic changes. Histological examination of RCC tissue enables the
determination of the Fuhrman grade. This system grades tumours from 1 to 4 and has been
shown to have prognostic significance. Grade is assigned based on the nuclear size, shape
and nucleolar prominence of cells (216). Grade 4 tumours of the same size and subtype as a
grade 1 tumour are more likely to metastasize.

The incidence of RCC has been increasing in recent years (217); this may be ascribed to a
number of factors including increasing use of imaging technologies in routine medical
practice leading to identification of smaller asymptomatic tumours. Furthermore, the risk of
RCC increases with increasing age, and the increased prevalence of smoking, obesity and
hypertension may have contributed to this increase in the incidence of RCC. (218) Individuals
on long term haemodialysis develop renal cysts (acquired renal cyst syndrome), these
individuals are four times more likely to develop RCC (218).

4.1.2 The diagnosis and staging of RCC

Diagnosis is determined partly by the means by which patients present with RCC. An
individual may undergo a diagnostic imaging technique such as ultrasound imaging or
computer assisted tomography (CT) and a lesion suspicious for a RCC can be identified
incidentally. This mode of presentation occurs in asymptomatic patients and can account for
up to 80% of new diagnoses (219). In those with symptomatic disease, the classical clinical
triad of pain in the flank, haematuria, and a palpable mass occurs in only 9% of cases. (219)
Other common clinical features include: fatigue, weight loss, anaemia, hypercalcaemia, raised
erthrocyte sedimentation rate (ESR), erythrocytosis, fever and symptoms derived from the
presence of metastatic disease such as a pathological fracture, cough and testicular
varicocele. (219).
Once RCC is suspected, the extent of disease is then assessed radiologically using CT to stage the disease according to the tumour nodes metastases (TNM) classification which characterizes tumours on the basis of the extent of the primary tumour, presence of nodal and distant metastases (figure 4.1.2)(220). Staging determined by radiological investigations is denoted by a prefix c and has been demonstrated to be upstaged postoperatively when the pathological staging is performed (denoted by a prefix p)(220) Imaging enables surgical planning to take place in order to establish the proximity of the tumour to other organs and vascular structures.(221) Typically, percutaneous biopsy is not carried out on presumed RCC due to the risk of seeding and primary surgical treatment is preferred.(221)

<table>
<thead>
<tr>
<th>Tumour</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Tx</td>
<td>Not assessed</td>
</tr>
<tr>
<td>T0</td>
<td>No tumour</td>
</tr>
<tr>
<td>T1a</td>
<td>≤4 cm in greatest dimension limited to the kidney</td>
</tr>
<tr>
<td>T1b</td>
<td>≥4cm and ≤7cm in greatest dimension limited to the kidney</td>
</tr>
<tr>
<td>T2a</td>
<td>≥7cm and ≤10cm in greatest dimension limited to the kidney</td>
</tr>
<tr>
<td>T2b</td>
<td>≥10cm in greatest dimension limited to the kidney</td>
</tr>
<tr>
<td>T3a</td>
<td>Extends into renal veins or its segmental veins, perirenal and/or renal sinus fat but not beyond Gerota’s fascia or below</td>
</tr>
<tr>
<td>T3b</td>
<td>Extends into the IVC below the diaphragm</td>
</tr>
<tr>
<td>T3c</td>
<td>Extends into the IVC above the diaphragm or invades the IVC wall</td>
</tr>
<tr>
<td>T4</td>
<td>Extends beyond Gerota’s fascia</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nodes</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Nx</td>
<td>Not assessed</td>
</tr>
<tr>
<td>N0</td>
<td>Absent</td>
</tr>
<tr>
<td>N1</td>
<td>Present</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Metastases</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>M0</td>
<td>None</td>
</tr>
<tr>
<td>M1</td>
<td>Present</td>
</tr>
</tbody>
</table>

Table 4.1.2. The staging of RCC. A table summarizing the TNM staging of renal cell carcinoma as described by the American Joint Committee on Cancer (AJCC). IVC is the inferior vena cava.(220)
4.1.3 The treatment of RCC

4.1.3.1 Surgical management of RCC – radical nephrectomy
Management of localized sporadic (non-inherited) RCC is largely surgical with the aim of removing all macroscopic disease resulting in the best possible overall survival (OS). For many years the gold standard of treatment has been the radical nephrectomy described by Robson(222) in 1969. This involves removal of: the kidney with Gerota’s fascia, ipsilateral adrenal gland and regional lymph nodes. This remains the most commonly performed surgery for RCC with nephron sparing treatment being reserved for specific clinical situations described in section 4.1.3.2. After radical nephrectomy OS is typically between 71-97% for pT1 and pT2 tumours and 20-53% for tumours which have spread locally.

4.1.3.2 Surgical management of RCC – nephron sparing treatment
Nephron sparing treatment involves removal of the renal cancer with an appropriate margin as opposed to the entire kidney. This preserves renal function and can be particularly useful in the management of small cancers, in those with impaired renal function or a solitary kidney and is the preferred option in patients who are at risk of developing multiple primary tumours. In T1 tumours, partial nephrectomy is currently the standard of care as cancer specific survival rates are similar to those after radical nephrectomy, however the morbidity associated with renal impairment is reduced.(223, 224). Furthermore OS in these patients is around 90% (225). In older patients who could have been treated by either a partial or radical nephrectomy, survival rates were higher in those who had a partial nephrectomy presumably due to the preservation of more functioning nephrons (225). In patients with sporadic RCC with pre-existing renal impairment, for example those with acquired renal cysts, partial nephrectomy is preferred to help maintain renal function. In those with familial RCC (see section 4.2) clinical features of renal cancer are different. Patients are prone to recurrent, multi-centric tumours and thus there is a need to preserve renal function. Individuals with
familial tendencies are therefore screened for the development of tumours and these are resected once the tumour reaches a particular size (usually 3cm in the case of VHL disease associated tumours) (226).

### 4.1.3.3 Surgical management of RCC – minimally invasive treatment

In the 2000s more patients were undergoing minimally invasive treatment using laparoscopic surgery. (223) This technique had the advantages of decreased post-operative morbidity and shorter hospital stays. A number of studies have demonstrated that cancer specific outcomes in laparoscopic partial nephrectomy are equivalent to those in open nephrectomy (223). Thus, this technique is commonly used in the management of RCC. Recently, robot assisted total and partial nephrectomies have been performed using the da Vinci Robotic Surgical system. (227) The robotic system is believed to provide better three dimensional visualization than laparoscopic systems, in addition robotic wrists allow easier removal and suture of tumours. Robot assisted and laparoscopic excision have been compared and had been shown to have comparable outcomes. (228) Currently laparoscopic surgery is more widely available due to logistical and training considerations. However, proponents of robot assisted therapies suggest that this technique may be less demanding and perhaps particularly useful for complex lesions.

### 4.1.3.4 Surgical management of RCC – the role of cytoreduction

Prior to the widespread use of targeted agents (described in section 4.1.3.5) the therapeutic options for metastatic RCC were limited. Metastatic RCC is occasionally associated with spontaneous regression post resection of RCC (229) and therefore, nephrectomy was carried out even in metastatic disease. Two randomized controlled trials carried out in the early 2000s revealed that nephrectomy combined with immunotherapy (the then standard of care) in the setting of metastatic disease RCC improved survival by between three (230) and ten
months (231). Hanna et al, have retrospectively evaluated cytoreductive nephrectomy in the targeted era and found that it nephrectomy is associated with a significantly improved median OS (17.1 months compared to 7.7 months) (232).

### 4.1.3.5 Targeted therapies in RCC

Scientific understanding of the molecular pathology of RCC has been translated into novel treatments. In RCC, agents directed against the angiogenesis pathways driven by the pseudohypoxic pathway and activated mTOR have led to tangible changes in patient outcomes (these pathways are described in more detail in section 4.2.1). One such drug, sunitinib targets VEGF, PDGFRα, FLT3 and c-kit has been shown in clinical trials to more than double PFS from five to eleven months (30). In the second line setting and in the first line in those with poor prognostic features (on clinical evaluation), mTOR inhibitors have been used effectively. Temsirolimus is used in individuals with high risk RCC and is the only targeted agent shown to improve OS (HR=0.75) in advanced disease (233). Everolimus is an oral preparation used in the second line setting and has also improved PFS from two months to five months (234). Other agents such as the tyrosine kinase inhibitors (TKI) pazopanib (235) and axitinib (236) have been licensed in RCC. Carbozantinib is a MET, VEGFR and AXL inhibitor which has been evaluated in a phase III patients with RCC previously been treated with a TKI. In comparison with everolimus, the PFS was 7.4 months compared with 3.8 months and the hazard ratio for death or progression was 0.60 (p=<0.001)(237). Targeted agents have also been utilized in ‘orphan’ cancers where historically there has been less investment in drug development for example sunitinib and everolimus are now used in neuroendocrine cancers (238, 239), and sorafenib in hepatocellular carcinoma (240). These drugs are also being used in common cancers, for example everolimus is efficacious in hormone receptor positive breast cancer (241). Thus, understanding the molecular biology of
RCC has helped improve the care for individuals with other cancers. Although, the PFS data described above suggests there is still more work to be done to improve outcomes for patients.

4.1.3.6 Immunotherapy in RCC
Historically RCC has been shown to be amenable to immunotherapy in the form of IL-2. Recently, immunotherapies directed towards antigens on T-cells (cytotoxic T lymphocyte antigen (CTLA-4), programmed death-1 (PD-1)) and tumour cells such as programmed death ligand 1(PD-L1) have demonstrated considerable efficacy in lung cancer and melanoma. Agents directed towards these targets are known as immune checkpoint inhibitors. These agents are being investigated in RCC. Nivolumab is a completely humanised anti-PD-1 antibody(242). Motzer et al, compared nivolumab with everolimus in metastatic RCC within a phase III trial, nivolumab was associated with an increased OS (25 months versus 19.6 months), HR for death 0.73 and 25% overall response rate compared with 3% with everolimus(243). There were no clear biomarkers indicating a response to nivolumab although in other cancer sites expression of PDL-1 on tumour cells and the presence of tumour infiltrating lymphocytes are thought to be of significance.

4.1.4 The prognosis of RCC
The outcome for individuals with RCC is very much dependent on the stage of the disease. In those without distant disease at presentation, five year survival is around 50%. (221) However, in those with metastatic disease the prognosis remains grave with only around 2% of those with the condition achieving complete remission even in the era of targeted treatments (244). In this setting, five year survival is approximately 10%. (221) Given that up to a quarter of all patients with the condition present with metastatic disease (245) the management of RCC represents as a significant clinical challenge and further developments in the treatment of RCC are urgently required. To date, the most recent developments in the
management of RCC have been derived from improved understanding of the molecular pathogenesis of RCC. The pathogenesis of RCC has largely been elucidated by research into the molecular abnormalities in familial RCC. Thus, studies of familial RCC are crucial to understanding RCC.

4.2 Familial RCC
Familial RCC syndromes represent a small proportion (~3%) of all kidney cancers (76, 214), however individuals with a first degree relative with RCC are twice as likely to develop RCC suggesting a heritable tendency (218). Furthermore, research into the genetic causes of familial RCC has led to the elucidation of the key cell signaling pathways which are aberrant in sporadic RCC. Inherited kidney cancer disorders may be divided into syndromic and non-syndromic kidney cancer. The clinical and molecular features of the main kidney cancer syndromes are described in table 4.2.1.1. In many cases (but not all) of inherited kidney cancer, patients will have a family history of RCC or extra-renal features of one of the specific syndromes listed and multi-centric/bilateral tumours are frequent. Familial kidney cancers typically present at an earlier age of diagnosis than sporadic RCC. A specific disorder may be suggested by the presence of extra-renal features and RCC histopathology. Thus, hereditary papillary carcinoma is associated with a c-met mutation and type I papillary cancer and RCC associated with VHL disease are always clear cell. The known syndromic causes of familial RCC are inherited in an autosomal dominant manner and segregation analysis of non-syndromic RCC families indicates the most likely mode of inheritance to be a single gene autosomal dominant model with age related penetrance.(246) Up to 10% of individuals with apparently non-syndromic RCC have mutations in SDHB or folliculin (FLCN).(133) Thus, individuals in whom genetic susceptibility to RCC is suspected, can be offered genetic testing. If a genetic cause is identified, predictive testing can be offered to at-
risk relatives and appropriate surveillance initiated for mutation carriers. Even if a known genetic cause is not identified, individuals in whom there is a strong suspicion of an inherited disease, and their close relatives, should be offered annual surveillance as currently known inherited RCC genes can only explain a minority of familial RCC cases. Owing to the propensity of individuals with inherited RCC to develop multiple tumours, the surgical management of such cases is geared around the principles of early detection by screening and limited surgical resection ("nephron sparing") aimed at preserving renal function.

4.2.1 von Hippel Lindau Disease (VHL)
VHL disease is the commonest cause of inherited RCC with an incidence of about 1 in 35,000 live births and is caused by germline mutations in the VHL TSG. The lifetime risk of developing a clear cell RCC is >70% in many cases (76). Other clinical features include: retinal and cerebellar haemangioblastomas, phaeochromocytoma, renal, pancreatic and epididymal cysts. About 20% of VHL mutations occur de novo and such individuals do not have a family history (114). Molecular genetic testing has been available since 1993 (77) and is indicated in all suspected cases. Once a mutation has been detected, family members can be tested and those testing negative for the gene mutation do not require surveillance. In contrast, individuals testing positive for VHL mutations are offered regular screening for retinal and central nervous system haemangioblastomas, RCC, pancreatic tumours and phaeochromocytomas (247). The precise VHL mutation detected may predict risk for some tumours (e.g. phaeochromocytoma or RCC described in section 3.2.3.1) and this allows more personalized screening protocols.
<table>
<thead>
<tr>
<th>Familial RCC Syndrome</th>
<th>Histological classification of RCC</th>
<th>Clinical Features</th>
<th>Gene</th>
<th>Molecular Pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>VHL</td>
<td>Clear cell</td>
<td>Retinal and CNS haemangioblastoma, phaeochromocytoma, pancreatic tumours, visceral cysts.</td>
<td><em>VHL</em></td>
<td>Accumulation of HIF leading to increased production of HIF target genes, causing angiogenesis and oncogenesis</td>
</tr>
<tr>
<td>HRPC1</td>
<td>Papillary type I</td>
<td>Multiple micropapillary tumours. Breast, stomach and pancreas cancer</td>
<td><em>MET</em></td>
<td>Increased MET-HGF signalling</td>
</tr>
<tr>
<td>HLRCC</td>
<td>Papillary type II</td>
<td>Cutaneous and uterine leiomyomas, leiomyosarcoma</td>
<td><em>FH</em></td>
<td>Oncometabolite (fumarate) accumulation leading to a pseudohypoxic response</td>
</tr>
<tr>
<td>SDH</td>
<td>Variable</td>
<td>Extra adrenal and adrenal phaeochromocytoma, head and neck paraganglioma</td>
<td><em>SDHB SDHD SDHC</em></td>
<td>Accumulation of the oncometabolite succinate leading to a pseudohypoxic response and altered HIF mediated transcription</td>
</tr>
<tr>
<td>BHD</td>
<td>Variable</td>
<td>Fibrofolliculomas, lung cysts, pneumothorax and colorectal polyps</td>
<td><em>FLCN</em></td>
<td>Presumed mTOR activation, altered TGF-β signaling</td>
</tr>
</tbody>
</table>

Table 4.2.1.1. The clinical and genetic features of the main familial kidney cancer syndromes.

Abbreviations: VHL-von Hippel Lindau, HRPC1-Hereditary Papillary RCC, HLRCC-Hereditary Leiomyomatosis RCC, SDH-succinate dehydrogenase subunit mutations, BHD-Birt-Hogg-Dube syndrome
RCC and other VHL-related tumours from individuals with VHL disease show biallelic \textit{VHL} inactivation (a germline mutation in one allele and somatic loss, mutation or epigenetic silencing of the other allele). pVHL has multiple functions but the best characterized is the regulation of HIF-1 and HIF-2. HIF-1 and HIF-2 have \(\alpha\) and \(\beta\) subunits. HIF1\(\alpha\) and HIF2\(\alpha\) have two transcription activating domains which initiate transcription of target genes on binding to DNA(248). They act as heterodimeric DNA binding transcription factors which regulate >200 genes with key roles in tumourigenesis including: angiogenesis, glucose metabolism, cellular growth, metastasis and apoptosis(249) These include: transforming growth factor (TGF), EGFR, VEGF, platelet-derived growth factor B (PDGF-\(\beta\)), interleukin - 8 (IL-8), glucose transporter 1, stromal cell derived factor involved in chemotaxis, and cyclin D1 associated with cell cycle (250) (see Figure 4.2.1.2). Expression of HIF-1 and HIF-2 is dependent on the stability of the HIF 1\(\alpha\) and HIF2\(\alpha\) subunits.(251) Under normal physiological conditions, the HIF\(\alpha\) subunits are hydroxylated on one or both prolyl residues (Pro405, Pro531) by members of the oxygen and 2-oxyglutarate dependent prolyl-hydroxylase family (PHD). There are least three PHD proteins, PHD1 (EGLN2), PHD2 (ELGN1), and PHD3 (ELGN3). PHD2 enables HIF1\(\alpha\) and HIF2\(\alpha\) hydroxylation, whereas PHD3 is responsible for HIF2\(\alpha\) hydroxylation. Hydroxylation of one or both proline residues creates a high affinity pVHL binding site. pVHL forms a multi-subunit ubiquitin ligase with elongin-B, elongin-C, Cullin-2 and ring-box 1 (RBX1). pVHL allows the ubiquitin conjugating machinery to come in close proximity with HIF allowing polyubiquitinisation and destruction of HIF. When oxygen levels are low (i.e. in hypoxia), the PHD enzymes are not activated, and HIF1\(\alpha\) and HIF2\(\alpha\) do not interact with VHL and are thus able to accumulate, translocate into the nucleus and heterodimerise with HIF\(\beta\) activating their target genes. If \textit{VHL} is inactivated, lack of pVHL has a similar effect to that of hypoxia
with HIF1α and HIF2α stabilization allowing transactivation of HIF target genes. (248) This response that occurs in cells with inactivated VHL is called the pseudohypoxic response. (248)

Both HIF-1 and HIF-2 degradation is regulated by pVHL but there is evidence that HIF-2 overexpression is more important for RCC development. Factor inhibiting HIF (FIH) has also been identified in RCC. FIH interacts with pVHL/HIF1α, and decreases HIF1 transcriptional activity. (252, 253). Low nuclear levels of FIH are associated with poor prognosis in RCC (254). VHL inactivation can also contribute to tumourigenesis in a HIF independent manner by suppression of p53 ubiquitinisation and so enhancing its transcriptional activity (16).

These activities exemplify the key role of VHL in RCC tumourigenesis.

Figure 4.2.1.2. VHL and pseudohypoxia. The role of pVHL in the hypoxic response and the effect of inadequate pVHL producing a pseudohypoxic response.
4.2.2 Birt-Hogg-Dube Syndrome

Birt Hogg Dube (BHD) syndrome is an autosomal dominant condition characterized by skin lesions, multiple lung cysts, spontaneous pneumothorax and kidney cancer. A classical triad of skin lesions can occur in BHD syndrome, these consist of fibrofolliculinomas, trichodiscomas and achrocordomas. This syndrome was first described in 1977 and twenty-five years later was found to be caused by mutations in a gene called folliculin (FLCN). Clinical diagnostic criteria for BHD are available for diagnosis (Figure 4.2.2) but a FLCN mutation can be detected in most cases.

The first clinical presentation of BHD syndrome may be to the urologist as a renal tumour, or to the acute physician with a spontaneous pneumothorax. BHD is likely to be under-diagnosed as the dermatological features (typically facial fibrofolliculomas) appear after 20 years of age but may be absent or, if sparse, overlooked. Individuals with BHD are estimated to be at a seven fold increased risk of RCC. In contrast to VHL disease, BHD is associated with a variety of histological subtypes of RCC including; clear cell carcinoma, chromophobe RCC and a hybrid of oncocytoma and chromophobe RCC.
study of individuals with familial kidney cancer without known mutations in VHL, MET and FH, FLCN mutations were found in 4.3% of cases. Though there are no universally agreed guidelines for surveillance, annual renal MRI (or ultrasound) is recommended from age 20 years. If a renal tumour is detected, the management is generally similar to that in VHL disease i.e. removal when 3 cm in diameter by nephron sparing surgery. Often there is considerable phenotypic variation within families (e.g. some individuals have only cutaneous lesions, others have pneumothoraces and others have RCC). In addition to the three major features of BHD syndrome, there is also a reported association with colorectal cancer although this is not universally accepted. It has been suggested that only some FLCN mutations may predispose to colorectal cancer.

FLCN encodes a 579 amino acid protein which is evolutionarily well conserved. There are >150 reported variants of FLCN but the vast majority of pathogenic mutations lead to truncated proteins. FLCN is believed to act as a TSG and somatic FLCN mutations can be detected in RCC from individuals BHD syndrome - in keeping with Knudson’s two hit hypothesis. Mutations in rat and canine orthologues of FLCN lead to renal tumours similar to those seen in BHD. A kidney specific Flcn knock-out mouse had enlarged cystic kidneys with cystic RCC. Flcn inactivation was associated with activation of the mTOR pathway suggesting that some of the TSG activity of FLCN was achieved by mTOR pathway inhibition. A FLCN interacting protein FNIP1 has been identified, this interacts with 5′-AMP–activated protein kinase [AMPK], an important energy sensor in cells that negatively regulates the mTOR (268-270). Treatment of Flcn knockout mice with mTOR inhibitors led to decreased tumour growth and increased survival but in other models the effect of folliculin deficiency on mTOR activity has been inconsistent and folliculin regulation of mTOR may be context dependent. Folliculin
may also have a role in regulating TGF-β pathway signaling, (272) mitochondrial function (273) and HIF transcription activity.(274)

4.2.3 Hereditary leiomyomatosis and RCC
Hereditary Leiomyomatosis (HLRCC) is a dominantly inherited disorder associated with cutaneous leiomyoma, uterine leiomyomas and in some individuals an aggressive type II papillary RCC (275). RCC is seen in 15-20% of HLRCC families(276). HLRCC is caused by mutations in the fumarate hydratase (FH) gene. Biallelic inactivation of FH occurs in almost all HLRCC associated renal tumours.(276) Fumarate hydratase is an enzyme within Kreb’s cycle that catalyses the conversion of fumarate to malate. FH inactivation increases cellular fumarate which inhibits the PHD enzymes. Proline hydroxylation of HIF1 and HIF2 are critical for pVHL regulation of HIF transcription factor levels. FH inactivation causes HIF stabilization and up-regulation of HIF-target genes (mimicking the effect of VHL inactivation) (277, 278). HLRCC associated RCC are typically solitary, unilateral, have a high nuclear grade and are aggressive. Most patients will die of metastatic disease within five years of diagnosis - even with small T1 tumours. Thus, unlike in other familial cancer syndromes, urologists should not wait until a threshold of 3cm has been reached but should intervene when a tumour is detected. RCC can occur in early adulthood so surveillance may be indicated from late childhood.(279)

4.2.4 Succinate dehydrogenase (SDH) subunit disorders
SDH disorders have been discussed in detail in section 3.2.3.1. Briefly, SDH is a heterotetrameric protein located on the inner mitochondrial membrane consisting of four subunits (A, B, C and D). It has a critical role in cellular energy metabolism. SDH catalyses the conversion of succinate to fumarate (280). Mutations in SDH subunit genes (most
commonly SDHB and SDHD) are associated with predisposition to familial phaeochromocytoma, paraganglioma and head and neck paraganglioma syndromes (73). Individuals with SDHB mutations have ~15% lifetime risk of developing RCC (133, 281) and about 4% of individuals with features of non-syndromic inherited RCC have a SDHB mutation (133). SDHB associated RCC may be associated with a distinctive histopathological appearance and a relatively good prognosis. (282) Paraganglioma and phaeochromocytoma associated with SDHB mutations have been shown to have increased HIF-1 and VEGF expression suggesting a shared pathophysiology with FH deficiency and linking SDHB mutations to the pseudo-hypoxic pathway.(283) Interestingly, in phaeochromocytoma SDHB mutations are associated with metastatic disease and worse outcomes.(134, 284) Inhibition of SDH has been shown to: increase succinate, cause inhibition of PHD and thus lead to stabilization of HIF1α (285). Renal tumours may occasionally be associated with SDHC and SDHD mutations. (280)

4.2.5 Hereditary papillary RCC (HPRC)

HPRC occurs in approximately 1 in 10 million persons (76) and has a high penetrance (90% likelihood of developing RCC at 80 years of age) (286). It is caused by activating mutations in the c-Met (MET) proto-oncogene. HPRC is inherited in an autosomal dominant manner and is associated with the development of multiple bilateral type I PRCC (287). 13% of sporadic papillary RCC also contain a c-Met mutation (288, 289). Identification of the c-Met mutation was facilitated by the fact that type I papillary RCC are associated with trisomy 7, and thus linkage analysis was performed and the proto-oncogene identified on 7q34.(289, 290) Breast, pancreas, and stomach cancers have been associated with HPRC in some families (279) (291). By fifty years of age, only around 30% of MET mutation carriers have
developed renal cancer. Individuals with inherited papillary RCC can have over 1000 microscopic papillary carcinoma in resected specimens, (292) though HRPC tumours have a better prognosis than those associated with HLRCC. Met is the tyrosine kinase receptor for hepatocyte growth factor. (293) Point mutations occur in both sporadic and inherited papillary RCC leading to constitutive activation of the receptor pathway. Interestingly, MET expression is up-regulated in some clear cell RCC models (293, 294), suggesting that the MET/HGF pathway may have more general relevance in the pathogenesis of RCC. (295)

4.2.6 Translocation associated RCC

Inherited RCC may be associated with a constitutional translocation, most commonly involving the short arm of chromosome 3 (3p) and thus karyotyping is a standard investigation for patients suspected of inherited RCC. Translocation associated cancer is an important clinical problem accounting for approximately 15% of cancers in those aged under 45. (296) Initially, autosomal dominantly inherited familial RCC was reported in association with a constitutional t(3:8)(p12;q24) and since then eleven further RCC-associated translocations have been identified. (297) Investigation of these translocations has led to the identification of candidate TSGs for example; FHIT, TRC8, LSAMP and NORE1. In some cases it has been suggested that the genes disrupted by the translocation function, like VHL, as TSGs with a ‘two hit model of tumourigenesis’, but in others predisposition to renal tumorigenesis appears to result from instability of the translocated chromosome such that 3p is lost from the cell and then a somatic VHL mutation occurs on the remaining chromosome 3. In a recent population based study, individuals with translocations were of a similar age to individuals with FLCN or VHL mutations, and were more likely to have multiple tumours (297).
**Low penetrance RCC susceptibility genes:** *MITF* gene was reported to predispose to RCC and melanoma (76). *BAP1* which is described in more detail in section 4.3.1 has also been identified as a cancer pre-disposition gene.(298, 299) *TMEM127* has also been described in 1.86% of RCC (in 2 mutations in early onset cases, 2 mutations in a cohort containing a wide range of histological subtypes).(300)

There is some interaction between the mechanisms that cause oncogenesis in familial RCC cancer for example, impaired HIF regulation is described in a number of different disorders, *FH* mutations cause excessive fumarate and *SDH* mutations cause excessive succinate. Accumulation of these ‘onco-metabolites’ inhibits PHD enzymes driving the pseudo hypoxic pathway. The interaction of different alterations in RCC oncogenesis is described in figure 4.3.

4.3 The molecular genetics of sporadic kidney cancer

4.3.1 Chromosomal changes in sporadic kidney cancer

As discussed previously, the role of VHL in sporadic kidney cancers is pivotal with >80% of clear cell RCC demonstrating *VHL* inactivation (301). *VHL* resides on chromosome 3p and early cytogenetic studies indicated 3p allele loss in over 60% of sporadic kidney cancers (302). Detailed mapping of the 3p area did not demonstrate a single area of critical allele loss, and 3p was found to contain a number of TSGs.(303) Important TSGs on 3p include *RASSF1A*, which was silenced by methylation in 24-91% of clear cell kidney cancers (98, 304) and *PBRM1* which is mutated in about 40% of RCC (305). Microarray-based studies have identified areas of increased copy number (1q, 2q, 5q, 7q, 8q, 12p and 20q) and decreased number (1p, 3p, 4q, 6q, 8p, 9p and 14q)(306). Gerlinger *et al* (307) found 3p loss in all tumours studied, losses on chromosomes 4q, 8p and 14q were seen in some tumours as was gain of 5q. Data from the TCGA suggested that 14q loss (associated with *HIF1A* loss)
was associated with more aggressive disease (308). These differences in copy number can help identify possible genes of interest in the pathogenesis of kidney cancer for example: *MDM4* on 1q is a p53 regulator, *MYC* at 8q, are amplified and putative tumour suppressor *NEGRI* at 1p is deleted.(308)

A specific somatic cytogenetic abnormality, (X;1)(p11.2) translocation, occurs in a rare subset of papillary RCC. (X;1)(p11.2) translocation positive tumours account for up to a third of paediatric or young adult RCC cases but are rare in older cases. Overall, Xp11.2 translocation positive cancers account for <1% of all RCC and have an aggressive course. The translocation breakpoint involves the *TFE3* gene at (X;1)(p11.2). *TFE3* is part of a family of transcription factors known as the MiT transcription factors. These share a homologous basic-helix-loop-helix leucine zipper DNA binding and dimerization domain. The (X;1)(p11.2) translocation is associated with the formation of a fusion gene between *TFE3* and a variety of partners including PRCC (papillary RCC (translocation-associated)) (1q21), *ASPSR1* (ASPL, alveolar soft part sarcoma chromosome region, candidate 1) (17q25), *SFPQ* ((PSF, splicing factor proline/glutamine-rich) (1p34), *CLTC* (clathrin heavy chain) (17q23) and *NONO* (non-POU domain containing octamer-binding) (Xq12). The translocation results in overexpression of the fusion protein and although Xp11.2 translocations are rare, expression of *TFE3* is a more common finding and can result from *TFE3* amplification(296)

4.3.2 Somatic mutations in sporadic kidney cancer

4.3.2.1 *PBRM1*

Following the identification of frequent *VHL* gene mutations in sporadic clear cell RCC it was more than 15 years later that a second frequently mutated gene was identified. Exome sequencing revealed truncating mutations in the *PBRM1* gene on chromosome 3p21 in 41% of
clear cell RCC. (305) Most mutations in PBRM1 involve a loss of protein. PBRM1 encodes BAF180 the chromatin targeting subunit of the PBAF SWI/SNF chromatin remodeling complex. SWI/SNF complexes alter accessibility to DNA by changing the dynamics of nucleosome occupancy. (309) BAF180 has six tandem bromodomains, two bromo-adjacent homology domains and a high mobility box. (310). These bromodomains target acetylated lysines and an alteration in one domain can impair TSG function. (310) The complexes target many genes and signaling pathways involved in cell proliferation including: Wnt, Erb, p53, MAPK and processes involved in cell cycle regulation and apoptosis. (309) PBRM1 behaves as a classical TSG and the second allele is frequently co-deleted with VHL (310).

4.3.2.2 SETD2 and other genes associated with histone modification
Prior to the identification of PBRM1, re-sequencing of 3500 candidate cancer genes in RCC identified three genes were each mutated in ~3% of sporadic RCC and which were each implicated in histone modification (87). These were the histone three lysine demethylases UTX/KDM6A and JARID1C/KDM5C and the H3 lysine methylase SETD2. Mutations in SETD2 have been identified in 10-15% of sporadic clear cell RCC. (310) SETD2 causes trimethylation of lysine 36 on histone 3, this is associated with transcriptionally active genes and may play a role in transcriptional elongation and regulation of alternative splicing of target genes. (309) These findings suggested that disruption of normal patterns of chromatin modification play a key role in RCC development. Interestingly NF2 mutations were detected in a small subset of tumours without VHL mutations. (311) Alteration of histone modifying genes in RCC have now been confirmed by a number of key publications (307, 312)

4.3.2.3 BAPI (BRCA associated protein 1)
BAPI mutations were identified in 14% of sporadic clear cell carcinomas (313). BAPI is associated with other cancers notably mesothelioma and melanoma (313) It encodes a nuclear
ubiquitin carboxyterminal hydrolase (UCH). The UCH protein has a number of functions including: (i) interacting with BRCA1 causing enhanced BRCA1 mediated cell growth suppression (298) (ii) forming part of the Polycomb group Repressive De-Ubiquitinase Complex which aids the repression of the HOX transcription factor by chromatin remodification (iii) binds to transcription factor HCFC1.(298)

Figure 4.3. Molecular pathophysiology of familial and sporadic RCC. The interactions of different familial and sporadic mutations in the development of renal cancers. Different molecular pathologies can be involved in one or more pathway aberrations e.g. VHL inactivation/loss influences pseudohypoxia, wnt signaling and inhibits p53 induced senescence. The main processes are boxed.
Subsequently, \textit{BAP1} alterations have been identified in a number of familial RCC cases (298, 299). Evaluation of a cohort of individuals with familial RCC alone failed to identify any \textit{BAP1} mutations. However, 18\% of individuals with a family history of either uveal melanoma, cutaneous melanoma or mesothelioma harboured a \textit{BAP1} mutation. Within these families there was an increased predisposition to RCC (8 fold increase relative to the population), suggesting \textit{BAP1} is a renal cancer pre-disposition gene within a ‘\textit{BAP1} syndrome’ (298). \textit{BAP1} mutations tend to be subclonal with focal loss in 2-3\% of clear cell RCC (310). If there is a \textit{BAP1} mutation it is less likely that there will be a co-existent \textit{PBRM1} mutation (310). However, cells that lost both \textit{BAP1} and \textit{PBRM1} have a particular rhabdoid morphology, and an aggressive de-differentiated appearance (313). This suggests that both genes effect different epigenetic pathways (42). \textit{BAP1} mutations were found in somatic tissue with co-existent \textit{VHL} inactivation (314), indicating that \textit{BAP1} mutations occurred in \textit{VHL} null clones and contributed to tumour progression (314). \textit{BAP1} loss is associated with a high Fuhrman grading of the tumour, \textit{mTOR} activation (313), metastases at presentation (315) and poor prognosis (316). \textit{BAP1} associated tumours have a 2.5-3.0 fold higher hazard ratio for death and \textit{BAP1} mutations are an independent poor prognostic factor in RCC (310). Given the adverse outcome associated with \textit{BAP1} mutations, these patients may benefit from different therapeutic strategies for example initial treatment with \textit{mTOR} inhibitors.

\textbf{4.2.3.4 Other somatically mutated genes}

\textit{TCEB1}, the gene which encodes Elongin C a vital part of the \textit{VHL} complex (see figure 4.2.2). This was found to be mutated in 3.3\% of tumours (314). No alterations were noted in other components of the \textit{VHL}, Elongin B, Elongin C and catalytic RING subunit (RBX1, which binds to the ubiquitin-conjugated E2 component) complex. \textit{VHL} and \textit{TCEB1} mutations were
mutually exclusive emphasizing the critical role of the VHL complex in renal oncogenesis.\(^{314}\) \textit{TCEB1} gene mutations were associated with loss of 8p producing complete loss of wild type \textit{TCEB1}.

\textit{TET2, KEAP and MTOR} are also mutated in RCC.\(^{314}\) \textit{TET2} encodes \(\alpha\)-ketoglutarate dependent oxidase which has been inactivated in other malignancies, the enzyme is believed to be critical in DNA demethylation. \textit{TET2} was mutated in 5.7\% of cases and deleted in 10.4\% of cases. There was no loss of heterozygosity seen, indicating that loss of one allele is sufficient for tumour development. PI3K have a catalytic subunit and a regulatory subunit, the \(p100\alpha\) subunit encoded by the \textit{PIK3CA} gene is mutated in 2 to 5\% of ccRCC.\(^{310}\) \textit{TSC1} a gene which forms a complex with \textit{TSC2} to act as a TSG and has also been found to be mutated in 4\% of RCC\(^{310}\).

### 4.3.3 Epigenetic changes in sporadic kidney cancer

Epigenetic modifications have been implicated in the pathogenesis of many cancers. The best studied feature of cancer epigenetics is epigenetic silencing of TSGs by \textit{de novo} promoter region hypermethylation \(^{97}\). \textit{VHL} was one of the first human TSGs in which epigenetic inactivation was demonstrated. Though genetic changes (mutation or allele loss) are more common sources of \textit{VHL} inactivation than \textit{de novo} DNA methylation which is seen in 7-15\% of cases \(^{312,317}\). The \textit{RASSF1A} TSG is inactivated much more frequently by promoter region hypermethylation than by intragenic mutations. More than forty candidate TSGs genes are reported to be methylated in more than 20\% of kidney cancers (see \(^{78}\)) and this number will expand as the results of large scale cancer genome projects are released. Data from TCGA \(^{312}\) showed 289 genes are epigenetically inactivated in at least 5\% of RCC. Interestingly, \textit{SETD2} mutations were associated with increased loss of methylation of DNA at
non-promoter regions.\textsuperscript{(312)} \textit{SETD2} encodes H3K36 methyltransferase which may be involved in the maintenance of the heterochromatic state. This altered methylation may lead to switching on of pathways which are advantageous to tumour growth. More detailed knowledge of the patterns of epigenetic inactivation of TSGs in RCC will offer opportunities for therapeutic intervention. Thus, a key feature of epigenetic TSG inactivation is that it is potentially reversible (promoter hypermethylation may be reversed by demethylating agents). In addition, delineation of the key signaling pathways that are dysregulated by epigenetic TSG inactivation will offer new possibilities for targeted therapies.

Integrative analysis of sequencing, methylation, copy number and RNA data held within the TCGA \textsuperscript{(312)} suggested there are some other important pathways implicated in RCC oncogenesis. 28\% of tumours had alterations in components of the PI(3)K/AKT/MTOR pathway. As discussed, mTOR inhibitors \textsuperscript{(234, 318)} play a role in kidney cancer and therefore individuals with tumours with aberrations in this pathway may preferentially benefit from treatment with these agents.

Overall, in sporadic RCC there are two main molecular pathophysiological processes (i) ‘Oncogenic metabolism’ by which there is altered cellular metabolism due to genetic changes in cancer cells, for example stabilization of HIF complexes due to \textit{VHL} mutations leading to a pseudohypoxic response (ii) ‘Epigenetic reprogramming’ changes in gene expression due to alterations in chromatin remodeling genes such as \textit{PBRM1}.\textsuperscript{(312)}

\textbf{4.4 Approaches to identifying new familial RCC genes}

\textbf{4.4.1 The hypothesis driven approach}

Until the identification of \textit{PBRM1} in 2011, \textsuperscript{(305)} other than \textit{VHL} most RCC associated genes were altered in only ~5\% of cases.\textsuperscript{(319)} Before NGS became widely available, focused
hypothesis-based approaches were used to identify potential new genes in kidney cancer. This involved utilizing data from a variety of sources to identify possible genes of interest. One approach is to study genes associated with the \textit{VHL} pathway, for example \textit{HIF1A} and \textit{HIF2A}. Mutational analysis of these genes revealed one functional mutation in \textit{HIF1A} only (320). Similarly, the \textit{PHD} genes were studied for potential inactivation in familial renal cancer (167) and no mutations found. \textit{FIH}, \textit{SDHB} and \textit{FH} were studied in sporadic renal cancer with no detectable mutation. (252) \textit{SDHB} mutations were known to be involved in phaeochromocytoma pathogenesis and were studied in familial RCC and found to have a causative role, (133) similarly \textit{TMEM127} was also found to be associated with RCC. (321)

\textit{RASSF1A} was identified as a kidney cancer associated gene because 3p21.3 was frequently deleted in RCC and \textit{RASSF1} was identified in this position. This gene was a TSG in breast and lung cancer. Thus, it was investigated in RCC and found to be inactivated by methylation in 91% of clear cell kidney cancers, (304) Another approach is to study families with an inherited cancer predisposition and perform genetic linkage analysis described in detail by Pulst (322) to determine the area of a chromosome in which the cancer gene was positioned. This area could then be interrogated to determine the nature of the causative gene. Co-segregation of the gene can be used in order to confirm its significance.

The approaches discussed above are time consuming and can yield negative results particularly as there is a degree of human bias regarding how to choose a ‘candidate’ gene. They may lead to genes not part of the current paradigm, and those whose function is not characterized to be overlooked. Thus, an alternative approach to identifying novel candidate genes is to use genomic platforms to identify genes of interest.
4.4.2 Utilizing a hypothesis generating approach

Given the high failure rate of finding CPG with candidate gene approaches, recently there has been a shift towards screening a large number of genes or the genome to identify areas and genes of interest (figure 4.4.). These screening methods use genomics platforms such as arrays that can interrogate numerous genes by looking at many single nucleotide polymorphisms (SNP) or NGS investigating the whole genome or exome. The use of NGS is discussed in section 4.7 and has led to the identification of more CPG in the last few years (see figure 4.5).

Array technologies enable candidate gene identification by determining which genes are differentially expressed between RCC cells and normal cells or which genes undergo copy number changes. Thus, one methodology is to look for differences in copy number between normal cells and cancer cells. This can highlight areas of the chromosome which may contain cancer associated genes. For example, as discussed previously these can identify areas of chromosome loss which may indicate the location of potential TSG.(323) Once these areas are identified then important cancer associated genes for example known TSG, or members of key pathways can be studied. Alternatively regions of copy number gain may indicate the location of candidate oncogene.

As many RCC cells have inactivated VHL, another approach to identify kidney cancer associated genes is to look for changes in methylation seen between cancer cells and paired normal cells. In order to evaluate a large number of genes, these are initially evaluated on an automated platform such as the Illumina beadarray platform which analyses >400,000 CpG sites (324) and/or an expression microarray can identify which transcripts are silenced in tumour cells (50, 96). If these screening methodologies suggest preferential methylation of
specific genes in cancer cells, these are confirmed by a more sensitive methodology and evaluated in a larger cohort of patients. This has led to the identification of some potential kidney cancer associated genes such as; \textit{OVOL1, DLEC1, TMPRSS2, SST} and \textit{BMP4}\textsuperscript{167}.

Another method for identifying possible candidate genes is by considering gene expression levels after treatment with a demethylating agent such as 5-azadeoxycytidine. RNA can be extracted from cells treated with the demethylating agent and analyzed on an array. Genes with increased gene expression post treatment would be, by inference, genes that might be epigenetically silenced by methylation in cancers. Confirmation of differential expression can be achieved using RT-PCR and PCR based assays to confirm promoter methylation e.g. by using methylation specific primers\textsuperscript{50}. This methodology led to identification of \textit{SPINT2} \textsuperscript{50} as a potential kidney cancer associated gene.

These methods enable researchers to make a rational choice of a gene for further study and reduce the likelihood of bias. They are initially relatively expensive but have the advantage of focusing researchers’ time and effort.
Figure 4.4 Changes in the methodology used to identify cancer pre-disposition genes (CPG) in all cancers since 1982. Initially CPG were identified by performing linkage analyses amongst individuals with strong cancer clustering within families. Then, more CPG were identified using candidate gene approaches based on knowledge of pre-existing genetic associations with the cancer. However, with the improvements in genome sequencing technology most recently NGS approaches have led to the identification of more CPG resulting in the second peak seen at around 2009. (Taken from Rahman (325))

4.5 Using exome sequencing to identify novel familial RCC associated genes

4.5.1 Next Generation Sequencing (NGS) technology

Over recent years massive technological advances have enabled vast amounts of the genome to be investigated faster than ever before using NGS which has a higher throughput and sensitivity than Sanger sequencing. (326) NGS generates large amounts of sequencing data as instead of a single read taking place, multiple short strands of DNA are amplified and read at the same time in a ‘massively parallel fashion’. There were three different platforms for NGS sequencing available at the time of this study (see figure 4.7.1). These methods use parallel sequencing technology and have led to a fall in the cost of sequencing from up to $3
billion (at the time of the Human Genome project) (327) to $1000 for a genome. (328) In this study, NGS was performed in collaboration with King’s College London on an Illumina Sequencer. The depth of coverage of the genome (50-100x) means that most single nucleotide variants (SNV) are detected, however copy number variation, structural rearrangements and small deletions may be missed. (329) Whole genome sequencing (WGS) is superior to Sanger sequencing for the detection of large deletions and duplications (329). Detection of deletions has been shown to be equivalent to MLPA. (329)

4.5.1.1 A summary of the processes involved in NGS
Prior to being sequenced the DNA requires to be prepared for amplification. The DNA sample of interest is sheared into fragments of 75-150bp (330) and then ligated with adapters. The DNA is then applied to a flow cell containing single stranded fragments ‘primers’ on its surface. Subsequently, ‘bridge-amplification’ takes place as unlabeled nucleotides and DNA polymerase are placed on the flow cell. A cluster of nucleotides contains millions of copies of the original fragment of DNA. The nucleotide chain attached to each primer is then removed by denaturing. These clusters of nucleotide (‘copies’) represent the single molecule that initiated the reaction. (331).

In the Illumina system sequencing occurs using ‘sequencing by synthesis’. Each cluster is incubated with DNA polymerase and nucleotides in a flow cell. Each nucleotide has a specific fluorescent label and the 3’-OH group is chemically blocked such that each incorporation is a unique event. The reaction is then imaged. After the imaging step, the 3’ blocking group is removed and the PCR reaction is allowed to continue. This process continues for a predefined number of steps controlled by the user which permits discrete read lengths. When data is analyzed, the bases are assigned to the reference sequence and the quality of the sequence
assessed. Poor quality sequence is removed. At present, Illumina is the dominant sequencing platform being used (330). The Illumina platform provides more data and is cheaper per run than the Roche and SOLiD platforms described below(332).

The Roche/454 sequencer was the first NGS platform available; it uses pyrosequencing to detect nucleotides. Fragmented DNA is ligated to a specific library of adapters, this is incubated with agarose beads that contain complementary nucleotide sequences to the adapters. As these beads are in excess, each bead is bound to a specific complementary fragment. The beads-fragment complexes are incubated in a micelle with DNA polymerase and reactants. Thermal cycling takes place (emulsion PCR) thus; each fragment is amplified into hundreds of copies attached to the bead. Subsequently, the beads are placed into a plate containing several hundred thousand wells. Each well contains a bead and then the plate is incubated with enzyme containing beads that catalyze pyrosequencing. A solution of each nucleotide is then added step-wise to the plate. Every time a nucleotide is incorporated, light
Figure 4.5.1. A summary of the processes involved in NGS. A fragment library is prepared by fragmenting DNA, end repair and ligation with adaptor sequences. Libraries are then amplified by (1) water in oil bead emulsion PCR (Roche and SOLID sequencing) or (2) solid surface bridge amplification (Illumina). Flow cell signaling then produces a signal which is processed to produce a primary sequence. (Adapted from Voelkerding(333))
is emitted and this is imaged as it is proportional to nucleotide length. This technique finds it difficult to read runs of the same nucleotide accurately (homodimer runs) however, because each nucleotide is added step wise substitution errors are very rare. (331)

The SOLiD sequencer, uses a fragment library similar to those described with the Illumina and Roche platforms. The platform also uses emulsion PCR on beads to amplify the DNA. Two flow cells are used per run. Each cell contains a library comprising different DNA sequences, the cell is incubated with DNA ligase and octomers with fluorescent labelled 4th and 5th bases. At each ligation step the fluorescent signal is detected and then some bases (including the fluorescent ones) from the octomer are removed. Subsequently a second ligation step occurs with this now longer primer. Each fluorescent nucleotide is therefore read twice decreasing the chance of base calling errors. This system is believed to be 99.99% accurate (326) but is more time consuming. (331) The SOLiD sequencer has the shortest run reads and therefore requires more computational analysis to align fragments to the reference sequence.

Although the methodology differs between different NGS platforms (figure 4.5.1), in general NGS differs from conventional sequencing because the sequencing product has a shorter read length and different types of error. NGS requires greater computational and bioinformatics input. (331) Furthermore, interpretation of data through analysis pipelines are more important. (67)

4.5.2 Future techniques in sequencing
Within this project, NGS using the Illumina platform was utilized as it is the most validated and widely used sequencing platform at the time of writing. Newer sequencing technologies,
the so-called ‘third generation sequencing’ also exist (72) such as the PacBio RS, Ion Torrent and Oxford Nanopore technologies platforms. These platforms do not use amplified DNA but read a single DNA molecule. The Ion Torrent system measures an electrical change every time a nucleotide is added.(72). In the Oxford Nanopore system, the DNA molecule passes through the nanopore. Each nucleotide produces a specific change in current allowing the nucleotide sequence to be read as it passes through the nanopore. The PacBio system images dye labelled nucleotides every time they are incorporated into a single strand of DNA by a polymerase.(72)

4.5.3 Applications of NGS

4.5.3.1 Multiplex gene panel testing

NGS can be used to sequence DNA in different forms; these will be briefly discussed below.

A collection of genes of interest can be analyzed on a ‘chip’ using NGS platforms, this method is called multiplex gene panel testing.(334) This method can be useful for investigating conditions for which there are a large number of known disease causing genes as a number of genes can be screened for simultaneously. This technology has been discussed in section 3.7.4. Multiplex testing can be advantageous because a ‘result’ can be obtained more speedily. However a disadvantage of this methodology is that multiple variants of uncertain significance can be detected and these lead to uncertainty for clinicians and patients. Furthermore, as more genes are found to be associated with a condition, the panel will have to be changed. The panel methodology also does not allow for novel gene discovery. Therefore, as exome and genome sequencing improve technically and become cheaper they may supersede gene panel testing.(204)
4.5.3.2 Whole Genome Sequencing (WGS)
Sequencing technology also enables the genome of organisms to be sequenced. Given the technical limitations of NGS, using this method alone ~95% of the genome can be sequenced (48). Areas with homodimers, high GC content and repetitive sequences are more difficult to sequence. WGS provides the most complete picture of the genome including structural variations in non-coding regions. WGS is more expensive than exome sequencing and requires more time consuming bioinformatics, filtering and processing. Owing to this, currently exome sequencing is preferentially used in many centres to enable gene discovery. However, genome sequencing can provide additional information for example in breast cancer, WGS was performed in twenty one samples and detailed analysis performed on the types of nucleotide substitution and deletion.(335) This evaluation revealed five different mutational signatures which occurred in different cancers and therefore represented different mutational processes e.g. UV exposure or deamination. These insights derived from mutational signatures may help tailor treatment on the basis of the tumour signature.
Furthermore, WGS may help drive research into the reversal or eradication of mutational spectra. Enthusiasts for genome sequencing argue that important mutations can be missed with WES approaches as only 0.6% of the point mutations detected were in the exome of lung cancer samples.(336) However, given the time and costs of investigating mutations it can be argued that investigating many more mutations that may be detected by WGS may be counterproductive as it could be argued that mutations in non-coding areas are less likely to be clinically significant.(6)

4.5.3.3 Whole Exome Sequencing (WES)
Exome sequencing involves sequencing all known coding regions (exons) of the genome. The advantage of WES is that by sequencing only 1% of the genome (326) 85% of all disease causing mutations could be identified.(337) In order for WES to be performed, sheared whole
genomic DNA must be selected in order to evaluate those sequences which can be transcribed into protein. Most commonly this technique known as ‘exon capture’ is achieved using a commercially available system for example Agilent Sure Select or Nimblegen SeqCap EZ(338). Different exome capturing technologies capture the exome in slightly different ways. Some areas of the exome are over-represented as there are numerous probes over a certain region. On the other hand, other areas of the exome may have fewer probes representing them. This lack of coverage can lead to false negative results. Insufficient depth of coverage (typically preferred to be x 20) can also lead to false positive results. This emphasizes the need for good quality control and bioinformatics filtering in NGS. Furthermore, WES is limited by technical considerations described previously for WGS. Typically there is ~95% coverage of target sequences (337). Once a potential disease associated variant is identified, it must be confirmed using a different methodology usually Sanger sequencing. Exome sequencing has been used to identify disease causing alterations in a number of different conditions. Initially WES was used to identify monogenic Mendelian disorders (339), however more recently it has been used to describe the somatic mutations in numerous cancers including common cancers such as prostate (340) and kidney (305, 314) in addition to rarer malignancies such as craniopharyngioma (341).

In both exome and genome sequencing 80% of the variants identified will be missense.(44) A key challenge for scientists is to try and identify the driver mutations from these. Of these missense variants, it is important to determine the influence of the substitution on protein function. It is believed that driver mutations influence protein function whereas passenger mutations are neutral in nature.(44)
4.5.3.4 Transcriptome Sequencing
The transcriptome is the portion of the genetic code that is transcribed into RNA and represents ~2% of the genome (342). Cellular RNA is converted into cDNA and is then sequenced using a NGS platform. Sequencing of the transcriptome enables sequencing of DNA which is actually transcribed within the cell. This provides more data than WES because an individual gene can give rise to numerous RNA molecules as different initiation and termination sites can be used, RNA can be edited and there is alternate spicing of genes. Transcriptome studies aid the detection of intergenic fusions, somatic mutations and alternative splice transcripts (326). Furthermore, chimeric transcripts, novel transcripts and differentially expressed transcripts (102) can be identified. Thus, there is better characterization of the molecular pathophysiology of a tumour.

4.5.3.5 Whole Genome Bisulphite Sequencing
In order to identify methylation in sporadic tumours, cellular DNA can be modified using bisulphite. This technique converts cytosine to uracil, after PCR amplification the uracil is converted to thymidine. However, methylated cytosine remains as cytosine. In whole genome bisulphite sequencing cellular DNA is sheared and ligated to methylation adapters. Then size selection and bisulphite conversion occurs. A library is then prepared and NGS takes place. To date, this technique has not been widely used due to its cost, need for large amounts of DNA and technical issues with bisulphite conversion. (343) However, in colorectal cancer this technique has been performed (344) and over 80% of the CpG islands were included. The study revealed areas of hypermethylation and hypomethylation in colorectal cancer indicating a role for chromatin reorganization in gene silencing. (344)
Aim

To identify novel causes of familial RCC. This study aims to use two strategies:

(A.) A candidate gene approach – this involves evaluating a gene associated with other cancers and see if the gene can cause a familial RCC phenotype, this approach has been successfully used in determining the role of SDHB in familial RCC(133).

(B.) An unbiased approach by evaluating whole genome or exome data to identify genes of interest. This type of approach has successfully been used in phaeochromocytoma. WES was utilized in this study because a pragmatic approach was taken; WES is cheaper to perform compared to WGS. Furthermore, bioinformatical analysis required to evaluate the data is much reduced and the majority of the coding regions can be interrogated. This approach is believed to identify a substantial proportion of cancer causing driver mutations.(48) The disadvantage of WES over WGS in this setting is that there is the potential that variation in intergenic areas, for example in promoter regions may be missed. However, typically the exome is sequenced to a greater depth than the genome.

Results

4.7. PTEN as a candidate gene for familial RCC

PTEN (phosphatase and tensin homologue deleted from chromosome 10) was chosen as potential kidney cancer associated gene to study as a cause for familial RCC. PTEN is a TSG that was identified by investigation of brain, breast and prostate cancer.(345) It lies on chromosome 10q23 and includes nine exons. The gene has two main domains: C2 the lipid binding domain and a phosphatase domain. Both domains are required for its tumour suppressor activity (figure 4.7.1). The lipid phosphatase activity dephosphorylates the 3-
phosphoinositide products of phosphatidyl-inositol 3,4,5, triphosphatase (PI3K).(345)

Usually 3-phosphoinositide products activate survival kinases including the AKT pathway. $PTEN$ negatively regulates the AKT pathway, this pathway controls a number of important cellular features such as cell cycle progression, metabolism, migration, apoptosis, transcription and translation.(345) Loss of $PTEN$ leads to excessive PI3K which activates AKT and subsequently mTOR.(346) mTOR activation has been previously demonstrated to be associated with poor prognosis in kidney cancer (347). $PTEN$ also binds to centromere proteins resulting in protein stability and nuclear localization is required for DNA double stranded break repair (345).

Until recently the role of $PTEN$ in RCC was unclear. Germline mutations in $PTEN$ are associated with a specific clinical syndrome known as Cowden syndrome (348). Cowden syndrome is an autosomal dominantly inherited condition where there is an increased risk of breast, endometrial, thyroid, benign and malignant brain tumours and skin tumours. The syndrome is also associated with overgrowth of the bone causing macrocephaly. Overgrowth of the skin can lead to the presence of benign tumours (348) causing the clinical features of: oromucosal papillomatous papules, and cutaneous verrucous papule(346).

Given the role of $PTEN$ in a number of inherited cancer syndromes it was investigated as a candidate TSG in familial kidney cancer. When this work was carried out the association between Cowden syndrome and RCC had not been characterized. The gene was sequenced in 44 patients who had been seen by a consultant clinical geneticist and diagnosed with an inherited renal cancer but no known features of Cowden syndrome. These patients had no known mutations of other kidney cancer genes. Two sources of familial RCC DNA were
studied, cases from a West Midlands-ascertained series and another cohort of samples from a collaborator were studied. Readable sequence was achieved for all nine exons that covered the amino acid coding sequence using Sanger sequencing including 50bp intronic sequence at the 5’ and 3’ end. All nine exons were sequenced in forward and reverse. The plan was to identify truncating mutations or missense mutations that had been found in Cowden’s syndrome or other tumour types. Any other missense variants identified were to be assessed regarding whether they involved conserved residues, did not occur in the general population or had an allele frequency of <1%. However, no variant was identified in any of exons, thus no further patients were evaluated.

4.8 CDKN2B as a potential new kidney cancer associated gene

4.8.1 Bioinformatic analysis of exome sequencing data
Exome sequencing was performed as described in section 2.3.1 in collaboration with Michael Simpson’s group. Exome sequencing data was collated on eight individuals with RCC who had seen a consultant cancer geneticist and identified to have an inherited RCC. These individuals had RCC, no known mutations in familial RCC carcinoma associated genes and multiple tumours and/or family histories of RCC. Prior to further analysis by myself, this data had undergone in house bioinformatics performed by Michael Simpson and group as described in the methods (section 2.3.1). Figure 4.8.1.1 summarises the bioinformatics pipeline WES performed at King’s College London.

Post processing by King’s College, further analysis was performed (see Figure 4.8.1.2). 1392 novel variants were identified. There were 22 stop gain, 827 single nucleotide variants, 503 splicing variants, and 40 frameshift changes. The novel variants were then compared to the COSMIC database of somatic mutations (319), and TCGA(349). If a gene was already known to be associated with sporadic cancer it was deemed of interest due to the previously
described cross-over between cancer causing genes in sporadic and familial cancers. The filtered data was also compared with methylation array data previously obtained by our group (96, 324). Variants were also evaluated on the NHLBI Exome Variant server, if they were common germline variants they would be excluded from further evaluation. (350)

Initially, in order to prioritize variants of interest, a shortlist was made of genes which fulfilled one the following criteria: (i) contained a stop-gain variant (truncating mutations), (ii) contained a frameshift variant, or (iii) were mutated in more than one individual. A total of 23 genes were subsequently shortlisted: **BRAF, c10orf71, CDKN2B, DHAH8, DPP4, PARK2, PPIR10, PTPN18, SLc6a18, USP36, XPC, AGAP9, ALMS1, c14orf135, MTSS1, PCDH12, SMAD9, ST7L, CEP290, CSMD1, KNTC1, MUC4, MYO18b**.

Gene function was then summarized by searching for the gene on NCBI PubMed. If a gene was described to play a role in an important cellular process e.g. cell signaling or cell cycle it would be considered for further evaluation. Genes with no known function, or those with a function not clearly currently thought be associated with a key cellular function were also excluded. Furthermore, whether the gene had been identified by COSMIC, TCGA or the group’s methylation array was considered. Of the twenty three genes, three were chosen as potential candidate genes owing to their known function in cancer associated pathways. As Sanger sequencing was to be used in candidate gene analysis, the size of the gene was also a factor. The three candidate genes chosen were: **CDKN2B, PARK2 and SMAD9**. **CDKN2B** is a known tumour suppressor coding for p15, a key regulator of the cell cycle (351), **PARK2** is an E3 ubiquitin ligase and has also been identified as a TSG (352). **SMAD9** forms part of the TGF-β pathway which is implicated in **VHL** inactivated renal cancer (353, 354). **CDKN2B** was chosen as the initial candidate gene to study due to its known role as a TSG. (355, 356) **PARK2, SMAD9** and **BRAF** were evaluated by another member of the group.
Figure 4.8.1 A. The process of preparing genomic DNA and bioinformatic analysis. These processes were performed by Michael Simpson’s group.
8 Exomes
WES performed on 8 individuals with RCC, no known mutation in familial RCC genes with multiple tumours or a strong family history

1392 novel non-synonymous variants identified
827 single nucleotide variants,
403 splicing variants,
40 frameshift variants
20 stopgain/nonsense

Filter 1
Exclude if allele frequency > 1% on NHLB1 exome variant server n=38

Analysis 1
1354 variants remaining
Identify the following:
• Methylated in house methylation array n=13
• Present on COSMIC/TCGA n=61

Filter 2
if one of the following:
Stop gain mutation, Frameshift mutation, Present in 2 or more patients n=23

Analysis 2
23 variants remaining
Consider whether: identified in analysis 1, or known TSG, or part of a cancer pathway n=3 (CDKN2B, PARK2, SMAD9)

CDKN2B

Figure 4.8.1.2 Bioinformatic filtering of exome sequencing data performed leading to the identification of CDKN2B as a gene for further study.
4.8.2 Confirmation of mutation and segregation analysis
Germline DNA from the patient with a germline stop-gain variant in CDKN2B was obtained. DNA was sequenced in forward and reverse and the stop-gain mutation G103T, pE35X in CDKN2B was confirmed (Figure 4.8.2 B). Clinical notes from the index patient were retrieved and evaluated (these are summarized in figure 4.8.2A illustrating the family tree). The germline DNA of the patient had previously evaluated for the presence of FLCN, VHL and SDHB mutations. The patient was diagnosed with a clear cell RCC aged 57, two brothers had clear cell RCC aged 54 and 60 respectively. His mother had a RCC of unknown histology. Germline DNA was obtained from 4 siblings, one of the siblings had a clear cell RCC, the patient’s other three siblings were unaffected. The same mutation was demonstrated in the affected individual but not in the three unaffected individuals.

4.8.3 Screening of CDKN2B mutations in familial RCC
In order to investigate the influence of CDKN2B mutations in familial RCC, mutational screening was performed in DNA from patients who had been seen by a consultant cancer clinical geneticist and identified to have an inherited RCC, have no mutations in known RCC associated genes, a personal history of RCC and either a family history of RCC or multiple RCC. Owing to the scarcity of available DNA, initial screening was performed on whole genome amplified DNA. A total of 97 germline samples were evaluated, these included 77 derived from the West Midlands Familial Renal Cell Carcinoma Repository and 20 samples donated to the research group for study. Three further variants were identified in these patients (4% overall mutation rate). Variants were confirmed in the germline stock DNA. The variants identified were missense mutations: P40T, A23E, D86N (figure 4.8.3.1). In silico analysis using the Polyphen(90) and SIFT algorithms(91) suggested these non-synonymous changes were probably damaging based on the influence of the amino acid change on protein structure and on the evolutionary conservation of the amino acid. For all
three missense changes, the amino acid altered was well conserved amongst numerous
organisms (figure 4.8.3.2).

Figure 4.8.2. The CDKN2B mutation identified in the index case.

A family tree indicating the other members of the family with a renal cell carcinoma in the
index family (A). The mutation in CDKN2B noted in this family (B), this was seen in both
reverse and forward sequencing of stock DNA.
Figure 4.8.3.1 Variants of CDKN2B in familial RCC. Electropherograms demonstrating the variants identified in the cohort of patients with familial renal cell carcinoma with corresponding normal sequences. Sequencing was performed in forward and reverse (not shown).
Figure 4.8.3.2 Conservation of variants identified

A  Missense variants identified in CDKN2B (highlighted in yellow) are also present in conserved homologous residues in CDKN2A.

B  Amino acid homology of p15 across different species, changes are highlighted representing the amino acid change occurring due to the following variants (a) c.68C>A, p.A23E (b) c.118C>A, p.P40T (c) c.256G>A, p.D86N. The amino acids are well conserved across species. (Figures obtained from Clusta Omega (http://www.ebi.ac.uk/Tools/services/web_clustalo) accessed 13/3/14)
4.8.4 Screening of CDKN2A mutations in familial RCC

CDKN2B is closely associated with CDKN2A (see sections 4.9.1 and 4.9.2). It resides within 35kb to CDKN2B. CDKN2A encodes p16. The first 50 amino acids of p15 and p16 have 44% sequence homology and the following 81 amino acids have 97% sequence identity (357) after which the sequences diverge. Both p15 and p16 inhibit cyclin dependent kinases cdk4 and cdk6. p15 and p16 cause a G1 cell cycle arrest by inhibiting pRb phosphorylation (358). However, CDKN2A has the ability to produce alternative transcripts, the smaller transcript p14ARF can also cause G2/M cell cycle arrest by inhibiting the destruction of p53 (358). In addition to their physical proximity, and similar function, the frequent deletion of the 9p21 has been observed in numerous human cancers (359), thus genetic variation in both genes was evaluated to determine co-segregation. Sanger sequencing was used to evaluate WGA for mutations in CDKN2A. DNA from the familial RCC cohort described in section 2.2.1 was used to examine all three exons that coded for CDKN2A protein. Briefly, these patients had been diagnosed as having an inherited RCC and had no known RCC associated gene mutation. They also had either a family history of RCC or multiple RCC. DNA was assessed for known mutations as described in CDKN2A as described on the COSMIC database or truncating mutations. Any missense variants would be evaluated using in silico tools to determine whether the corresponding amino acid change was conserved. Missense variants would also be assessed for their frequency in the general population. Any missense variants with a frequency of <1% were believed to be significant. Mutational screening was performed in forward and reverse for all three exons of the gene in the 97 germline samples. Readable sequence was obtained for all three exons, however no variants were detected in the samples evaluated.
4.8.5 Screening of CDKN2B mutations in familial phaeochromocytoma

As discussed previously, RCC and phaeochromocytoma share common susceptibility genes e.g. VHL and SDHB (104). SDHB mutations were initially described in familial phaeochromocytoma/paraganglioma (132, 360) and later observed in familial RCC (133). The role of CDKN2B variants was investigated in familial phaeochromocytoma. A total of 52 germline DNA samples from individuals with phaeochromocytoma and a family history of malignancy were analyzed for variants in CDKN2B, one variant was identified. This was confirmed in both directions and in the stock DNA. The alteration was c.116 G>A, p.D39N, this was predicted to be benign by PolyPhen(90) and tolerated by SIFT(91) (figure 4.8.5).

Clinical records were retrieved for this patient to determine whether there were any distinguishing clinical features. The patient had a benign extra-adrenal para-aortic paraganglioma aged 52. He had no syndromic features on clinical evaluation by both clinical endocrinologists and clinical geneticists. Both his mother and sister had previously had breast cancer but no DNA was available from them as they were both deceased and thus segregation analysis was not performed.

4.8.6 Screening of CDKN2B mutations in sporadic RCC

Clinically significant genes in sporadic RCC have been identified by studies in familial RCC for example VHL. It has been estimated that 40% of mutations of CPG identified in germline DNA also occur in somatic tumours (325). Given the overlap between somatic and germline mutations, variations in CDKN2B were investigated in a panel of sporadic RCC derived from the UK population from individuals with a sporadic, single RCC with no family history of cancer and single solitary RCC was analyzed. The plan was to determine whether any of these individuals had a truncating mutation in CDKN2B or a known mutation in the gene. If any missense variants were identified, their effect on protein function would be ascertained. If the change was non-synonymous, the frequency of the change in the general population
would be determined and identified as possibly significant if its frequency was <1%. \textit{In silico} tools would be used to determine whether the alteration was in a conserved amino acid. A total of 52 DNA samples from sporadic tumours were screened for variations in \textit{CDKN2B} using Sanger sequencing. Sequencing was performed in both directions on both exons from gel extracted samples. Readable sequence was obtained from all samples in both directions for both exons, however no variants in the gene were identified.

\textbf{4.8.7 Investigation of CDKN2B and CDKN2A mutations and expression of CDKN2A and CDKN2B in cell line DNA}

The COSMIC database (319) was interrogated for the presence of any known variants in \textit{CDKN2B}. Six mutations were recorded however these mutations were confined to lung, large intestinal and oesophageal cancers, recent data from the TCGA (figure 4.8.7.1) confirms that \textit{CDKN2B} is infrequently mutated. Our group had access to additional RCC derived cell lines and therefore, further evaluation of \textit{CDKN2B} was performed in 12 renal cell lines. DNA from the following cell lines was evaluated: SKRC 45, SKRC54, RCC4, 796-P, KTCL-26, KTCL-140, UMRC2, UMRC3, RCC1, RCC12, RCC11, SKRC47. No candidate mutations were identified by sequencing the gene. We also investigated whether \textit{CDKN2B} expression was altered for example by epigenetic changes in a number of RCC lines. Immunoblotting was performed on protein lysates from the following cell lines: Caki I, RCC 4, KTCL-26, SKRC47, RCC11, SKRC39, ACHN (figure 4.8.7.2). \textit{CDKN2B} expression was seen in all cell lines.
Variant identified in familial phaeochromocytoma patient. An electropherogram showing a variant identified in a familial phaeochromocytoma patient (c.116 G>A, p D39N). *In silico* analysis was equivocal with the variant being benign by PolyPhen (score 0.00) and tolerated by SIFT (score 0.19).

The frequency of genetic alterations in *CDKN2B*. Figure taken from cBioPortal (361) accessed on 19/8/2016 (http://www.cbioportal.org). Deletions are more common (blue) compared with amplification (red) and mutation (green). Mutation frequency is <5% in the samples studied.
Figure 4.8.7.2 CDKN2B expression (15kDa) in renal cell carcinoma cell lines by immunoblotting (overnight incubation with primary antibody concentration 1:1000 at 4°, 1:10,000 secondary antibody). Cell lines tested were: Caki I, RCC 4, KTCL-26, SKRC47, RCC11, SKRC39, ACHN from left to right.

Figure 4.8.8. Electropherograms from two sporadic phaeochromocytoma patients. These variants were (A) c.T80>C, pV27A and (B). c.A40>G, pS14G. These were predicted to be tolerated by both predictive algorithms (C). Sanger sequencing was performed in forward and reverse in both directions on stock DNA.
4.8.8 Screening of CDKN2B mutations in sporadic phaeochromocytoma

CDKN2B was sequenced in 21 DNA samples derived from sporadic phaeochromocytoma. Two variants were identified: c.T80>C, pV27A and c.A40>G, p.S14G, these were predicted to be tolerated by the SIFT algorithm (91) and benign by Polyphen (90) (Figure 4.8.8).

4.8.9 Investigation of inactivation of CDKN2B by copy number abnormalities

Data from a 250k SNP array (362) indicated that of 90 sporadic RCC samples evaluated, 7 had deletion in the region of chromosome 9p. The only genes present in the area of deletion were CDKN2B and CDKN2A indicating deletion maybe responsible for CDKN2B inactivation in a proportion of RCC. A further 250k SNP array study (323) also suggested deletion of chromosome 9p albeit failing to reach statistical significance. Deletion of one allele may be ‘missed’ by sequencing analysis due to amplification of the genomic sequence of the other allele. WES can also fail to identify small indel (insertions and deletions), large deletions and duplications. Thus, inactivation of CDKN2B may occur by these mechanisms. In order to investigate this further, MLPA analysis was performed which included all genes on the 9p chromosomal area including CDKN2A and CDKN2B. Resources allowed for 7 paired tumour: normal sporadic RCC samples and 15 sporadic RCC samples to be analysed. In each MLPA run there were five normal controls. 7 sporadic phaeochromocytoma samples were also analysed. No large deletions were identified in either the RCC or phaeochromocytoma samples.

4.8.10 Investigation of the functional effect of detected variants.

In order to determine the functional significance of the CDKN2B variants in vitro studies were used to determine growth suppression function. The pVHL null cell line KTCL-26 and expressing cell line SKRC-47 were chosen for study. These cell lines were transfected with either empty vector (EV) or wild type CDKN2B (WT). Colony formation assays performed
demonstrated that cell lines transfected with empty vector had statistically more colonies than those transfected with wild type CDKN2B (figure 4.8.10.1 A and B). This is suggestive of CDKN2B having TSG activity in RCC as super-expression of wild type CDKN2B decreases tumour cell growth.

SKRC47 and KTCL26 cells were transfected with CDKN2B plasmid containing the following variants: p Glu.35 Stop variant in CDKN2B (pE35X), pAla23Glu variant in CDKN2B (pA23E), pPro40Thy variant in CDKN2B (pP40T), pAsp86AsN variant in CDKN2B (D86N). These were compared to cells transfected with EV. The number of colonies produced by cells transfected with the variants noted in RCC were not significantly different to those produced by cells transfected with EV suggesting the variants led to a loss of CDKN2B associated tumour suppressor activity. CDKN2B expression was equivalent in the plasmids containing CDKN2B other than a decrease in the truncated protein, possibly due to decreased protein stability (Figure 4.8.10.2). Each experiment was repeated three times on three different occasions.
Figure 4.8.10.1 Colony formation assays using cells transfected with different CDKN2B vectors. Representative colony formation assay plates stained using crystal violet from colonies derived from the pVHL null renal cancer cell line KTCL-26(A) and pVHL positive cell line SKRC-47 (B). Cells were transfected with empty vector (EV), wild type CDKN2B (WT), vector containing the following variants: p Glu.35 Stop variant in CDKN2B (pE35X), pAla23Glu variant in CDKN2B (pA23E), pPro40Thy variant in CDKN2B (pP40T), pAsp86AsN variant in CDKN2B (D86N). The experiment was performed three times on three different occasions (n=3)
Comparison of empty vector (EV) and wild type CDKN2B expression in KTCL26 (A) and SKRC47 (B) RCC cell lines. Transfection with wild-type CDKN2B significantly reduced the number of colonies formed ($t=3.936$, df = 6 $P=0.007$ and $t=18.2$ $P=0.0002$ respectively). (C).

Comparison of growth suppression (by colony formation assay) of empty vector, wild type CDKN2B and three candidate missense mutations (p.Pro40Thr (pP40T), p.Ala23Glu (pA23E) and p.Asp86Asn (pD86N)) in KTCL26 RCC (VHL null) and SKRC47 (VHL positive) cell line. There were no statistically significant differences ($P>0.4$ for KTCL26 and $P>0.1$ for SKRC47) between the number of colonies in cell lines transfected with empty vector compared to those transfected with mutants containing variants (CDKN2B expression levels for the three missense mutations were $\pm 10\%$ of wild-type expression level). (D)

Representative CDKN2B expression levels for colony formation assays. Expression levels for missense mutations were comparable to that of wild type protein (data represents mean of three experiments ($n=3$), (error bars represent standard error).
**4.8.11 Investigation of downstream effects of CDKN2B over-expression**

*CDKN2B* binds to and inhibits the D type cyclin dependent kinases\(^{(363)}\) thus we investigated whether super-expression of *CDKN2B* altered the expression of cyclin D1. Cyclin D1 is an important regulator of the cell cycle \(^{(250)}\), and is overexpressed in over 70% of RCC suggesting its importance in the condition.\(^{(364)}\) *CDKN2B* was overexpressed in SKRC47 RCC cell lines and KTCL-26 RCC cell lines by stable transfection. Protein lysates were analyzed for differential expression of cyclin D1 protein, no difference was seen (figure 4.8.11 A). Clear cell RCC has a long latency and variable penetrance amongst individuals with von Hippel Lindau disease suggesting *VHL* inactivation alone is insufficient for RCC development. This indicates that other alterations are required for tumour development, alterations in *CDKN2B* may represent one of these changes. HIF2\(\alpha\) is believed to be more important than HIF1\(\alpha\) in RCC oncogenesis for a number of reasons including the fact that HIF2\(\alpha\) is noted in pre-malignant tumours \(^{(365)}\), VHL null tumours produce HIF1\(\alpha\) and HIF2\(\alpha\) or just HIF2\(\alpha\) alone \(^{(366)}\) and thirdly HIF2\(\alpha\) elimination can suppress tumour growth in pVHL null models \(^{(367)}\). Thus, the effect of *CDKN2B* overexpression on HIF2\(\alpha\) levels was studied and no difference in protein expression demonstrated (figure 4.8.11 B).

**4.8.12 Depletion of p15 in the HK-2 cell line did not lead to alteration in cyclin D1 and HIF2\(\alpha\) protein levels**

The HK-2 cell line is a proximal tubular cell line derived from normal adult tubular epithelial cells.\(^{(368)}\) The cell line is phenotypically similar to differentiated proximal tubular cells and thus is a model for a ‘normal’ kidney cell. HK-2 cells were transiently transfected with either scrambled siRNA or siRNA against p15 to determine whether depletion of p15 altered protein expression of cyclin D1 or HIF2\(\alpha\). The transfection was performed three times. There was no obvious alteration of either cyclin D1 or HIF2\(\alpha\).
Figure 4.8.11 The influence of CDKN2B over-expression (WT) compared to endogenous expression (EV) on (A) cyclin D1 and (B) HIF-2α in SKRC-47 and KTCL-26 cell lines. These are representative blots from three separate experiments (n=3). Overexpression of CDKN2B was not associated with differences in cyclinD1 or HIF2-α expression.

(A) 

KTCL-26 SKRC-47
EV WT EV WT

(B) 

KTCL-26 SKRC-47
EV WT EV WT

Figure 4.8.12 The influence of CDKN2B depletion in HK-2 cells on (A) HIF2-α and (B) Cyclin D1. The blots above are representative of three separate experiments (n=3). Depletion of CDKN2B was not associated with differences in cyclinD1 or HIF2-α expression. + represents HK-2 cells transfected with scrambled siRNA (i.e control), - represents HK-2 cells transfected with siRNA targeting p15.
Discussion

4.9.1 The role of PTEN in familial RCC
Evaluation of the role of PTEN in familial RCC was carried out in section 4.7 in probands with predominantly clear cell RCC. Subsequent to this work, Eng’s group found there was an increased risk of RCC, particularly papillary and chromophobe RCC amongst individuals with Cowden syndrome. Kidney cancers associated with Cowden syndrome included papillary (72.7%), chromophobe (18.2%) and clear cell (9%). Given the relative rarity of RCC it was not surprising that the association with RCC and these genetic alterations has only recently been identified. The failure to identify a PTEN mutation in described in section 4.7 may have been due to a number of possible reasons. As described previously the commonest type of renal cancer is clear cell RCC. It is likely that most of the individuals involved our cohort had this type of cancer. Thus, as was subsequently determined by Eng’s and Linehan’s group PTEN mutations in the context of Cowden syndrome were most likely to be associated with papillary and chromophobe tumours. Thus, our sample was inadvertently depleted of individuals with possible PTEN mutations. Furthermore, even in individuals with Cowden Syndrome the lifetime risk of developing renal carcinoma is relatively low (33.6% (10.4-56.9%). Thus, it may only take place when other features associated with a germline PTEN mutation such as hamartoma are present. This would make it even less likely that a PTEN mutation would have been identified in the individuals evaluated in this thesis as none of the patients in the study had any discernible clinical features of Cowden syndrome. To date, no germline PTEN mutations have been identified in individuals without a cluster of symptoms associated with a syndrome.

Somatic PTEN mutations have been demonstrated in 30 different tumour types (345). These include cancers not associated with Cowden syndrome such as prostate cancer, lung cancer
and pancreatic cancer. There are >1600 recorded mutations in the gene. There are mutational hot spots within the gene, however, these are not associated with a specific tumour type (figure 4.9.1). Furthermore, recent data from COSMIC indicates that PTEN is mutated in 2% of sporadic kidney cancers. Thus, although its role in inherited RCC may be limited to syndromic disease it is becoming an increasingly important gene in sporadic cancers. Biallelic PTEN loss is a poor prognostic factor in RCC, is associated with high grade tumours and invasive phenotype. Furthermore, loss of PTEN is associated with increased Akt/PI3K signaling which amongst other functions activates the mTOR pathway.

Figure 4.9.1. Mutations in the PTEN gene. A schematic diagram showing the mutational hotspots of PTEN in both the lipid binding and phosphatase domain (taken from COSMIC accessed on 19/3/14, when there were 1668 mutations on the database).
4.9.2 CDKN2B as a kidney cancer associated gene

4.9.2.1 CDKN2B structure and function
The CDKN2B gene is found on chromosome 9p21 where it is co-localised with CDKN2A.

This region of the genome is known to be subject to genomic rearrangements such as translocation, inversions and deletions (372, 373). CDKN2A and CDKN2B are found together with a third gene ARF. ARF is derived from a unique exon (1β) spliced to part of the second exon of CDKN2A. The gene is transcribed at an alternative reading frame (ARF) and thus the subsequent protein has no homology to CDKN2A (374) interestingly the biological activity of the ARF is derived from the amino acids encoded in exon 1β. The arrangement of these three genes is conserved within mammals and is unique (Figure 4.9.2.1). These genes play an important role in cell cycle control (see later) by regulating the key orchestrators of cell cycle progression; pRb (CDKN2A and B) and p53 (ARF). CDKN2A and CDKN2B together with CDKN2C and CDKN2D encode the INK4 family of proteins (375) which contain numerous ankyrin repeats which are responsible for the complex three dimensional structure of the proteins. In the case of p15 (encoded by CDKN2B) this consists of four helix turn helix repeats which are held in position by hydrophobic bonds.(376) The conformation of these proteins allows them to bind to cdk4 and cdk6 inhibiting their interaction with cyclin D. This allows them to manipulate pRb mediated cell cycle control.(375, 377)

4.9.2.2 Evolution of the CDKN2B gene
The CDKN2B and CDKN2A are closely related and are believed to be derived from a common precursor (374, 377). Two separate genes appear to be conserved in most mammalian species however, in fish there is one single gene which is believed be derived from a common ancestral gene (374). The ankyrin residues seem to be particularly conserved between species, however upstream of these residues there is significant variation.(377) The
proposed theory regarding the development of two separate genes is outlined below in figure 4.9.2. The presence of two genes in mammals enables more complex tissue specific regulation of the cell cycle and sensitivity to growth factors. This has led CDKN2B and CDKN2A to have different cancer associations.

Figure 4.9.2.1 The position of CDKN2A and CDKN2B on chromosome 9p

4.9.3 CDKN2A and CDKN2B and the regulation of the cell cycle
CDKN2A encodes p16 and partly transcribes ARF. p16 causes cell cycle arrest by binding to cyclin dependent kinases (CDK) 4 and 6. These inhibit the phosphorylation of pRb causing cell cycle arrest in G1 as cells do not enter the S phase (378). CDKN2A is frequently inactivated by deletions and inactivating mutations, cells with this alteration have a selective advantage and thus can go on to become tumour cells (378). Variations in the sequence of CDKN2A which are shared with ARF do not lead to cell cycle arrest. CDKN2B encodes the p15 protein which binds to and inhibits CDK4 and CDK6 (363). CDK4 and 6 usually phosphorylate pRb causing it not to bind to E2F allowing cell growth. Super-expression of p15 causes cell cycle arrest in G1 phase in a pRb dependent fashion by inhibiting CDK4 and 6 mediated inactivation of pRB. Overexpression of p15 leads to redistribution of CDK4 from cyclinD-CDK4 complexes to CDK-p15 complexes, this leads to unbound cyclin D to be rapidly degraded by a ubiquitin dependent proteasome degradation pathway. (363)
Figure 4.9.2.2 The evolution of CDKN2A and B as distinct genes in mammals. A single precursor CDKN2A/B gene is found as precursor in fish. This ancestral gene is on a different chromosome to the pre-ARF gene (A). Genomic rearrangement places the pre-ARF gene with its transcriptional regulator 3' of the ancestral CDKN2A/B gene (B). Gene duplication results in another CDKN2A/B gene upstream of the ancestral gene and includes the 1β exon from pre-ARF which lies between the two genes. (C) The duplicated genes then diverged with CDKN2A gaining an extra exon and the pre-ARF sequences were lost. The splice donor of 1β splices to the closest acceptor splice site in the second exon of CDKN2A (dashed line) to produce the ARF in mammals. Adapted from Gilley and Fried(374)
p15 is an effector of TGF-β (357). TGF-β has two roles in cancer development. Initially, TGF-β signalling inhibits cell growth via p15 inhibiting CDK4 and CDK6, causing G1 phase arrest. However, in later stages of cancer development, the signalling pathway is associated with cell proliferation, cell invasion, EMT and poor prognosis. These changes are associated with an alteration in gene expression which is partially reversed by addition of functional pVHL in RCC cell lines (379).

4.9.4 The association of CDKN2B and malignancy.
CDKN2B has previously been demonstrated to be a TSG in a number of human cancers. Germline variants causing truncated CDKN2B have recently been associated with ovarian carcinoma. Furthermore, a SNP in CDKN2B has been associated with poor prognosis in glioma. Copy number data has previously suggested that genes encoded on chromosome 9p are important in RCC. Work presented in this thesis has demonstrated that CDKN2B to be inactivated by mutation in a proportion of familial RCC.

4.9.4.1 Inactivation of CDKN2B by deletion
CDKN2B inactivation is commonly achieved by deletion rather than mutation in sporadic tumours. Owing to the close proximity of CDKN2A to CDKN2B, inactivation of one gene by deletion in cancer is also associated with inactivation of the other gene. This is seen in some cancers for example in diffuse large B cell and cutaneous T cell lymphoma deletion of CDKN2A and CDKN2B can occur, this inactivation is associated with a poor prognosis. Co-deletion also occurs in another rare brain tumour. In other cancers such as acute lymphoblastic leukaemia CDKN2B is selectively deleted. Deletion of CDKN2B is associated with poor prognosis in squamous cell carcinoma of the lung. We did not find this in sporadic RCC however a relatively small number of samples were analysed.
4.9.4.2 Inactivation of CDKN2B by epigenetic inactivation.

CDKN2B is also inactivated by epigenetic silencing in the form of methylation. The most commonly described cancers which are associated with CDKN2B methylation are leukaemias in particular those of the myeloid lineage.\cite{388} One method by which silencing can occur is that the anti-sense RNA to p15 can bind to DNA and this can lead to gene silencing. This has been demonstrated in leukaemia and has been thought to be a possible trigger to hypermethylation.\cite{389} 46\% of paediatric lymphoblastic leukaemia are associated with methylation as assessed by MS-MLPA.\cite{390} Solid tumours are also associated with methylation of CDKN2B. Lindberg et al,\cite{391} found 2/18 pancreatic neuroendocrine tumours had over 50\% of their CPG islands methylated. Methylation was associated with decreased p15 expression. Over 20\% of epithelial ovarian cancers contained methylated CDKN2B.\cite{392} CDKN2B was also methylated in 28\% of laryngeal squamous cell carcinoma cases\cite{393} this mechanism could potentially be important in RCC.

4.10 Possible mechanisms of CDKN2B alterations causing renal cancer

Inactivation of VHL in cell line models is associated with increased levels of cyclin D1 mRNA via a pseudo-hypoxic pathway induced mechanism\,(250, 251, 394). It maybe postulated that by overcoming p15\textsuperscript{INK} induced inhibition of cyclin D1, there may be loss of pRb induced cell cycle arrest in some kidney tissues leading to increased cell growth. Interestingly, 54 of 66 non-papillary RCC (presumably the majority of these were clear cell carcinoma) displayed overexpression of cyclin D1 emphasizing the potential importance of cyclin D1 in RCC oncogenesis\cite{395}. Thus, inactivation of CDKN2B by mutation or methylation may be a significant oncogenic event in a small but significant proportion of both familial and sporadic renal cancers. Immunoblotting from CDKN2B over-expressing models did not demonstrate lower cyclin D levels in cell lines with excessive wild type CDKN2B however, this may be due to differences being obscured by the presence of endogenous
CDKN2B in those cells transfected with empty vector. Similarly, the absence of a difference in HIF2α protein levels could also be explained by this. A more sensitive assay than western blotting may demonstrate the influence of p15 depletion on HIF2α and cyclin D1, furthermore other genetic aberrations associated with cell lines may influence the expression of these proteins.

CDKN2B may also play a key role in RCC development by influencing TGFβ activity. As the CDKN2B variants were identified in familial germline DNA, this suggests that CDKN2B inactivation may influence the TGF pathway in early tumours. TGFβ has been shown to upregulate p15^INK levels(357), and subsequently in individuals with inactivated/mutated CDKN2B abnormal p15 is produced and growth arrest will not take place.

Another putative mechanism for CDKN2B mediated RCC oncogenesis is that CDKN2B has an important gatekeeper effect through its involvement in pRb mediated cell cycle arrest. pRb causes growth inhibition predominantly in response to extracellular stimuli (24). If CDKN2B is inactivated, cell cycle arrest cannot take place and thus cells continue to divide in adverse situations potentially allowing more genetic alterations to accumulate including some with the ability to drive tumour growth. Cells with inactivated CDKN2B may lack an effective gatekeeper function and thus gain an evolutionary advantage by increasing genomic variation which allows them to evade normal cell cycle control activities. Supporting evidence for this hypothesis comes from murine models produced by Latres et al (396), as cultured mice embryonic fibroblasts from p15 null mice had a proliferative advantage that enabled them to be more susceptible to transformation by H-ras.

The D86N alteration in CDKN2B identified in RCC has been described sporadic parathyroid carcinoma (1/85 patients). Costa-Guda et al demonstrated that p15 protein containing the
D86N alteration had impaired binding to CDK6(381). This supports the hypothesis that this variant is pathogenic as impaired CDK6 binding would allow ongoing phosphorylation of pRb and thus its activation. Therefore, cellular proliferation would be increased providing cells with the alteration a selective advantage. Further evidence for the pathogenic nature of this mutation was the loss of heterozygosity seen in the tumour.

Recently, CDKN2B has been demonstrated to be down-regulated in hypoxia(397) as cells cultured in hypoxic conditions had decreased CDKN2B mRNA and protein. Potentially, in pseudohypoxia as seen in RCC, CDKN2B can be downregulated promoting growth. This may be achieved by transcriptional repression, as in normoxic conditions siRNA directed against myc, HIF1α and ARNT led to decreased transcription of CDKN2B(397). Thus, inactivation of CDKN2B may mimic some of the changes seen in the pseudo-hypoxic situation promoting cellular growth.

4.11 Limitations of this study

4.11.1 Limitations of the exome sequencing approach

The ability of exome sequencing libraries to capture the exome has improved since this study was performed. A recent publication compared different exome capture platforms, and suggested mutations failed to be identified due to incomplete coverage of a gene of interest (204). For example, the Agilent Sure Select captured 90% of most genes but missed a significant proportion of other important phaeochromocytoma associated genes (SDHD, SDHC and SDHA genes had 32%, 60% and 49% coverage respectively). Newer exome capture systems e.g. the Illumina Nextera Rapid Capture Exome had improved coverage of 100% for all phaeochromocytoma genes investigated but did not cover 5’UTR which the Agilent Sure Select system did.(204) WES does not accurately identify structural variants and therefore MLPA is recommended. Although, MLPA for CDKN2B loss was performed in
sporadic samples, due to cost factors it was not performed in the familial cohort and therefore some cases of deletion could have been missed.

As with all exome sequencing approaches, this study may have missed alterations in the non-coding regions e.g. gene promoter regions which were not sequenced. The role of the non-coding region in disease has not been fully evaluated and represents an area for future study for genomic researchers. These areas may have hitherto undefined roles as GWAS have identified >90% of risk variants reside in non-coding regions.(67) The ENCODE project(40) identified that 8.5% of the genome was associated with specific protein DNA binding and approximately 80% of DNA participates in at least one biochemical or RNA event in one type of human cell. This suggests that more of the genome is associated with gene regulation and therefore alteration in the intronic sequences may have a biological significance. Furthermore, clinically significant rearrangements such as the ALK fusion may be missed as re-arrangements can take place in intronic regions.(398) It is likely that as the costs associated with NGS fall, WGS will replace WES allowing better characterization of non-coding sequences and thus rearrangements and other variants maybe identified.

The bioinformatics pipeline used in this study may have led to some bias against the detection of potential genes associated with cancer. Firstly, alterations in genes which were already detailed on dbSNP were excluded from study. This included rare variants and the dbSNP database does include known potential mutations such as the D68N alteration in CDKN2B. More sophisticated data-analysis pathways now available include minor allele frequencies which help quantify rare alterations. Such pathways can also differentiate between different
types of variants within dbSNP e.g. only filter out variants with a minor allele frequency of greater than 1\%.(399)

The methods used to choose genes for further study were biased. Firstly, known genes were preferentially identified for study, this was because there would not be sufficient time and resources available to characterize an entirely new gene. Furthermore, as there were multiple genes with variants it was important to focus limited resources on those most likely to be significant (e.g. known TSG). This could have led to important CPG not being characterized as mutations in these genes may not fit the prevailing paradigm and they are not thought to be ‘candidate genes’. An example of this is IDH1 mutations occurring in glioblastoma multiforme (GBM) which were identified using integrative genomic analysis (400) and were hitherto not known to be associated with the condition. This gene is now well characterized in GBM and other gliomas and associated with a good prognosis (401) and is used to make clinical decisions (402).

Furthermore, the gene selection method used in this study was crude and lacked pathway analysis. This was partly because the analysis was manual and required inputting data regarding variants in webtools such as Ensembl (87), NCBI(89) and SIFT (91). This was time consuming and laborious, newer bioinformatics tools have now made this type of analysis much quicker and easier (44). The genetics of kidney cancer has previously been identified to involve a number of important signaling pathways for example; the pseudohypoxia, mTOR and wnt pathways. Some of the key publications regarding NGS have confirmed the significance of key signaling pathways in cancer, for example in pancreatic cancer there are alterations in 12 core signaling pathways (403) and in prostate cancer the
importance of the PTEN and wnt pathways was highlighted as many mutations occurred in genes within these pathways. (404) Therefore, the importance of signaling pathways may have been underestimated, for example other genes in the TGF-β pathway may have been altered. However, given relatively small number of samples which underwent exome sequencing in this study, this effect is likely to be reduced.

4.11.2 Assessment of the significance of CDKN2B variants in RCC
One criticism of this work is the relative infrequency of CDKN2B variants in RCC. In her critique of CPG, Rahman argues that CPG should increase the relative risk of cancer 2-3 fold and 5% of individuals with the alteration should develop a cancer. (325) Variants of CDKN2B described on dbSNP and on the exome variant server are infrequent suggesting there is not a significant proportion of individuals with the mutation with a ‘normal’ phenotype, suggesting the gene is important and alterations in the gene are functionally significant. Some authors argue that rare variants identified in exome/genome sequencing projects are not causative as they can occur in ‘normal’ individuals and that because a variant is not seen in controls does not mean it is causative (325). Furthermore, variants which are truncating or non-sense may not necessarily be pathogenic as they occur in normal individuals. This can be the case in some genes, however given that CDKN2B is a known TSG, suggests that the presence of the variants identified in this study is significant.

Genomic studies of cancer have suggested that there are a few cancer genes mutated in a high proportion of cancers evaluated (typically one to three genes in >20% of tumours), several genes mutated in 10-20% of cases (the shoulder) and many more mutated at lower frequencies, these make up ‘the long tail’ (42). Some genes mutated in low frequencies in
some cancers are mutated in higher frequencies in other tumours e.g. *KRAS* is infrequently mutated in breast cancer but more commonly mutated in colon cancer (41). Thus, the frequency of mutation prevalence does not mean that the gene is unimportant. Furthermore, currently not all the genes associated with ‘the long tail’ have been evaluated because most studies have not had the correct amount of power to detect low level changes due to sample size and stromal contamination in sporadic studies may have meant there was insufficient depth to identify mutations (41). This problem is likely to be overcome in the next few years as more large scale sequencing projects are being performed. In this study Sanger sequencing was used to evaluate sporadic tumour DNA for mutations, as a ‘normal’ allele is preferentially amplified during a PCR reaction it may be difficult to identify heterozygous changes. Newer NGS techniques including those which enable sequencing of a single DNA molecule may increase the likelihood of somatic changes being identified. Although it should be noted that some key publications in sporadic tumour mutation have used Sanger sequencing to identify novel mutations (311, 403).

*CDKN2B* mutations may represent a CPG which is part of the “long tail”. These CPG are believed to be important in the understanding of cancer biology (42) as they can provide insights in to particular signaling pathways for example *KRAS* mutations can lead to failure of EGFR inhibition in colon cancer. Redundant mutation in cancer pathways is increasingly seen, they may help classify tumours and help tailor treatments (42). Genes mutated less commonly i.e. those within the long tail may also combine into common pathways and thus highlight their role in cancer development i.e. RNA binding proteins were found to be mutated in lung cancer and CML suggesting RNA processing pathways may play a role in cancer development (42). The evaluation of family studies is important in the determination
of high risk CPG. This is the approach that was taken in this study, however for genes with modest effects on cancer risk, population based studies such as GWAS play a role in identifying such genes, such studies have identified a 500bp area in chromosome 9p which is important in multiple cancers (including breast, melanoma, glioma and leukaemia) as well as type II diabetes and myocardial infarction. This area contains CDKN2B and CDKN2A suggesting that they have an important role in cancer and this may be achieved by altering cancer metabolism. (41)

Evaluation of the importance of missense variants is not clearly defined however, conventionally the functional significance of missense variants can be determined by the assessment of the variant on protein structure and on function. In the case of TSG, the effect of missense mutations on function can be attributed to the influence of these alterations on characteristics typical of cancer cells. Classically, this can involve loss of inhibition of cell growth demonstrated by colony formation assays, loss of anchorage dependent growth demonstrated by soft-agar assays and increased cellular migration (i.e. that seen in metastasis and cell invasion) demonstrated using the monolayer wound healing assay or transwell invasion assay. (405) In this study, colony formation assays were used, however it would have been advantageous to utilize another in vitro assay to demonstrate pathogenicity and other members of the group are continuing this work.

The importance of missense mutations is also determined by whether amino acids affected by the alteration are evolutionarily conserved as protein function is strongly associated with well conserved amino acids (406). In this study, missense variants were associated with well
conserved amino acid changes. Furthermore the amino acid change associated with protein function e.g. catalytic sites or sites where protein-protein interactions take place are more likely to be associated with a mutation. We used two previously cited and peer-reviewed *in silico* prediction tools, SIFT and Polyphen-2 in order to determine whether these changes were functionally significant.(90, 91). SIFT uses evolutionary conservation to determine the probability that the change is tolerated.(406) These tools appear to be the most commonly used to evaluate missense variants.(407) Furthermore, one variant (D86N) has been demonstrated independently to alter protein-protein interactions.(381) Although these are commonly used, other prediction tools are available investigating protein functions e.g. SNAP (406) and mutation assessor (407, 408) look at conservation, other tools look at amino acid change and protein stability (409). Given the quantity of data recently available from NGS technologies, bioinformatics approaches to assign significance to mutations require further development and assessment.

Professor Sir Tom Blundell’s group worked in collaboration with us and carried out computer modelling on the effect of protein alterations on CDKN2B in complex with CDK4 or CDK6 based on the X-ray crystal structure of CDK6 in complex with p19. They found that the variants caused an alteration in amino acid at different positions in the protein. pAsp86 and pAla23 are close to the surface along the interface with CDK4 and CDK6, pPro40 is buried distal to the interaction surface. pAsp86 makes strong intermolecular bonds with CDK4 and CDK6, if it is replaced with an asparagine these interactions are weakened and therefore binding affinity is reduced. pAla23 is on the α helix where it makes strong inter and intramolecular bonds with CDK 4 and 6, if it is replaced by glutamate there is reduced affinity for CDK4 and 6. pPro40 is located on a hydrogen bonded turn allowing interactions with the
first and second ankyrin repeats within the protein which enable interaction with CDK4 and 6. Substitution of the proline relaxes the loop decreasing the stability of the protein. Altered interaction of mutated CDKN2B with CDK4/6 may enable individuals with CDKN2B alterations to be amenable to treatment with CDK4/6 inhibitors which have demonstrated promising in vitro results in cell lines with aberrations in mTOR and PI3K signaling(410). These pathways are both commonly altered in RCC. Thus use of CDK4/6 inhibitors in RCC cell models, particularly in the setting of p15 depletion would be a fascinating extension to this work.

4.13 Conclusion - The putative role of CDKN2B in RCC.

This study represents a significant development in the understanding of the molecular pathology of RCC. This is partly due to the study design focusing on familial RCC. Familial RCC represents a small proportion of RCC in total (2-3%) (246) but studies of these conditions have enabled seminal insights into the pathophysiology of sporadic RCC. Despite intensive research in the field, only two genes VHL and PBRM1 have been associated with >30% of kidney cancers. Studies of non-syndromic familial RCC patients have indicated that the pattern of inheritance is most likely to be autosomal dominant (246), suggesting hitherto unknown TSG are responsible for a significant proportion of familial RCC. Furthermore, as has occurred in phaeochromocytoma, as further research is carried out on RCC genetics, an increased proportion of cases may be attributable to genetic predisposition. As CDKN2B is a known TSG(351) (356, 380, 411), the mutations described are likely to represent ‘driver’ mutations in RCC. This work demonstrated CDKN2B is associated with familial RCC in 4% of cases investigated. This is a clinically significant proportion and is similar to the proportion of individuals with non-syndromic RCC with mutations in the BHD predisposition gene FLCN (4.3%). Though somatic CDKN2B mutations appear to be rare in sporadic RCC,
previous studies using copy number analysis have shown a loss of gene expression in the 9p area. (323, 362). CDKN2B involvement in familial RCC is a novel and important finding in the field. In the future, as costs of NGS decrease patients’ tumours may be sequenced using WGS at diagnosis, or CDKN2B should be incorporated in the cancer gene panel testing. Further evaluation of CDKN2B and its associated pathways in RCC may enable better understanding of the molecular pathogenesis of the condition and may aid the development of personalized therapies such as the use of CDK4 and 6 inhibitors.
CHAPTER 5. INVESTIGATING NOVEL TREATMENTS IN RCC – EXPLOITING SYNTHETIC LETHALITY

5.1 Development of therapies for RCC – targeting known and unknown pathways in RCC - ‘Synthetic Lethality’
As described in chapter 4, there have been considerable developments in the treatment of RCC due to improved understanding of some of the key pathways associated with RCC. Most notably, TKI were developed as a result of improved understanding of the downstream consequences of inactivating the VHL TSG. However, there is a widely held view that on the whole, despite better understanding regarding which mutations drive cancer progression this has not always been translated into the development of agents which directly target cancer cells (412).

Cancer cells accumulate numerous genetic alterations over time. The cancer phenotype occurs due a loss of TSG function and the products of activated oncogenes (413). Although cancer cells may contain only a few ‘driver’ mutations these are usually part of a small number of well conserved key pathways (403) such as; cell cycle control, DNA damage response, integrin mediated cell adhesion, apoptosis and TGF-β signalling (413). Driver mutations include commonly mutated genes such as TP53, BRCA1, BRCA2, NF1, and RB1 (414) These pathways have been intensively investigated for possible therapeutic uses. Cancer cells cannot survive without some of the products of oncogenes due to their intrinsically abnormal physiology. This process is called ‘oncogene addiction’. Normal healthy cells are not dependent on these gene products. Therefore, this ‘addiction’ can be exploited to identify possible new therapeutic targets. The key aim of this strategy is to identify ‘critical functional nodes in the oncogene network’ (415), once these are inhibited there is a presumption that the oncogenic system will collapse and the cancer cell will die, apoptose, differentiate or go into senescence (25). The advantage of the principle of synthetic
lethality is that even ‘passenger mutations’ can be interactors resulting in potentially novel treatments.\textsuperscript{(412)}

This difference in cellular physiology between normal and cancer cells due to oncogene addiction is utilised in strategies using the concept of synthetic lethality (figure 5.1). Two genes are synthetically lethal if a mutation in either gene alone is not lethal but mutations of both cause cell death. In addition, interactions can be synthetically ‘sick’ when mutations of one gene cause no change in cellular wellbeing but simultaneous mutations are deleterious to cell health.\textsuperscript{(413)} Synthetic lethality is a particularly important concept in cancer medicine as TSG are crucial to cancer cell function. Over 80\% of mutations seen in cancers are in TSG\textsuperscript{(416)}. \textit{In vitro} studies and murine models have demonstrated that reactivation of TSG can cause widespread apoptosis or senescence of tumours dependent on the tissue being investigated.\textsuperscript{(413)} However, in humans it is usually not possible to re-activate a TSG (unless the TSG is epigenetically inactivated) however the ‘undruggable’ TSG can become druggable if one of its synthetic lethal interactors can be targeted\textsuperscript{(413)}.

Synthetic lethal interactions can also provide important insights into genetic interactions within the cell. Furthermore, many genes within the genome have not had their function characterised, in 2012 only \(~2\%\) of genes had a defined function\textsuperscript{(414)}. Within the cell there are numerous genes with similar functions, so that key characteristics such as mitosis are not vulnerable to the loss of one gene (so called ‘buffering’).\textsuperscript{(417)} By considering their interactions, the functional annotation of unassigned genes can be performed.\textsuperscript{(417)}
Two genes are defined as having a synthetically lethal interaction if inactivation of one gene does not adversely influence cellular growth but simultaneous inactivation of both genes leads to cell death. This concept can also include 'sickness' of the cell with two inactivated genes.
5.2 BRCA associated cancer- the exemplar of the therapeutic role of synthetic lethality

The most clinically relevant use of synthetic lethality is the use of Polyadenosine diphosphate [ADP] ribose polymerase 1 (PARP1) inhibitors in BRCA-1 and BRCA-2 mutation positive breast and ovarian cancers. PARP1 activity is essential in DNA damage repair as it is required for base excision repair. Base excision repair is important for errors in a single DNA strand. The proteins encoded by the BRCA1 and BRCA2 genes are essential for error free homologous recombination repair (HR). HR is required when there are double stranded breaks in DNA. Double stranded break repairs are the most serious type of DNA damage which, when un repaired, can trigger apoptosis. Cells which have BRCA1 or BRCA2 deficiency have to repair double strand breaks using alternative, more error prone, mechanisms such as non-homologous end joining or single strand annealing. These methods are less accurate than HR (418) and can lead to increased genomic instability (413).

Inhibition of the PARP pathway in vitro results in DNA damage that must be repaired by the homologous repair pathway. Thus, when cells deficient in BRCA1 and BRCA2 are treated with PARP inhibitors they are unable to repair DNA and subsequent DNA damage causes cell death due to chromosomal instability. Cells without BRCA1 or BRCA2 inactivation are less sensitive to the inhibitors. Fong et al (420) have shown that PARP inhibitors cause tumour stabilisation/regression only in those individuals with BRCA mutations. PARP inhibitors are currently being further evaluated in a series of phase III clinical trials in BRCA-associated breast and ovarian cancer.

5.3 Combining synthetic lethality with chemotherapy or radiotherapy

Alkylating agents such as doxorubicin and DNA adduct forming drugs like cisplatin (described in section 1.1.3) cause DNA damage and corresponding cellular stress. When cancer cells which have lost both copies of a particular TSG are exposed to external cellular
stress such as chemotherapy there could be an effect on cell death. However, if another synthetic lethal interactor of the TSG is also inhibited pharmacologically this may increase the effect of the chemotherapy on cell death. (413) This interaction has been utilised in a mouse model of p53 deficient tumours. When murine p53 deficient cancer cells were treated with a MK 2 inhibitor (inhibitor of a cell cycle checkpoint pathway) there was mitotic catastrophe associated with regression of tumours if the cells were also treated with cisplatin or doxorubicin. (422) In another study, when p53 deficient cells were exposed to ionising radiation there was increased expression of PLK1. These p53 deficient cells were also exposed to PLK1 inhibitors and this inhibition was associated with cell death (418).

5.4 Using synthetic lethal interactions to overcome chemo-resistance
The RAS proto-oncogene is mutated in a large number of cancers including 25% of lung adenocarcinomas. (423) 90% of these mutations are within KRAS, and individuals with KRAS mutations have a worse prognosis, resistance to EGFR inhibitors and have less benefit from chemotherapy. (423) Although, KRAS mutations have been well characterised in lung cancer there are no effective targeted agents directed against mutated K-RAS. Thus, downstream effectors of the RAS could be inhibited to decrease RAS activity. One downstream activator associated with RAS is MEK, selumetinib is a MEK inhibitor. Selumetinib was given with docetaxel in patients with KRAS mutations in a phase II randomised trial and compared to docetaxel alone. This trial indicated that dual therapy was associated with better PFS and response rates, suggesting a potential clinical improvement associated with using an interpretation of a synthetic lethal approach, however further evaluation of this strategy is required. (423)
5.5 Synthetic lethality screens

Synthetic lethality interactions are being evaluated on a large scale using synthetic lethality screens utilising the principle of RNA interference (RNAi). The principle of RNA interference is that double stranded RNA causes sequence specific degradation of complementary mRNA, thus decreasing protein expression. Double stranded short interfering RNA (siRNA) molecules are 21-23 nucleotides long with a 2 nucleotide overhang on their 3’ end. They can be synthetic siRNA molecules which typically form part of library assays or short-hairpin RNA (shRNA). shRNA can be constitutively expressed using RNA polymerase and are then cleaved intra-cellularly to form short siRNA effectors.

Synthetic siRNA are transported into cells using transfection reagents so that they are not damaged by nucleases in the extra-cellular media. The intracellular half-life of synthetic siRNA is approximately 24 hours, however the duration of gene silencing in rapidly growing cells achieved by siRNA is usually a week. Knockdown lasts longer in non-dividing cells as it is believed the length of knockdown is due to ‘dilution’ of siRNA as cells increase in number. The consequent duration of protein knockdown is dependent on the half-life of the protein but typically occurs within a week. Synthetic lethality screens allow multiple potential targets and gene interactions to be investigated simultaneously. They provide a functional, systems biology approach to identify cancer cell vulnerabilities. Most synthetic lethality interactions identified in mammalian systems have been derived from large-scale RNAi libraries which have a high throughput and each gene is interrogated in at least a single well. Many siRNA library studies have identified non-oncogenes that are synthetically lethal to cancer cells suggesting that they can also act as cancer targets.
In order to study more long term gene depletion, pooled shRNA assays have been used by Elledge’s group (415). Isogenic cell lines were infected with pooled shRNA by viral vectors. Each shRNA contained a unique barcode of 60 nucleotides. After the cells were cultured in selective culture medium, the genomic DNA containing integrated shRNA was isolated. The DNA underwent PCR amplification and DNA microarray analysis to consider differential expression of genes (416), the amount of each barcode reflects the abundance of each shRNA evaluated. Lou et al performed parallel screening of >30,000 shRNA in isogenic colorectal cancer cell lines which contained either wild type RAS or mutant RAS. 26% of transcripts showed differential expression and RAS mutant cells were particularly sensitive to loss of PLK1. This suggested that cells with mutated RAS were dependent on PLK1 for cell cycle progression (415) and a synthetic lethal interaction between mutated RAS and PLK1. Bommi-Reddy and co-workers (425) used shRNA vectors to identify genes which may be synthetically lethal with VHL, these results are discussed in section 5.9.6.

5.6 Aims
As discussed in previous chapters, the application of genomics has greatly facilitated the diagnosis of genetic diseases and the discovery of novel genes. One of the biggest criticisms of the genomics revolution is the lag between gene discovery and tangible clinical treatments. In this chapter, a functional genomics approach is taken in order to highlight potential pathways of importance in RCC which may be useful therapeutically. The aim of work in this chapter is to use synthetic lethality screens to identify potential synthetic lethal interactors with VHL in a RCC model.
5.7 Methods

5.7.1 The methodology of the synthetic lethality screen

In vitro studies have utilised synthetic lethality with two aims: (i) to identify new genes and pathways associated with cancer and (ii) to find new therapeutic targets. Despite the importance of VHL in RCC, synthetic lethal interactions with VHL have not been extensively evaluated. In this study, the SKRC-39 cell line was used as the parental line for the isogenic lines because though a VHL mutation has not been identified, it is known to be deficient for pVHL expression. SKRC-39 had been previously transfected within our group to stably express either wild type VHL or an empty vector. The paired isogenic lines were thus further named SKRC39ev or SKRC39wt. The expression of pVHL was confirmed using immunoblotting (Figure 5.7.1). Introduction of VHL into the cell lines did not influence their ability to proliferate in standard in vitro conditions.

RNAi libraries have been used to conduct loss of function screens in human mammalian cell systems and their methodology is summarised in figure 5.7.2. As described previously, dysregulation of the HIF pathway (described in section 4.3) leads to upregulation of a number of protein kinases associated with cell growth. Protein kinase inhibitors have been previously shown to be effective in the treatment of RCC (30, 334). They are logical targets for investigation as they are eminently ‘druggable’. Therefore, the isogenic renal cell lines expressing VHL or not expressing VHL were interrogated for synthetic lethal interactions with human kinases.

The Silencer Select human siRNA library was used in this study and the methodology used is described in section 2.1.2. The library contains 2130 unique siRNA targeting a total of 710 human kinase genes which have been validated (99). Previous work within our group has
demonstrated that SKRC-39 cells are transfected easily with the INTERFIRin transfection agent. In this experimental model, SKRC-39 which stably expressed wild type VHL were compared with those which expressed an empty vector (PC DNA 3.1). These cells are denoted SKRC39ev and SKRC39wt respectively.

RCC cells were assayed for viability using the cell titre blue assay. This assay quantifies living cells by looking at their ability to convert resazurin into a fluorescent end product (resorufin) which is measured using a plate-reading fluorometer. Non-viable cells are unable to perform this conversion as they have lost metabolic activity and thus the read-out represents living cells (426). Fluorescence levels were determined for each well treated with an oligonucleotide. Each kinase gene was evaluated in three wells each containing a different oligonucleotide. The influence of the oligonucleotide on cell viability was established using the following calculations:

(i) Normalised value = fluorimeter reading in well treated with oligonucleotide /mean fluorimeter reading in cells from the same cell line treated with siRNA against luciferase
(ii) Comparison was made to identify those oligonucleotides causing a selective decrease in viability amongst cancer cells by dividing the normalised SKRC39wt value (calculated above) by the normalised SKRC39ev value for each individual oligonucleotide.

Therefore, siRNA causing a selective decrease in the viability of RCC cells had a normalised SKRC39wt/SKRC39ev value of >1. Those genes which caused fold changes ≥1.2 were selected for further investigation.
Figure 5.7.1 A representative immunoblot from 3 experiments demonstrating absence of pVHL expression in SKRC39ev and presence of pVHL in SKRC39wt cell lines.

Figure 5.7.2. A schematic diagram explaining the process of evaluating interactions using a siRNA library
5.7.2 Determination of the depletion of STK10 and PLK1 on global protein expression of SKRC39ev and SKRC39wt cells using the Kinexus KAM-850 protein array.

As described in section 2.18, the Kinexus Microarray was used to determine changes in global protein expression in SKRC39ev and SKRC39wt cells after treatment with Dharmacon smartpool siRNA directed against \textit{STK10} and \textit{PLK1}. Kinexus was provided with cell pellets from cells treated with siRNA and then labelled protein lysates were applied to the KAM-850 Antibody Microarray chip. The array contained includes 517 pan-specific antibodies for protein expression and 337 phosphosite-specific antibodies. Analysis of the data derived from the array was performed by Kinexus (see section 2.18 for a more detailed explanation). In brief, the protein targets included in the array were: proteins involved in apoptosis, cell proliferation, stress and kinases. Kinexus processed fluorescence data and used their standard algorithm to analyse the data. A fluorescence signal from each protein was measured in duplicate and the average figure taken. The average percent change from the control sample was expressed as a Z-ratio. Kinexus provided a shortlist of genes in which protein expression was altered significantly (Z-ratio of +/- 1.2-1.5) after depletion of either STK10 or PLK1. Antibodies included in the array have a degree of cross reactivity and therefore proteins identified by the array to be overexpressed may not actually be over-expressed. Furthermore, antibodies within the array may not bind sufficiently well to proteins resulting in false negatives. Therefore, immunoblotting must be performed to confirm results(100). In this study, the Kinetworks multi-immunoblotting analysis was performed by Kinexus and utilised to validate the findings of the array. This was performed as it was cost-effective and time efficient as Kinexus had access to the appropriate antibodies and also optimised the conditions for blotting. This system allowed for the analysis of 18 antibodies only.
5.8 Results

5.8.1 siRNA library identifies potential synthetic lethal interactors with VHL

The results of the kinase library were analysed within Excel spreadsheets. As described in section 5.7.1, fluorescence data derived after incubation with cell titre blue was converted into a ratio representing a change in viability of SKRC39wt:SKC39ev cells after treatment with specific oligonucleotides. Further analysis of this data was performed as summarised in figure 5.8.1.1 in order to identify genes for further study. Those genes with at least two fold change ratios of >1.2 seen with 2 oligonucleotides were defined as genes of interest. This proportion was similar to that seen in other studies. At least two oligonucleotides were stipulated to reduce the chance of false positives and this figure has been recommended by some key authors in the field. A shortlist of genes was made in order to prioritise genes of interest. From 710 genes, a total of 19 genes met these criteria. This was a larger number than some other studies which chose to investigate fewer genes in the initial setting (e.g. eight). Further transfection experiments were carried out to confirm whether inhibiting these nineteen genes was associated with a decrease in cell viability in SKRC39ev (Figure 5.8.1.2). The 19 genes (PIP5K1A, RYK, STK10, EIF2AK3, PLXNA3, CDC2L6, CDC42SE2, SGK269, GRIP2, NUAK2, DCLK3, SLAMF6, AK7, SIK1, NADK, WNK2, CLK4, PFTK2, LOC375133) identified from the first round screen were then re-examined by a further set of replication experiments. In these experiments, depletion using all three oligonucleotides was carried out in the 96 well setting three times. 16 of the 19 genes (PIP5K1A, RYK, STK10, EIF2AK3, PLXNA3, CDC2L6, CDC42SE2, SGK269, GRIP2, NUAK2, DCLK3, SLAMF6, AK7, SIK1, WNK2, CLK4) demonstrated a consistent preferential reduction in cell viability (measured by cell titre blue assay), at a threshold fold-change of 1.2, for the same minimum of two of three siRNA oligonucleotides identified through the original screening (Figure
5.8.1.2). The false positive rate may have been due to a degree of experimental error due to the small volumes of reagents.

Figure 5.8.1.1. A summary of the process used to select genes from the siRNA screening library for further evaluation.
involved. The decision to select genes on the basis of their relative effect (i.e. fold change) rather than statistical analysis was made during the experiment. This methodology had been adopted by others within the group and the rationale behind it was because of the small numbers involved, statistical analysis may not be reliable and false negatives/positives could occur. Retrospective analysis using t-tests failed to demonstrate statistically significant changes.

Figure 5.8.1.2 Confirmation of the kinase screen results.

Viability of SKRC39wt cells compared to SKRC39ev as assessed by the cell titre blue assay. The graph depicts the mean results of three experiments using three different oligonucleotides for each gene tested, error bars represent standard deviation. A ratio of the viability of SKRC39wt: SKRC39ev of ≥ 1.2 was classed as significant. 19 genes identified from the first round of siRNA screen were reassessed. 16 of these genes demonstrated a consistent preferential reduction in cell viability (measured by cell titre blue assay) at a threshold fold change of 1.2. Three genes did not show consistent results with the initial screen and were thus excluded.
5.8.2 Synthetic lethal interaction leads to activation of the caspase 3/7 pathway in RCC

In order to further focus the screen, 10 genes which had the highest ratio of decreased viability in SKRC39ev compared to SKRC39wt were shortlisted. These genes were: \textit{SLAMF6, PIP5K1A, SGK269, SIK1, GRIP2, DCLK3, STK10, RYK, CDC2L6, PLXNA3}. An apoptosis assay using the caspase 3 reagent was performed in cells treated with siRNA directed against 10 shortlisted genes in order to further confirm their significance (figure 5.8.2). Caspase 3 is the key effector of apoptosis in response to a number of pro-apoptotic signals. This assay measured caspase 3-7 activities by combining a pro-luminescent caspase 3/7, DEVD-aminoluciferin substrate and a luciferase enzyme. The mixture was added to cells. The cells underwent lysis and then caspase 3 lysed the substrate, resulting in free aminoluciferin which was used by luciferase. Thus, the luminescent signal was proportional to the caspase activity and apoptosis. The hypothesis being tested is that if two genes have a synthetically lethal interaction, the cell machinery would initiate apoptosis so that cells would die in an organised manner. Thus, genes which were synthetically lethal and caused apoptosis would have a greater fold change in luminescence signal. This methodology has been used by other researchers using siRNA screening libraries.\cite{428, 429} Data was displayed as fold change in apoptosis of VHL null cells to VHL positive cells (i.e. SKRC39ev:SKRC39wt).

Figure 5.8.2 shows that there was a consistent level of apoptosis (a greater than 1.2 fold change using 5nM siRNA) amongst the shortlisted genes across three experiments. This confirmed the cell viability data and suggested that the observed changes in viability may be mediated by apoptosis being preferentially induced in VHL null cells. As in section 5.8.1, statistical analysis was not performed on this data, as it was felt that with the small numbers involved, this method of analysis may not be reliable.
Figure 5.8.2. Depletion using siRNA directed towards ten shortlisted genes is associated with apoptosis.

The ten genes which were associated with the largest fold change in viable SKRC39wt compared to SKRC39ev cells was assessed using cell titre blue were selected for further study. The caspase 3 detection assay was used to determine whether siRNA mediated depletion induced apoptosis. The graph shows that the mean fold change in apoptosis of SKRC39ev compared with SKRC39wt cells over three experiments was greater than 1.2. The error bars represent standard deviation. These data indicate depletion of these ten genes using siRNA leads to increased apoptosis in VHL null cells compared to VHL positive cells i.e. SKRC39ev compared to SKTC39wt.
Prior to carrying out further investigations into genes of interest, it was important to determine whether these genes were expressed in normal kidney tissue and cancer cells. *In silico* analysis was carried out to determine whether these genes were expressed in renal tissues using Oncomine and Genecards. Oncomine ([www.oncomine.org](http://www.oncomine.org)) is a compendium of data from sources including TCGA, array data, gene copy number data which can be interrogated to assess whether genes of interest are expressed in a wide range of tissues. Genecards ([http://www.genecards.org/](http://www.genecards.org/)) is a database that considers transcriptomic, genetic, proteomic and functional data. Of the 10 shortlisted genes, four were shown to be expressed in normal kidney cells or RCC: *RYK, STK10, CDC2L6 and CDC42se2*. Two were chosen for further study: *STK10* and *RYK*.

### 5.8.3 Confirmation of differential growth and depletion of protein expression

In order to increase the scale of experiments, higher volumes of oligonucleotide were required. After shortlisting the genes of interest, in order to carry out further experiments, single oligonucleotides were used to inhibit each kinase gene. The choice was based on which siRNA within the library gave the most consistent results. Differences in cell viability induced by depletion of *STK10* and *RYK* were evaluated using oligonucleotides corresponding to those in the siRNA library (ASO0DLJS and ASO0DLK). Cell titre blue assays were repeated in SKRC39ev and SKRC39wt cell lines. These experiments using 5nM siRNA confirmed a fluorescence ratio in SKRC39wt: SKRC39ev of greater than/equal to 1.2 (figure 5.8.3.1a). Immunoblotting confirmed that RYK and STK-10 protein levels were reduced in cells treated with specific siRNA compared to siRNA targeting luciferase (figure 5.8.3b).
Figure 5.8.3.1 (a) The effect of siRNA directed against STK-10 and RYK on SKRC39ev and SKRC39wt cells. The change in viability of SKRC39ev cells compared to SKRC39wt cells as assessed by cell titre blue. Depletion of RYK and STK10 both demonstrate a fold change in viability of $\geq 1.2$ in VHL expressing cells compared to those with absent VHL. The graphs represent the mean of at least 3 experiments, the error bars represent standard deviation.

Figure 5.8.3.1 (b) Immunoblotting demonstrating decreased RYK and STK-10 protein levels in cells treated with siRNA directed against RYK and STK-10 compared to controls. The blots are representative taken from a mean of three experiments. (Abbreviations L=protein derived from luciferase siRNA treated cells, R=protein derived from RYK siRNA treated cells, S=protein derived from STK-10 treated cells)
Differences in cell viability were also demonstrated by transfecting cells in six well plates with either luciferase (control) *STK10* or *RYK* siRNA, this experiment was performed three times and the average number of cells determined from in 4 fields per well (figure 5.8.3 A and B). Photographs of representative fields were taken at 24 and 48 hours. These photographs show fewer cells in the SKRC39wt cells demonstrating the general influence of VHL on cell viability. Furthermore, there are fewer cells in the STK-10 and RYK depleted cells compared to those cells transfected with luciferase siRNA corroborating previous assay results. The effect was more marked at 48 hours. The mean number of cells in each well treated with siRNA was only statistically significantly different for SKRC39ev cells at 48 hours when those treated with control (LUC) were compared to STK10 or RYK directed oligonucleotide.
Figure 5.8.3.2. The influence of siRNA on cell numbers in SKRC39ev and SKRC39wt cells at 24 and 48 hours. (A) and (B), the mean number of viable cells/field (at x10 magnification) in SKRC39ev and SKRC39wt cells treated with control siRNA (LUC), or siRNA directed towards STK-10 or RYK for 3 experiments (error bars represent standard deviation). There significantly fewer (p=<0.05) cells in SKRC39ev cells treated with siRNA against STK-10 and RYK compared to control at 48 hours. There are also fewer SKRC29wt cells however the difference does not reach statistical significance.
Figure 5.8.3.2. The influence of siRNA on cell numbers in SKRC39ev and SKRC39wt cells at 24 and 48 hours. (C) Representative photographs from three experiments showing the numbers of SKRC39ev and SKRC39wt cells remaining after treatment with oligonucleotide directed against STK-10 and RYK compared to luciferase at 24 and 48 hours.

5.8.4. Depletion of STK-10 and RYK using different oligonucleotide doses
Differences in cell viability due to STK-10 and RYK depletion using siRNA were also evaluated in SKRC39ev and SKRC39wt cells using different concentrations of siRNA (1nM, 2.5nM, 5nM and 10nM). VHL null cells were preferentially influenced by 2.5nM, 5nM and 10nM of siRNA against the the two candidate genes compared to SKRC39wt cells reflected by an increased fold change in viability in the SKRC39ev:SKRC39wt cells (using cell titre blue) greater than 1.2. Increasing the concentration of siRNA did not decrease cell viability suggesting a maximal effect occurs at 2.5-5nM. There was no significant difference in the fold change values of cells treated with the standard experimental dose of 5nM and 2.5nM or 10nM. However, a significant difference in the viability of SKRC39wt/SKRC39ev cells

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occurred with both oligonucleotides when 5nM of siRNA was used compared to 1nM. Depletion using oligonucleotide directed towards STK10 led to a greater decrease in viability than depletion of RYK (figure 5.8.4a). Depletion of STK-10 and RYK were shown to decrease viability of cell lines by inducing apoptosis (figure 5.8.4b). There was some variability between different experiments however there was always more apoptosis in VHL null cells compared to VHL containing cells. This variability may have been due to the sensitivity of the test.
Figure 5.8.4 (a) Dose dependent responses for candidate synthetic lethality targets. Change in cell viability of SKRC39wt compared to SKC39ev cells was assessed using the cell viability blue assay for three experiments. Data represents the mean fold change of the normalised fluorescence value of SKRC39wt/SKRC39ev over three experiments, the error bars represent standard deviation. STK-10 and RYK siRNAs led to decreased viability of SKRC39ev more than SKRC39wt cells (denoted by a fold change >1), there was no significant change in viability at higher doses of oligonucleotide. T-tests showed the only significant difference in viability at doses other than 5nM (the standard dose used in the experiments) was at 1nM for both STK-10 and PLK-1 where viability changes appeared to be less influenced by depletion.
Figure 5.8.4. (b) Depletion of RCC cells with oligonucleotide directed against RYK and STK-10 leads to apoptosis of cells. The ratio of fluorescence measured after addition of the caspase 3 reagent in SKRC39ev cells compared to SKRC39wt cells. Data represents the mean and standard deviations of three experiments. There is increased fluorescence in SKRC39ev cells indicated by ratios over 1 compared to SKRC39wt cells. This suggests apoptosis is more common in VHL null cells. This data reflects viability data derived using cell titre blue, suggesting that the change in viability seen when SKRC39ev cells are treated with STK10 or RYK directed oligonucleotides may be due to increased apoptosis.

5.8.5 Depletion of STK-10 and RYK in different cell lines
To investigate the potential wider applicability of these findings, cell titre blue assays were performed after siRNA knockdown of STK10 and RYK in three further VHL-inactivated RCC cell lines (KTCL-26, RCC-4 and 786-O). Knockdown of STK10 significantly reduced the viability of KTCL-26 and 786-O cells (ratio control/ treated mean (±standard deviation): 1.27(±0.08) (t=3.85 P=0.03) and 1.12 (±0.02) (t=6.32 P=0.02) respectively) but no significant differences were detected for RYK siRNA knockdown (Figure 5.8 5a). Western analysis
demonstrated that both STK-10 and RYK were expressed in all three cell lines so that the observed variability in response to siRNA knockdown did not result from lack of STK-10 and RYK protein expression (data not shown). This data did use a single oligonucleotide and thus is limited by the possible influence of off-target effects and also possible poor depletion in specific cell lines.

5.8.6. STK-10 is depleted by siRNA oligonucleotide.
In order to determine the level of knockdown achieved using siRNA, real time experiments comparing cDNA levels in SKRC39ev and SKRC39wt cells treated with siRNA against luciferase and STK-10. STK-10 was chosen because the degree of knockdown seen on western blotting was more pronounced than for RYK and STK-10 appeared to be of more general relevance than RYK. Thus, it seemed to be a better ‘candidate’ for synthetic lethal interaction. RNA was extracted and then converted into cDNA, and the experiment conducted as described in section 2.16. STK-10 siRNA did produce down regulation of the STK-10 gene in both SKRC-39ev and SKRC-39wt cells (Figure 5.8.6). The level of inactivation did not differ significantly between SKRC-39ev and SKRC-39wt cells. Furthermore, there was not a significant dose response for siRNA concentrations of 5nM compared to 10nM. This reflected the cell viability data for siRNA concentrations of 1, 2.5, 5 and 10 nM (Figure 5.8.2) suggesting that maximal knockdown occurs at 5nM concentrations.
Figure 5.8.5. The effect of siRNA oligonucleotides directed against *STK-10* and *RYK* on RCC cell lines. The graphs show the mean ratios (± standard deviation) of cell viability of KTCL-26, RCC-4 and 786-O cell lines treated with siRNA oligonucleotides directed against *STK-10* and *RYK* compared to luciferase controls in three different experiments. One oligonucleotide was used against *STK-10* and *RYK* (chosen as described in section 5.8.3). A statistically significant (p<0.05) decline in viability (denoted by *) of the 786-O and KTCL-26 cell line was seen when STK10 was depleted but not RYK.
Figure 5.8.6. The percentage depletion of STK-10 in SKRC39ev and SKRC39wt cells. The percentage depletion of STK-10 RNA after treatment of SKRC39ev and SKRC39wt (denoted as EV and WT in the graph) with either control (LUC) or STK10 specific oligonucleotide seen in: (i) SKRC-39ev cells treated with 5nM STK-10 siRNA compared with 5nM LUC siRNA (ii) SKRC-39ev cells treated with 10nM STK-10 siRNA compared with 10nM LUC siRNA (iii) SKRC-39wt cells treated with 5nM STK-10 siRNA compared with 5nM LUC siRNA (iv) SKRC-39wt cells treated with 10nM STK-10 siRNA compared with 10nM LUC siRNA. The graph represents the mean change in DNA for three experiments.

5.8.7 Treatment of SKRC39 cells with an inhibitor of STK-10
As STK-10 had a greater influence on cell viability than RYK it was evaluated in further detail. STK-10 is a Serine/threonine kinase. Kapra et al, have described serine/threonine kinases as essential in cellular signalling and homeostasis. These functions are achieved through phosphorylation of transcription factors, cell cycle regulators, cytoplasmic and nuclear effectors. (430). Erlotinib is a orally available EGFR inhibitor licenced for the treatment of lung and pancreatic cancer. Erlotinib can inhibit STK-10 at clinically significant levels (431).
leading to increased T cell migration and IL-2 secretion. RCC is particularly susceptible to immunological therapies and in fact, high dose IL-2 treatment is associated with prolonged remission in a small number of individuals with metastatic RCC.\cite{432} Inhibition of \textit{STK-10} may have further anticancer effects to those described above by increased production of anti-cancer cytokines \textit{in vivo} by circulating lymphocytes. The SWOG S0317 trial evaluated the use of erlotonib in patients with papillary RCC providing some \textit{in vivo} evidence for \textit{STK-10} inhibition in RCC and corroborates our data as the SKRC-39 cell line is derived from papillary RCC.\cite{433}. However unlike SKRC-39 cells, the majority of individuals with papillary RCC do not have \textit{VHL} inactivation.\cite{434} Individuals in this phase II clinical trial were treatment naive. Only two patients had a mutation in \textit{VHL} (silencing by epigenetic inactivation was not assessed). These two patients had disease stabilisation as best clinical response. At the time of the trial activating mutations in \textit{EGFR} were not discovered, thus only EGFR expression was discussed, furthermore the influence of erlotonib on \textit{STK-10} was not known and therefore all responses were attributed to the influence of erlotonib on the \textit{EGFR} pathway. The trial demonstrated an 11% response rate and 64% disease control rate. The median OS in this cohort was 27 months, in the metastatic setting this survival is impressive and suggests that erlotonib has some impact on RCC progression. Some of this response maybe contributed to by \textit{STK-10} inhibition.

The influence of erlotonib on isogenic SKRC-39 cell lines was evaluated in order to see whether the effects are similar to those seen with \textit{STK10} knockdown by siRNA. A cell inhibition assay was performed using increasing concentrations of erlotonib. The GI50 evaluates the growth inhibitory effect of the erlotonib on SKRC-39 cells. There was no significant difference between the GI50 value of erlotonib in SKRC39ev and SKRC39 wt cells as shown in figure 5.8.7. There was no difference between in cell viability except at
high concentrations where the drug was likely to be toxic to cells. These results may be because erlotonib is not a ‘pure’ \textit{STK-10} inhibitor and there maybe some impact on EGFR inhibition on cell growth. \textit{EGFR} is believed to be important in RCC, it is upregulated by TGF-\(\beta\)\(435\) and has been demonstrated to be over expressed in RCC. Furthermore, EGFR over-expression is associated with higher tumour grade in clear cell carcinoma \(436\). Thus, it is potentially the case that the influence of EGFR inhibition which was equivalent in both cell lines overwhelmed the influence of \textit{STK-10} inhibition. Cell lines often contain multiple genetic aberrations and it maybe that another genetic alteration can lead to resistance to erlotonib.

![Graph showing cell viability vs. concentration of erlotonib]

\textbf{Figure 5.8.7}. The influence of increasing doses of erlotonib on cell viability of SKRC39ev (blue) and SKRC39wt cells (red). These data represent the mean data and standard deviations (error bars) of three experiments performed, each containing five replicates representing cells treated with five different doses of erlotonib \(0, 10\text{nM}, 100\text{nM}, 1000\text{nM}, 10000\text{nM and 100,000nM}\). There were no significant differences in cell viability between VHL containing and null cells except at high doses of erlotonib which are likely to be secondary to a direct toxic effect.
5.8.8. Depletion of STK-10 or PLK leads to decreased RCC viability and selective loss of VHL inactivated cells

In view of the potential relevance of STK-10 as a synthetic lethal interactor with VHL it was further evaluated. Other researchers had identified that siRNA induced knockdown of PLK1, a reported downstream target of STK-10, induced synthetic lethality in pVHL deficient RCC cell lines (437). STK-10 is known to phosphorylate PLK1 and inactivating mutations of STK-10 can lead to decreased phosphorylation of PLK1. (438) Review of the Silencer Select human siRNA library screening in SKRC39ev and SKRC39wt revealed PLK1 had not been shortlisted as only 1 of the 3 oligonucleotides directed against the gene altered cell viability above the threshold for selection. Thus, it may have been a false negative. Therefore, we investigated whether siRNA-induced knockdown of PLK1 might enhance or substitute for STK10 inactivation. The effect of depletion of one or both of these genes was evaluated in five RCC cell lines (SKRC39ev, SKRC39wt, RCC-4, KTCL-26, 786-O) using the Dharmacon SMARTPOOL on Target Plus system (which contains four pooled oligonucleotides against each target). Pooled oligonucleotides were used because of the lack of sensitivity to PLK1 previously encountered using single siRNA and this technique was being used to deplete PLK1 successfully by other colleagues. Depletion of STK10 and PLK1 using 5nM siRNA in 3 separate experiments led to a significant decrease in cell viability of all RCC cell lines (p=<0.05). Moreover, in the SKRC39ev and SKRC39wt cell lines there was also a selective decrease in cell viability of VHL null cells compared to VHL positive cells when treated with STK10 (mean 67%), PLK1 (mean 62%) and both STK-10 and PLK (mean 58%) (Figure 5.8.8a). Similarily depletion of STK-10 decreased cell viability in KTCL-26, RCC-4 and 786-O cells (mean 30.6%, 47% and respectively) and depletion of PLK1 also decreased cell viability of KTCL-26, RCC-4 and 786-O cells (mean 35%, 46% and 40%
respectively) (Figure 5.8.8b). These differences were statistically significant (p<0.05). Simultaneous depletion of both \textit{STK-10} and \textit{PLK1} did not have a more profound effect than targeting each gene individually (Figure 5.8.8).

![Graph A](image)

**Figure 5.8.8** Depletion of RCC cell lines with pooled siRNA. (a) Comparison of mean (±SE) percentage decrease in the cell viability of VHL null cells (SKRC39ev) relative to RCC cells containing VHL (SKRC39wt) when treated with pooled siRNA oligonucleotides directed against \textit{STK-10}, \textit{PLK1}, or both (compared with those treated with luciferase siRNA)(b) Comparison of mean (± SE) percentage decrease in cell viability of KTCL-26, RCC-4 and 786-O RCC cell lines when treated with pooled siRNA oligonucleotides directed against \textit{STK-10}, \textit{PLK1} or both (compared with those treated with luciferase siRNA). (* represents p<0.05 by t-test). Data represents the mean results of 3 experiments.
5.8.9 The global effect of STK-10 and PLK1 depletion on protein expression in SKRC39ev and SKRC39wt cells

In order to evaluate the influence of depletion of STK-10 and PLK1 on SKRC39ev and SKRC39wt cells, protein lysates from cells treated with siRNA directed against STK10, PLK1 and control were evaluated using a commercial protein microarray. There are two main types of protein microarray, reviewed by Zhang (439). Forward phase arrays use antibodies immobilised on a slide and incubate the slide with labelled protein lysate. The slide is washed and the captured fluorescent protein is then quantified. The second method, ‘reverse lysate’ technology uses immobilises protein lysates which are incubated with primary antibodies of interest. These are washed and then incubated with secondary antibodies. Forward phase technologies i.e. those with immobilised antibody are better established and were more popular at the time of this research, recently reverse lysate technologies are being utilised.

Traditional means of evaluating protein expression e.g. immunoblotting can be time consuming, costly, and require considerable quantities of protein. Microarrays have the advantage that they enable interrogation of hundreds of proteins using relatively small quantities of protein. The utility of microarrays is limited by the specificity of antibodies towards individual proteins and thus they require validation of their results. Antibody arrays are also less successful at identifying changes in intracellular proteins which are typically expressed at lower levels than structural proteins. (439) Their main use is as a screening technology to highlight potential proteins of interest, the Kinexus microarray was utilised because it had previously been successfully used by our research group. (440)

5.8.9.1 Global changes in protein expression in cells with STK-10 and PLK-1 depletion using the Kinex Antibody Microarray

The Kinex Antibody Microarray data underwent initial quality control and filtering by Kinexus. This involved determining the z-ratio for each antibody, percentage error range,
flag and median normalised signal intensity. The z-ratio was determined as described in section 5.7.2, the globalised signal intensity normalises the net signal of a comparison to that of all the signal values in a sample, the percentage error range is a measure of how close the normalised percentage error of adjacent spots for each protein are, flag indicates the morphology and background of each spot. On this basis, proteins were identified as being of potential interest by the company. The company defined a z ratio of at least ±1.2-1.5 as being significant. Further analysis then took place in order to determine the significance of the change in protein expression described by the shortlists of z ratios (see figure 5.8.9.1.1).

When protein expression in SKRC39ev cells treated with STK-10 siRNA was compared with those treated with control oligonucleotide, 22 antibody signals were higher and 19 were reduced compared to controls. In SKRC39ev cells treated with PLK1 oligonucleotides compared with control; 12 antibody signals were higher and 19 were lower than controls. In SKRC39wt cells treated with PLK1 oligonucleotides compared with control; 13 antibody signals were higher and 20 lower than controls. Arrays derived from SKRC39wt cells treated with STK10 oligonucleotides compared with control contained 13 antibody signals which were higher and 21 lower than controls. In order to determine which differences in protein expression may be of significance, a number of processes were applied to evaluate the list. These processes are summarised in figure 5.8.9.1.1.

The similarities and differences between cells depleted of STK-10 and PLK1 were analysed by individually interrogating each protein shortlisted, looking for proteins altered in both PLK1 and STK10 depleted cells. In SKRC39ev cells there were four proteins which featured in both shortlists for cells depleted with STK-10 and PLK1, no proteins featured in the shortlists for SKRC39wt cells.
Figure 5.8.9.1.1 Analysis of the data provided from the Kinexus protein microarray. The initial two filters were carried out by Kinexus. Further analysis took place using those proteins identified by filter 2 in order to identify which proteins could be chosen to validate the array.
Pathway analysis using STRING 9.1 (http://string-db.org/) and The Database for Annotation, Visualisation and Integrated Discovery (DAVID) version 6.7 (http://david.abcc.ncifcrf.gov/) was utilised to identify any pathways altered by STK-10 and PLK1 depletion. Specifically pathways in the Kyoto Encyclopedia of Genes and Genomes (KEGG) were analysed. Pathway analysis failed to indicate changes to specific pathways when SRC39ev or SKRC39wt cells were depleted of STK-10 or PLK1. For example; in SKC39ev cells treated with STK-10 siRNA there was upregulation of proteins involved in the MAPK pathway and downregulation of the ‘pathways in cancer pathway’, whereas those treated with PLK1 siRNA demonstrated upregulation of no specific pathway and down regulation of ‘cell cycle proteins’. Similarly, in the SKRC39wt model treated with STK-10 siRNA; the ‘TGF signalling’ was upregulated and ‘pancreatic cancer pathway’ downregulated. The SKRC39wt cells treated with PLK1 siRNA demonstrated increase in ‘the p53 pathway’ and ‘pathways associated with cancer’ and a concomitant decrease in the latter pathway also.

As no clear pathways or proteins appeared to be common to depletion of STK-10 and PLK1 cells, correlation analysis (figures 5.8.9.1.2 and 5.8.9.1.3) was performed using SPSS. All proteins in the shortlist were included and their corresponding z value was extracted from the raw data provided by Kinexus. This revealed similar proteins were up/down regulated in SKRC39wt cells (figure 5.8.9.1.2) however there was little correlation between proteins altered by STK-10 or PLK1 deletion in SKRC39ev cells (figure 5.8.9.1.3). This suggested in VHL inactivated cells PLK1 and STK-10 may have different mechanisms of action. Eighteen proteins were chosen for further validation studies as there is an associated false positive rate with the protein microarray technique(440). Eighteen proteins were chosen because this represented the number which were evaluable via the Kinetworks custom KCPS 1.0 screen. In order to validate the pattern of protein changes identified by the screen, proteins with z
values that lay clearly within all four quadrants of graph 5.8.9.1.2 were identified. Of these proteins, those proteins which represented important biologically or cancer associated functions were chosen for validation. Protein function was determined by performing PubMed searches. The proteins suggested for further screening were: eIF4G, ErbB3, HistoneH3, Cdk1C, Chk1, CDK5, CDK6, Hsp60, HDAC4/5/6, HistoneH3, IkbB, MEK1(MAP2K1), MEK2 (MAP2K2), STAT1a, STAT1b, NFκBp50 (two antibodies), Smad2, p18INK4c, PP2A/Ca, PP2B/Cb, RSK1.

Figure 5.8.9.1.2 A scatterplot demonstrating which proteins were up or down regulated by PLK1 depletion (x axis) and STK-10 depletion (y axis) in VHL positive cells. There was a correlation between proteins altered by depletion of STK-10 or PLK1 (Pearson’s correlation 0.786 p<0.01).
Figure 5.8.9.1.3 A scatterplot demonstrating which proteins were up or down regulated by PLK1 depletion (x axis) and STK-10 depletion (y axis) in VHL negative cells. There was no clear correlation between proteins altered by depletion of STK-10 or PLK1 (Pearson’s correlation 0.254 p=0.03). STK-10 depletion was associated with increased MAPK kinase signalling, and decreased pathways in cancer. PLK1 depletion was associated with decreased cell cycle proteins.

5.8.9.2 Validation of microarray results
The Kinetworks Custom Protein Screen was utilised to validate the results of the initial protein microarray. This screen confirms the results of the array using immunoblotting. The initial protein lysates and the same antibodies as were present on the array were used in this
Pooled protein from three experiments was used in the assay however, the assay was only performed once as per Kinexus’ standard protocol and no statistical analysis performed. Resources allowed for analysis of eighteen proteins. Proteins were chosen for validation on the basis of their position in the scattergraph i.e. representing large changes in expression and/or potential functional relevance. The final list was collated after discussion with Kinexus to identify candidates for which their assays were most accurate. The custom protein screen quantified the chemiluminescence signals of eighteen antibodies on a western blot. The chemiluminescence signal of experimental arms were compared to control i.e. the percentage change in chemiluminescence of each protein in STK10 or PLK1 depleted cells compared to controls. Figure 5.8.9.3 depicts the information derived from the screen. With regard to the SKRC39wt cells, the changes in z score from the screening data correlated with the same changes in chemiluminescence signal as compared to controls. The change in chemiluminescence signal for the eighteen proteins were similar in SKRC39wt cells depleted of either STK10 or PLK1 suggesting STK-10 and PLK1 work in similar pathways. However, in SKRC39ev cells the protein screen results appeared to be unreliable with comparatively low chemiluminescence counts (see Figure 5.8.9.2C) possibly due to increased cell death in this group. This may also have been due to technical problems with the immunoblot as it appears that even in the control cells, protein signals were very low as in those cells depleted of STK10. Thus, there may have been a problem with the concentration of the protein in the immunoblot or the technical process. Further information regarding the technical process was sought from Kinexus but not received. It was not possible to infer anything regarding protein expression due to STK-10 or PLK1 depletion in VHL null cells due to the quality of the data. However, in cells with intact VHL, overall depletion of STK-10 and PLK1 in cells had similar effects on protein expression.
Figure 5.8.9.2. Chemiluminescence data obtained on 18 antibodies chosen for validation in 5.8.9.1 Quantitative chemiluminescence signals for antibodies directed against 18 proteins on the vertical axis: (A) Signals from lysates from SKRC39wt cells transfected with control, PLK1 siRNA and STK10 siRNA, (B) Signals from lysates from SKRC39ev cells transfected with control and PLK1 siRNA, (C) Signals from lysates from SKRC39ev cells transfected with control and PLK1 siRNA. Data was analysed and provided by Kinexus and represents data from one immunoblot derived from proteins derived from three experiments using pooled siRNA described in (2.1.8).
5.9 Discussion
Elucidation of the genetic characteristics of familial RCC syndromes in particular VHL disease, has led to improved understanding of the biology of RCC. RCC is a disease entity characterised by aberrations in a number of key cellular pathways; such as the HIF and mTOR pathways. Inactivation of the VHL gene is implicated in over 70% of clear cell RCC and thus VHL was investigated as a key member of synthetic lethal interactions with RCC cells. Kinase genes were chosen for investigation as constitutive activation of receptor tyrosine kinases is a common mechanism in human cancers. Activation can occur directly e.g. by mutation or indirectly e.g. by genetic amplification, autocrine growth factor stimulation, dysregulation of receptor trafficking pathways, or crosstalk with other kinase signaling cascades. Activation can occur at multiple levels increasing signaling. Thus, a functional genomics approach was used to better understand the vulnerabilities of RCC cells by evaluating over 700 kinase genes. A screening protocol, identified two potential targets: STK-10 and RYK. These genes were confirmed to decrease cell viability in paired isogenic cell lines in a robust manner. Both RYK and STK-10 were present in SKRC-39 isogenic cell lines at a protein level. Depletion of STK-10 and RYK in the presence of inactivation of VHL led to increased apoptosis in this cell line model using the caspase glo assay. This provided a mechanistic explanation for the decreased viability, i.e. inhibition of both genes leads to increased apoptosis.

5.9.1 RYK as a possible synthetic interactor
RYK was identified as a possible synthetic lethal interactor with VHL. It was not investigated as intensively as STK-10 as the latter appeared to be a stronger candidate. RYK is an atypical growth factor receptor that has 2 transmembrane domains and one catalytic domain. It is not believed to be regulated by phosphorylation at its activation site. Its biological activity is likely to be determined by recruitment of a signalling component. RYK has been implicated
in atypical chronic myeloid leukaemia, where a translocation led to production of truncated 
*RYK* (443) which was thought to be leukaemiogenic. Furthermore, it has been shown to 
overexpressed in ovarian cancer cells (444), and mouse fibroblasts over-expressing Ryk 
developed into tumours when inoculated in Nu/Nu mice (444) mRNA for RYK has been 
localised to human kidney, brain, lung and colon tissue. (445) In mice, Ryk protein expression 
has been demonstrated in the tubular epithelium of the kidney (445), indicating it plays a role 
in kidney cellular physiology. However, this role is poorly defined and may represent a 
housekeeping function, or a widely transcriptionally active promoter whose activity is 
important in only a subset of expressing tissues.

*Ryk* is required for both canonical (*CTNNB1*) and non-canonical wnt signalling. The 
ultimate effector of the wnt pathway is β-catenin, the non-canonical pathway does not utilise 
β-catenin. The wnt pathway is responsible for a number of important cellular differentiation 
mechanisms in development and in adults is associated with tissue homeostasis, regeneration 
and stem/progenitor cell function (figure 5.9.1). (446). *RYK* has been demonstrated to interact 
with an E3 ubiquitin ligase MINDBOMB1 to activate the wnt signalling pathway. 
MINDBOMB1 also ubiquitinises Ryk, one hypothesis suggested by Berndt *et al*, is that by 
ubiquitinising Ryk, MINDBOMB1 causes the endocytosis of multimeric *RYK* which as in 
the case of other proteins (i.e. Frizzled and LRP6) is required for efficient wnt signalling 
(446). In recent years, the wnt signalling complex has been increasingly recognised in the 
aetiology of RCC. Wnt activates β-catenin which together with other transcription factors 
upregulates target genes such as the oncogene c-myc (*MYC*) which is increased in copy 
number in papillary RCC and a subset of primary RCCs. β-catenin is degraded by the *VHL* 
E3 ligase, thus in *VHL* inactivated RCC β-catenin function is increased. (447) Wnt also
activates the mTOR pathway. The mTOR pathway has established importance in RCC and mTOR inhibitors are clinically used in RCC(448). In RCC, autocrine secretion of VEGF and PDGF leads to these growth factors binding to their receptors causing activation of PI3K and then producing PIP3 which recruits Akt to the cell membrane. Akt is then activated by mTOR and PDK1. Akt promotes cell growth by inhibiting apoptosis and GSK3 whose function is to degrade cell cycle proteins. MTOR is also believed to increase HIF1 levels.(448) Wnt activates the mTOR pathway by inhibiting GSK3. Thus, RYK inhibition in the context of VHL underactivity may be synthetically lethal because it decreases both wnt and mTOR signalling required for cell growth. This is however a speculative argument as currently there is little direct evidence for RYK playing a role in kidney cancer. Most work on RYK has involved its role in the wnt signalling pathway in neuronal cells. Recently it has been proposed that RYK may play a key role in cancer invasiveness in human glioma cells (449), thus its role in cancer may yet be revealed.

5.9.2 STK-10 as a synthetic interactor and potential mechanisms of its action

STK-10 is a serine/threonine kinase, this class of kinases play an essential role in cell signalling and homeostasis by phosphorylation of transcription factors, cell cycle regulators, cytoplasmic and nuclear effectors as defined by Kapra et al,(430). STK-10 is an activator of PLK1 which is implicated in a large number of human cancers. PLK1 is a component of multiple checkpoints which are required for normal cell division (450). STK-10 has been demonstrated by Walter et al(450), to be expressed in a wide range of human tissues including kidney, to be present in the ACHN kidney cancer cell line and throughout the cell cycle. Furthermore, NIH-3T3 transfected with kinase dead STK-10 grow more slowly than
Figure 5.9.1 The RYK protein signalling network identified using affinity purified mass spectrometry taken from Berndt et al, 2011.(446)

Figure 5.9.3 The function of PLK1 in mitosis. PLK1 expression is associated with progression through the cell cycle. It begins to accumulate during S phase, peaking at the G2-M transition, plateaus throughout mitosis and has a sharp reduction upon mitotic exit. Taken from (451).
those transfected with wild-type, this corroborates our finding that knocking down the gene decreases cell viability. In NIH-3T3 STK-10 null cells, abnormal nuclei were seen indicating abnormal cell cycle progression and cytokinesis. It is hypothesized that STK-10 activates PLK1 and knockdown leads to partial inhibition of the gene, leading to abnormal cell division contributing to the clonal evolution and proliferation seen in cancer. PLK1 also activates APC (the proteosome) which is responsible for destroying mitotic proteins. This may provide a mechanism of interaction as inactivation of VHL leads to decreased ubiquitinisation of proteins and thus increased protein levels, decreased proteosomal degradation may further increase the life span of proteins important in cell division.

Another possible mechanism of interaction between STK-10 and VHL is through an effect on cyclin dependent kinase cdc2-cyclin B1. This protein is important in the G2/M transition of the cell cycle. Kim and colleagues transfected cells with an adenovirus containing VHL in order to determine the function of pVHL. In these cells, pVHL was shown induce cyclin-dependent kinase inhibitor (CDKI) p27Kip1 and inhibit CDK2 and cyclinB1-dependent cdc2 activities. Re-expression of wild-type VHL was associated with reduced cell growth (452). Thus, in VHL null cells, cyclin B1 dependent-cdc2 activity would be increased. Ellinger-Ziegelbauer and colleagues studied the function of STK-10 and postulated that it activated PLK1 which caused activation of cdc2-cyclin B.(453) Thus, if STK-10 is knocked down as in our cell model, there would be less active PLK1 and thus less active cyclin B1-cdc2 activity resulting in abrogation of some of tumorigenic function of mutant pVHL. TCGA data (454) shows that in most tumours STK-10 can be altered by mutation, deletion and amplification, however in RCC it is only amplified. It may be postulated that as most RCC contain inactivated VHL, such tumours may experience selective pressure so that cells which do not
contain an inactivation of STK-10 are more likely to be present. STK-10 has itself been implicated in cancer, Arora and colleagues found siRNA mediated STK-10 depletion was associated with decreased cell growth of Ewing’s sarcoma cell lines. (99) STK-10 is also mutated in a small proportion of testicular cancer cells (455), breast, lung, ovary and pancreatic tumours. (456).

5.9.3 PLK1 as a cancer target
STK-10 has been reported to activate PLK1 which has been implicated in a large number of human cancers including RCC (437). PLK1 is a serine-threonine kinase, with a kinase at the N terminus and 2 or more polo-domains involved in phosphopeptide binding at the C terminal (457). These functions enable PLK1 to be involved in every step of mitosis (figure 5.9.3). It is a component of multiple checkpoints which are required for normal cell division (450). In addition to its role in mitosis, PLK1 is involved with p27, regulatory loops with transcription factors, interplay with cdk1, phosphorylation of p53 family members; p63 and p73. (458) In cancer cells, PLK1 localises to the nucleus earlier in the cell cycle suggesting the protein has some cancer specific functions. Overexpression of PLK1 is associated with higher grade more aggressive tumours and correlated with poorer survival compared to tumours with normal PLK1 expression (437). Overexpression of PLK1 can cause transformation and anchorage independence of NIH3T3 cells and tumour formation in mice. (459) Silencing of PLK1 is associated with a G2/M cell cycle arrest and inhibition of tumour cell proliferation. (458, 459)

PLK1 interacts with a number of key cancer proteins including: PTEN, p53, pRb, BRCA2, Akt, myc and β-catenin. (458) Furthermore, PLK1 interacts with the 12 core signalling
pathways required for the three core cellular processes described by Vogelstein and colleagues (cell fate, genome maintenance and cell survival).\(^{(47)}\) Ding \textit{et al} \(^{(437)}\) reported that \textit{PLK1} decreased proliferation in RCC, although \textit{STK-10} was not demonstrated to be associated with synthetic lethality interactions in the setting of \textit{VHL} inactivation. Sustained inhibition of \textit{PLK1} using an inhibitor decreased RCC growth in a murine model, indicating that \textit{Plk1} may play a role in RCC growth. Although, the screen described in this chapter did not prioritise \textit{PLK1} for further investigation after the first stage screening experiments, retrospective inspection of the results revealed that two of the three \textit{PLK1} siRNA oligonucleotides preferentially reduced cell numbers in the SKRC39ev cells compared to SKRCwt cells (but only one of the siRNAs exceeded the threshold of a ratio of >1.2).

\textit{PLK1} has previously been noted as a commonly identified gene interaction described by synthetic lethality screens using siRNA (it is seen in 33\% of genome wide and 26\% of focused lists).\(^{(460)}\) Depletion of \textit{PLK1}, in the experimental model described above produced a similar effect on cell viability as the depletion of \textit{STK-10} indicating that the two genes may influence renal cell viability using a similar pathway. The effect was not additive suggesting that depletion of either gene produced the maximal biological effect on this pathway.

### 5.9.4 \textit{STK-10, PLK1 and VHL} expression in primary clear cell RCC

In order to investigate the potential role of \textit{STK-10} or \textit{PLK1} inhibition in primary clear cell RCC, RNAseq data from the TCGA portal for sporadic clear cell RCC (\(n=69\)) for which normalised data for both tumour and matched normal renal tissue was available was evaluated by a bioinformatician (Alexey Larinov, University of Cambridge). It was determined whether \textit{VHL}, \textit{PLK1} and \textit{STK-10} mRNA levels varied in tumour tissues compared to normal tissues.
The relationship between the three genes was also determined. Most sporadic RCC demonstrated increased STK-10 transcript levels in tumour compared to normal renal tissue (paired t-test p<0.001; 84% showed a >1.5-fold increase and 68% showed a >2-fold increase) (Figure 5.8.10). Similarly, most tumours demonstrated increased PLK1 transcript levels compared to matched normal renal tissue (paired t-test p<0.001; 88% showed a >1.5-fold increase and 87% showed a >2-fold increase). Overall 91.3% (63/69) of clear cell RCC demonstrated >2-fold increased expression of PLK1 or STK-10 (relative to matched normal tissue). Though most (65%, 45/69) tumours showed a >1.5 fold reduction in VHL expression there was no correlation between normalised mRNA expression in tumour and matched normal tissue for STK-10 and VHL (Spearman rank correlation coefficient (rho) =0.053 P=0.67) nor PLK1 and VHL expression (rho = -0.085 P=0.49); however there was a significant correlation between PLK1 and STK-10 relative expression (rho=0.455 P=0.0001).

Of the 69 cases with paired tumour-normal RNaseq profiling, 60 cases also had the somatic mutation data. This allowed sub-analyses within VHL- mutated and non-mutated sub-groups. The tumour-normal differences in VHL, STK-10 and PLK1 mRNA expressions, and the correlation between PLK1 and STK-10 expression, were present in both sub-groups – suggesting that changes in STK-10 and PLK1 expression in RCC are not a consequence of VHL gene mutations.
Figure 5.9.4. Expression of VHL, STK-10 and PLK1 transcripts in renal tissue compared to normal tissue using TCGA RNA-seq data. Renal tumours had decreased expression of VHL and increased expression of STK-10 and PLK1. (Analysis and graph produced by Alexey Larimov, University of Cambridge)

5.9.5 PLK inhibitors in cancer
The role of PLK1 in cancers is somewhat controversial. PLK1 is over-expressed in cancer cells and TCGA data suggests a genetic alteration of PLK1 occurs in numerous cancers including colorectal, stomach and renal cancer (454) however these changes are not commonly thought to be causal. PLK1, plays a key role in cycle regulation and is closely related to p53 and p21. It is expressed at a specific time in the cell cycle, mainly during the M phase and therefore only tends to be present in rapidly dividing cells(461). Although some
believe its overexpression is a consequence of excessive cellular proliferation associated with cancers. Initial evidence against *PLK1* being a cancer associated gene was the lack of point mutations or deletions in human cancers (458), however, more recently, this observation has been superceded by data from the TCGA which has demonstrated *PLK1* mutations in a number of cancers (e.g. colorectal, peripheral nerve sheath tumours, stomach and B-cell lymphoma) (454). *PLK1* expression may be reduced by other genes associated with cancer for example key interactors. Therefore, its role in key signalling pathways including the p53 pathway have made it a potential therapeutic target and thus *PLK1* inhibitors are being evaluated clinically(458).

*PLK* inhibitors function by inhibiting the production of the mitotic spindle and therefore causing cell cycle arrest mid-mitosis (461) However, mitotic arrest induced by *PLK1* inhibitors is short-lived lasting only 2-3 days. Thus, there is concern regarding their effectiveness in patients with solid tumours whose cells divide less rapidly and thus will have fewer cells in M phase. Consequently, fewer cells within a tumour would be susceptible to their action.(461) *PLK1* has been used in phase I clinical trials with limited results.(459, 462) In one trial, PLK1 inhibitors were associated with a risk of thromboembolism and the best response noted was stable disease.

Given the current lack of clinical utility seen with PLK1 inhibitors, the protein microarray study described in 5.8.9, was concieved to determine whether STK-10 depletion had a similar effect on protein expression as PLK1 depletion. The study did demonstrate that pathways were similar in cells with VHL intact, however the study was inconclusive for VHL null cells. This was likely to be secondary to increased cell death in these cells contributing to lower protein concentrations or a technical issue. Other protein analysis techniques which could have been utilised instead include: Enzyme Linked Immunosorbent Assay, immunoblotting
and mass spectrometry based techniques. The latter two are also limited by the protein concentrations required, the time and cost of interrogating large numbers of protein (463). Mass spectometry requires complex protein preparation which would have been practically difficult. (464) Mass spectroscopy combined with liquid chromatography in recent years has become the most popular methodology for evaluating large numbers of proteins and was instrumental in the production of the first draft version of the human proteome.(465) A further advantage of these techniques is that they facilitate understanding of protein-protein interactions and signalling pathways compared to a more static readout from microarrays.

5.9.6 A comparison of results from this synthetic lethality screen to data published from other screening libraries
The targets identified by this study are different to those identified by Bommi-Reddy and coworkers (425). There are two possible reasons for this: (i) Bommi-Reddy et al, used a different kinase library and methodology. Bommi-Reddy and co-workers used a high-throughput lentiviral screen using 100 lentiviruses studying 88 kinases from figure 2 in their paper it appears that they did not include STK-10, RYK or CDC2L6 in their study. (ii) The library also used different cell line models (786-O and RCC-4).

A limitation of the siRNA screening method is that the gene depletion has differing effects in different cell lines. For example in the paper by Bommi-Reddy et al, only four genes (CDK4, HER4, MET and IRR) preferentially decreased cell viability in VHL null 786-0 and RCC-4 cell lines. In our screen no significant effects of knockdown of CDK6, HER4, MET, IRR were detected. This may reflect less efficient knockdown or SKRC39 being less susceptible to the influence of depletion of these genes to differing genotypic features. Though STK10 was not included in the siRNA screen performed by Bommi-Reddy et al, (425) they found that
knockdown of \textit{PLK1} reduced cell viability in RCC-4 but not 786-O cells. We found that \textit{STK10} knockdown using a single siRNA reduced cell viability in pVHL deficient SKRC-39, KTCL-26 and 786-O cells suggesting that \textit{STK10} inhibition selectively affects VHL-deficient RCC. It is however interesting to note that knocking down of \textit{PLK1} in RCC-4 cells caused a decrease in viability, this may support our hypothesis that \textit{STK-10} inhibition is important in RCC cell viability in the absence of VHL as PLK1 is activated by STK-10.

Ding and colleagues used both gene expression arrays and siRNA screening to identify possible new interactors with VHL (437). Gene expression data was derived from 90 clear cell RCC tumours revealed \textit{PLK1} was associated with aggressiveness of RCC. This gene expression data was correlated with data from an \textit{in vitro} siRNA screen of the whole kinome and phosphatome using 786-O isogenic cell lines. The library used contained four unique siRNA duplexes targeting each gene. The group did not identify any novel synthetic lethal interactions and therefore chose to evaluate which genes influenced cell proliferation the most. They identified 35 genes that influenced cellular proliferation and then filtered them according to function. They then chose a panel of genes to evaluate in other parental kidney cell lines. Regardless of VHL status they identified depletion of seven genes including \textit{PLK1} was associated decreased cell growth of panel of RCC cell lines studied. They validated this using colony formation assays and real-time PCR. They then chose \textit{PLK1} for further study. \textit{PLK1} was inhibited using a commercially available inhibitor and was associated with decreased cancer cell growth both \textit{in vitro} and in a mouse model.

Taken together with the previously reported potential functional relationship between STK-10 and PLK1 and the finding that \textit{STK10} and \textit{PLK1} expression is increased in most sporadic
RCC, there is a strong case for further investigation of targeting the STK-10/PLK1 pathway as a potential synthetic lethal approach to the treatment of pVHL-deficient RCC.

5.10 Limitations

5.10.1. Limitations and controversies regarding synthetic lethality as a means of identifying potential candidate genes

Synthetic lethality aims to identify key genes which are necessary for cancer cells continued survival and growth. The attractiveness of this approach is its relative selectivity towards cancer cells. However, such targeted treatments can have their efficacy reduced due to the drug resistance caused by further genetic alterations. For example resistance to EGFR inhibitors occurs via numerous mechanisms including; the T790M mutation which attenuates inhibitor binding, amplification of the resistant allele and activating mutations in downstream kinases. (466) There is little understanding of how epigenetics, systemic signals and the local microenvironment influence synthetic lethal interactions. (412) Within cancers cells, signaling pathways are complex and intertwined. Inhibition of one component of a pathway can affect the activity of other pathways other its ‘key’ pathway. (414) This can lead to unintended consequences in cancer physiology.

A number of synthetic lethal interactions involve DNA repair pathways, inhibition of which in normal tissues might result in secondary malignancies. (418) An increased risk of malignancy has been associated with several types anticancer therapies. When BRAF inhibitors were used in melanoma, there was an increased incidence of squamous cell carcinoma. Squamous cell carcinomas in BRAF inhibitor treated patients were due to ‘paradoxical MAPK activation’ by the inhibitor. In cells with wild type BRAF, treatment with an inhibitor causes dimerization of RAF that can cause MAPK hyperactivation through
increased signaling through RAF. Skin cells can harbour HRAS mutations caused by UV exposure. Thus skin cells with a HRAS mutation developed into squamous cell carcinomas due to the MAPK hyperactivation.\(^{(467)}\) This effect was reduced by adding a MAPK (MEK) inhibitor to a BRAF inhibitor.

**5.10.2 Limitations and controversies regarding synthetic lethality screening using RNAi**

Although there are difficulties with the generalisability of the principle of synthetic lethality to cancers on the whole due to the dynamic genetic nature of cancers, RNAi screening libraries have been widely used as a means of identifying cancer genes. Despite this, there are a number of limitations of this strategy and its applicability to more advanced pre-clinical and clinical models.\(^{(427)}\) RNAi screening libraries usually utilize isogenic cell lines as a means of identifying synthetic lethal interactions. However isogenic cell lines do not strictly reflect a ‘normal’ cancer cell, for example they may contain specific mutations leading to the identification of synthetic interactors specific to that cell line only. Furthermore, some cell lines have been found to be different to their tissue of origin \((468, 469)\). Thus, synthetic lethal interactions identified in cell models may not necessarily be seen in primary cancer cells that harbor the genetic defect.\(^{(417)}\) In retrospect, it would have been helpful to investigate multiple isogenic cell lines (if sufficient time had been available) as it is possible that with only one isogenic cell line model, there may be a characteristic unique to this cell line that led STK-10 to be a synthetic lethal interactor, but that other kidney cancer cells may, not harbouring this change, be resistant to STK-10 inhibition (however subsequently additional RCC cell lines were shown to be sensitive to STK-10 depletion).

Furthermore, different isogenic cell lines are cultured separately, potentially there can be subtle differences in their growth environment causing the apparent synthetic lethal effect. This can be overcome by applying fluorescent labels to the different cells so they can be co-
cultured (416) Furthermore, intra-tumoural heterogeneity(470) means that even if a gene is a synthetic interactor with a TSG, some areas of a tumour may not contain the mutation in the synthetic lethal interactor and will have a survival advantage compared to cells with both mutations. This clone will not be susceptible to the ‘synthetic lethal interactor’ and can then go on to become the dominant clone as it will have a survival advantage. Intra-tumoural heterogeneity may partially explain why so few agents derived from synthetic lethal interactions are used clinically, as the dynamic tumour in humans is so much more complicated than in vitro models. In this study, the VHL gene was studied as this is a key driver mutation and thus less likely to be susceptible to genetic heterogeneity associated ‘resistance’ as a ‘truncal’ mutation it should be present in all subclones. Nevertheless, the principle of intra-tumoural heterogeneity has led to some authors suggesting that differential gene expression in human tumour cells rather than isogenic cell lines should be studied to identify synthetic lethal interactions.(412) Interestingly, Ding et al,(437) found no new synthetic lethal interactors in their VHL isogenic cell line, however used data from a gene expression array to determine which proliferation affecting gene to evaluate further (PLK1). Furthermore, cancer cells in vitro and tumours in pre-clinical animal models divide at a much faster rate than in vivo (some radiological studies have identified tumour doubling times to be ~100 days in some tumours), therefore some genes identified by screens may not be as important in vivo as the tumours do not divide as rapidly(461).

Another criticism of RNAi screening is that results derived from different screens can be variable, for example overlaps in RAS synthetic lethality screens are ~75%. (417) This is partly because there is an inherent variability in knockdown efficiency with rates being between 70-90%.(417) There is also the possibility of a false positive rate with siRNA depletion due to off-target effects(417), however results from the screen performed in the
study described in this chapter did account for off-target effects. There is some controversy regarding the best mechanism for in vitro post-transcriptional silencing. The screening library used a single oligonucleotide to knockdown genes, whereas subsequent experiments used pooled siRNA due to the lack of sensitivity of the PLK1 oligonucleotides in the screening library. The advantage of using pooled oligonucleotides is that by using three or four siRNA there is a higher percentage of silencing. The siRNA in the pool target multiple areas on the target mRNA, thus there is less of a chance of missing a target due to poor annealing secondary to differences in sequence due to mutations, polymorphisms and splice variants. (471) Pooled treatments are believed by some to have decreased off-target effects as less toxicity is seen when using individual siRNAs as off-target effects are believed to be concentration dependent. (471) Although others believe that off-target effects are greater in pooled systems compared to single systems. (471) Use of single siRNA in the library was the correct choice as each gene was interrogated by three siRNA reducing the chance of having too many false positive results. A possible criticism of this study is that for many of the studies, as with many other studies of this type, was that on one oligonucleotide was used. In part this was because of resource issues and also the desire to utilize the best oligonucleotide detected from the screening data. In addition, it can be argued that using a single oligonucleotide would be associated with fewer off target effects. Nevertheless, this is a limitation of this work, as this oligonucleotide may not have recognized STK-10 and RYK in all settings and some synthetic lethal interactions may have been missed. However, in an analysis of data published from siRNA libraries, Bhinder and Djaballah indicated that pooled siRNA libraries may be less reliable than single siRNA well experiments because 80% of the ‘hits’ described by these libraries were only seen in pooled libraries suggesting some of the effects may be due to off-target effects, or multiple integrations per cell. (460)
At the time of this study, the siRNA library approach was the most commonly used method available for post transcriptional gene silencing. However, newer methods of genome editing are being more established in the last few years. They include CRISPR-Cas and TALENS which enable genome editing by directing oligonucleotides to the gene of interest and splicing in an altered nucleotide. Although these techniques could not be used in high throughput screening method, they could be used to examine synthetic interactions in cancer cell models with the gene knocked down. CRISPR enables multiple genes to be silenced in the same cell and thus could be a very useful tool in modelling lethal interactions. Furthermore, it can facilitate in vivo research for example making it much quicker and easier to make knockout mice.

Other potential criticisms leveled at synthetic lethality screens is that they are frequently poorly reproducible, even when the same library is used on the same model. This can be due to differences in technique due to small volumes involved. There are also differences in the screening techniques used in different studies with different thresholds deemed as significant. At the time the screen was performed there was no gold standard regarding the best filtering method. Most published studies used a similar method to ours involving evaluation of fold changes in viability. A decision was made to select candidates from the screen used in this thesis using the relative effect on fold change. This was due to concerns that a statistical analysis based approach at the preliminary stage might lead to a high number of false negatives and thus potential candidates would be overlooked. For example STK-10 which was identified as a candidate using fold change criteria and then validated by demonstrating statistically significant changes. Nevertheless, in retrospect, it would have been interesting to compare the results of candidate selection using fold-change to those using
a statistical analysis based approach. This would have been best achieved using an automated, high throughput system, this would have enabled large numbers of replicates to be undertaken and allowed more robust statistical analysis.

This methodology may have meant ‘good’ candidates were overlooked and weaker genes could be chosen. This is exemplified by the fact that PLK1 was not the gene associated with the ‘best effect’ on cell proliferation in the lethality assay performed by Ding and colleagues, but was chosen for study due to corroborative data from gene expression arrays (437). Thus, in order to avoid ‘missing’ candidate genes it may have been better use an integrated approach including other available data to identify genes of interest. There are also differences in the ‘hit rate’ dependent on whether screens are genome wide or focused on specific genes of interest. The study described in this chapter used a targeted screen evaluating kinases. Targeted screens are more commonly used and were chosen in this study because of the desire to identify ‘druggable’ targets. A criticism of this approach is that in an analysis of published results, more hits are derived from focused screens indicating weaker hits may be highlighted.(460)

Another difficulty with using an siRNA approach is that it is difficult to block genes using siRNA in vivo as intracellular delivery of siRNA is difficult, requiring conjugation of the siRNA with a nanoparticle or liposome.(34) Although nanoparticle delivery vehicles for siRNA are being investigated in murine models(473) this technique has inherent difficulties. Thus, siRNA may be best used as a screen and once a lethal interactor is identified, translational studies could involve requiring mechanisms of inhibition such as small molecule inhibitors.(418) However, as described in 5.8.7 small molecule inhibitors can have multiple
targets leading to different results to siRNA knockdown. Defining inhibitors can be difficult; for example, iniparib was used in clinical trials as a PARP inhibitor but was later found to be a very weak inhibitor of PARP.\(^{474}\) Furthermore, it has been noted that ‘targets’ identified by academic institutions do not necessarily correlate with results achieved when the same target is knocked down by using inhibitors in industry.\(^{427}\) In order to verify targets better perhaps more functional work should routinely be performed for example by re-introducing the gene identified by the screen or to produce RCC by injecting cell lines into nude mice and evaluating the tumour growth in the presence of siRNA against the gene of interest compared to a control. These techniques though commendable are time-consuming and are not feasible for most targets identified.\(^{427}\)

5.11 Conclusion
The functional genomics approach utilized in this chapter was used to identify possible synthetic lethal interactors with pVHL. Although there are limitations to the technique of synthetic lethality, it still plays a role in candidate gene identification and determining pathways involved in cancer. The main novel candidate that was established by this study was STK-10. This gene was also identified by a siRNA screening library evaluating Ewing’s Sarcoma. It interacts with the PLK1 gene which is a key gene in the control of mitosis. PLK1 was identified by another screening library using a different methodology to have an important effect on kidney cancer growth.\(^{437}\) Thus, this work supports the currently available data that suggests the STK-10/PLK1 pathway is a key pathway in RCC. This indicates further work should be performed to evaluate this pathway in RCC in order to study novel therapeutic strategies.
CHAPTER 6. DISCUSSION

6.1 A summary of the work presented in this thesis
There is no doubt that cancer genomics is the future of cancer management; influencing
diagnosis, treatment and monitoring of disease. Developments in cancer genomics have been
rapid with many changes in the field and key developments occurring during the period of this
research.

At the time the work in chapter three of this thesis was performed, most clinical genetic
testing took place using Sanger sequencing. Each gene was tested sequentially which was
both costly and time consuming. Therefore, it was important to identify whom to test for
genetic predispositions due to resource implications and in order to manage patient
expectations. Given the time taken and the costs involved to sequence a gene for mutations it
is important to identify which genes to investigate. This thesis described the mutation
prevalence of SDHB, SDHD and VHL in phaeochromocytoma/paraganglioma/HNPGL within
a UK-based referral cohort with for the first time. Thirty-one percent of the cohort with PPGL
had a mutation in one of these genes. The likelihood for testing positive for a genetic
mutation was increased by having a positive family history, multiple tumours, or extra-
adrenal paraganglioma. Amongst those with HNPGL, sixty-three percent had a mutation in
these genes, this proportion was higher in those with a positive family history and multi-
centric tumours. However, 28% of those with a single sporadic phaeochromocytoma had a
mutation in SDHB, SDHD or VHL and 34% with a single, sporadic HNPGL had a mutation.
This suggested a relatively high mutation rate in individuals with low clinical risk factors for
genetic predisposition. ROC analysis evaluated different age cut offs for testing in those with
single, sporadic phaeochromocytoma, and found that even when evaluating all of those aged
under 60 years of age, 23% of mutations would be missed. This suggested that more comprehensive cheaper testing strategies should be utilised. This includes use of NGS to evaluate multiple phaeochromocytoma associated genes simultaneously either by using a multiplex testing or WES. Both of these techniques are being evaluated in the diagnostic setting (205, 206). At present universal testing of all individuals with phaeochromocytoma is not the standard of care in the UK, however as the price of genetic evaluation and time taken for analysis fall this may well change.

The next theme studied in this thesis evaluated the identification of new CPG in familial RCC. Studies of familial kidney cancer are the exemplar of how studies in rare diseases such as VHL can lead to improvements in the understanding of cancer biology and consequently in the treatment of sporadic kidney cancer. Thus, two different approaches were used to evaluate new familial RCC associated genes. In a hypothesis driven approach, the gene PTEN was evaluated in familial RCC cases. This gene is known to be associated with Cowden syndrome which is associated with benign hamartoma, breast cancer, endometrial cancer and uterine cancer. PTEN has also been found to be mutated in 3% sporadic RCC (319, 475). In the cohort of individuals with a family history of RCC, no germline mutations were identified in this gene. Recent developments in this field have led to the identification of PTEN mutations in individuals with papillary RCC associated with Cowden syndrome (346, 369). This suggests that PTEN mutations are associated with RCC in individuals with some syndromic features of Cowden syndrome. The second approach used in this study was to use a hypothesis generating approach by performing an unbiased evaluation of WES in familial RCC patients. For the first time, a truncating mutation in the known tumour suppressor CDKN2B was identified in a patient with familial RCC. The mutation segregated between
family members with RCC. Three further missense alterations were identified in the gene and they were predicted to influence protein function using *in silico* tools and computer based NMR modelling. The tumourigenic potential of these missense variants was confirmed by performing colony formation assays. This work suggests that *CDKN2B* mutations may be significant driver mutations in a subset of familial RCC patients. *CDKN2B* mutations in this population require further evaluation in a larger cohort of patients (for example using NGS) to determine their prevalence. Furthermore, phenotype-genotype correlations and supporting data from other genomic studies may enable *CDKN2B* associated tumours to be further characterised. Potentially, this may lead to different treatment strategies directed towards *CDKN2B* associated tumours such as has been proposed for *SDHB* associated malignant phaeochromocytoma. In particular it might be predicted that CDK4 inhibitors such as palbociclib(476) might be appropriate for *CDKN2B* mutation carriers with advanced RCC as *CDKN2B* (p15) is an inhibitor of CDK4(477).

The final theme of this thesis was to identify potential novel therapeutic approaches for RCC using a functional genomics approach. A synthetic lethality screen was used to evaluate VHL+/− isogenic cell lines. This screen identified three targets of which one was chosen for further evaluation. This study demonstrated that STK-10 depletion was associated with decreased viability of VHL inactivated cells. STK-10 depletion has also decreased the viability of Ewing’s sarcoma cell lines.(99) The STK-10 protein has been reported to be associated with PLK1 and this protein has previously been identified as being associated with RCC in other screens (437). The effect of STK-10 depletion seemed equivalent to PLK1 depletion in this experimental model. Data from a protein expression array suggested that depletion of PLK1 and STK10 in VHL positive cells had a similar mechanism of action,
whereas the pathway may be different in VHL inactivated cells. PLK1 and STK10 represent novel therapeutic targets in RCC, in fact early clinical studies are currently underway using PLK1 inhibitors (478), although the efficacy of PLK1 inhibitors as single agents may be limited. (479) Work in this thesis suggests that there is a strong case for further investigation of targeting the STK10/PLK1 pathway as a potential synthetic lethal approach to the treatment of pVHL-deficient RCC.

6.2 Stratified medicine in the era of genomics
The management of cancer is undergoing a paradigm shift, diagnosis and treatment are moving away from being based on phenotype and are increasingly being determined on genotype. This change from phenotype to genotype is being incorporated into stratified medicine which is defined as a process where interventions either pharmacological or non-pharmacological are targeted to a subgroup of patients according to biological or risk characteristics. (480) The basis of ‘stratified medicine’ is to identify individuals in whom treatment will provide the most benefit and/or least harm. (480)

The simplest model of this is that described in chapter three of this thesis, where individuals with known genetic propensity to a condition are diagnosed with a genetic aberration. They then undergo a specific screening regimen and treatment for the associated condition. In sporadic cancers one of the challenges to personalised medicine is the tumour heterogeneity. Within in any tumour there are subclones with different genetic mutations, this can lead to differential responses to treatment and resistance occurring (56).
6.2.1 Intratumoural heterogeneity

In this thesis, the research performed focused mainly on the investigation of familial RCC. In familial cases, the genetic aberration is ‘pure’: the germline mutation is present in normal cells and (for TSGs) inactivation of the wild-type allele (by mutation, loss of heterozygosity, etc.) initiates carcinogenesis. Subsequently other mutations are acquired and these may be present in all tumour cells or only some (depending when in tumour evolution the mutation occurred). However, the majority of cancers are sporadic and in these cases even if a TSG is found to be inactivated it may be unclear whether or not it the initiating event (and so will be present in all tumour cells) or occurred later in tumour evolution. There is increasing evidence that for most cancers there is extreme complexity in the genetic picture (481) TCGA Research Network’s integrative analysis into different renal cancers suggested that the tumour landscape is complex with a high level of heterogeneity between tumours.(312)

Driver cancer mutations were found in only a subset of cells within a RCC tumour. It is believed that mutations form a ‘trunk and branch’ pattern analogous to a phylogenetic tree (307, 470). In a study of sporadic renal tumours from ten individuals, nine clear cell carcinomas had VHL inactivation by mutation and one had inactivation by methylation(307). VHL was suggested to be the key truncal mutation, but overall 67% of mutations were not present in all areas of the tumour and there was heterogeneity even in tumour samples from patients who were treatment naïve. This suggests that during the evolution of the cancer, some cells gain different mutations and therefore are separated in both time and space. In order to explain this phenomenon easily, some have proposed a ‘mountain and hills’ analogy. Mountains represent mutations present in most cells of a tumours whereas hills are mutated in only a few cancer cells. The mountain mutations are similar to truncal mutations and, when selecting genetically-guided therapies, these should therefore be focused upon.(482)
After *VHL*, *PBRM1* was the second most common ‘truncal’ mutation identified. Of the six tumours with a *PBRM1* mutation, the mutation was ‘truncal’ in three cases. This indicated that in some tumours *PBRM1* mutation is an early event. Other mutations in ‘driver’ genes were seen in subclones, for example *TP53* and *BAP1* mutations. Within tumour subclones, parallel evolution occurs with different genetic mutations. However, within the evolution of tumour subclones, there are some gene types which are preferentially mutated notably alterations in genes involving the SWI/SNF chromatin remodelling complex (*SETD2, BAP1, PBRM1, ARID1A* and *SMARCA4*). Another gene *AHNAK* is believed to be involved in chromatin remodelling and was found to be a key mutation by another group investigating RCC. The convergence on this gene set indicates the importance of this process in RCC development. Interestingly, the mutations seen in the CpG islands were more commonly C>T transitions and the branch mutations had fewer A>G mutations compared to truncal mutations. Xu *et al.*, confirmed C>T transitions were commonly seen in RCC, particularly in driver and rare mutations. Interestingly, common mutation sites had increased transversion mutations. Gerlinger *et al.*, suggested that this may mean that a specific process leads to these mutations taking place. Such processes may be particular to renal cancer as in breast cancer CpG islands in fact have lower C>T transitions.

As *VHL* represents a ‘trunk’ mutation being present in most cancer cells and therefore it seems appropriate that current therapies are directed towards these truncal mutations e.g. targeted treatments associated with increased survival in RCC are directed towards *VHL* (multi-targeted tyrosine kinases and bevacizumab). As chromatin remodelling is suspected of importance in RCC oncogenesis, this pathway represents a good target for further drug development. Despite most available evidence indicating the key roles of *VHL* and *PBRM1* in
RCC development, single cell exome sequencing (482) has revealed that some tumours lack these mutations (although VHL inactivation by methylation was not studied in this patient). To further complicate matters, it has been hypothesized that some ‘truncal’ mutations may not always remain truncal as after treatment some subclones may not be dependent on the ‘truncal’ mutation i.e. resistance occurs.(483) In colorectal cancers, there is intra-tumoural heterogeneity within driver genes (KRAS, BRAF, and PIK3CA) in at least 1-8% of cases suggesting heterogeneity even in some early ‘truncal’ mutations(484). Owing to this diversity, cancer treatment may require multiple agents to target different pathways as has been exemplified in melanoma (see section 6.2.3).(66)

6.2.2 Technical issues regarding sequencing of tumours
As analysis of cancer genomes becomes routine clinical practice there are some technical aspects of NGS to consider. Currently, the systems in place for sequencing mean that the turnaround time for genomic analysis from sample taking to result is four to eight weeks, this is too long for a patient to wait before starting treatment.(483) It is hoped that this will be improved to a more acceptable two weeks. As analysis becomes standard of care, clear standards are required regarding the depth of sequencing so that the number of false positives (usually associated with limited depth) and false negatives (associated with poor sensitivity) are minimised(483). In addition, sensitive and cost effective methods for detecting copy number abnormalities require development. There may also be a need for a change in methods used for the pathological evaluation of surgical specimens. Most samples are fixed in formalin and paraffin embedded. DNA and RNA from formalin fixed specimens are of a poorer quality than that derived from fresh (or fresh frozen) tissue. Furthermore, tumour samples are frequently ‘contaminated’ with normal tissue, this makes it harder to determine heterozygous
changes which may represent only 25% of a sequencing bandwidth rather than 50% in a
germline sample as normal tissue contaminating the tumour contains two normal alleles with
the tumour containing 1 normal allele and one mutated allele.(483) Although laser
microdissection can reduce contamination, it is time consuming, can damage nucleic acids
and yield low levels of DNA, thus further work is needed to improve this technique or better
bioinformatics support may reduce the chances of changes being ‘missed’. It is likely that if
sequencing of solid tumours is going to become the norm, in order to account for
intratumoural heterogeneity, deep sequencing of driver genes associated with the hallmarks of
cancer will be required to detect changes in key genes.

6.2.3 Sampling of tumours to determine mutational complement
Even though stratified medicine is currently in its infancy, there are indications that treatment
with targeted agents may prove to be more complicated than initially believed. *BRAF* was the
first oncogene identified by the human genome project in melanoma.(64) Treatment of
melanoma patients with the *BRAF* inhibitor vemurafenib in *BRAF* mutation positive patients
has been associated with resistance in 20%.(466) Multiple mechanisms are responsible for
this such as amplification of mutant *BRAF*, expression of truncated *BRAF*, mutations in MEK
and RAS, loss of PTEN and hyperactivation of the PI3 kinase pathway.(466) Thus, as
tumours may develop further mutations and there is significant tumour heterogeneity.
Archival tissue specimens may not provide the most appropriate source of genetic information
for evaluation. In leukaemia, cancer cells are easily obtainable; however in solid tumours
repeat biopsies may not be feasible or accepted by patients. Given the increasing number of
treatment lines available to patients there may be a need to analyse the genomics of a patient’s
cancer on numerous occasions. Furthermore, there may be a need for multiple biopsies at the
same time as different metastases may represent different tumour clones. One possible method to overcome this complication is the possibility of a ‘liquid biopsy’.

One possible form of a ‘liquid biopsy’ is using circulating tumour cells (CTC). These are tumour cells that circulate freely in the blood of patients, these can be derived from primary tumours, metastases or recurrences. They can be shed from small localised tumours and can be associated with a poor prognosis.(485) These can now be efficiently captured using high throughput systems based on the expression of a marker on their cell surface for example epithelial cell adhesion molecule. One such system the Cellsearch, has been approved by the FDA to allow monitoring of CTC in metastatic breast and prostate cancers.(486) CTC from treatment naïve small cell lung cancer patients can be placed into immunodeficient mice and produce tumours typical of the small cell lung cancer in the patient. The tumours in mice were also treated with chemotherapy and had a similar response to that seen in the patients from whom they were derived. Thus, CTC represent a potential new model for drug discovery as CTC explants can be treated with a variety of agents to determine new more effective treatments.

Another form of liquid biopsy is the use of circulating tumour DNA. This DNA can be derived from cancer cells that die in situ or as part of a dissemination cascade.(484) Analysis of plasma free DNA is particularly attractive as it represents the entire tumour genome mixing together variants originating from multiple independent tumours obviating the effects of tumour heterogeneity.(487) Sequencing of free plasma DNA could be used to diagnose cancer therefore reducing the risks of surgical biopsy.(488) In individuals with a high tumour load,
i.e. advanced metastatic disease, deep sequencing of plasma free DNA may allow assessment of clonal heterogeneity and drug selection (487). Free DNA could be sequenced to identify any resistance associated mutations, potentially this could aid treatment decisions as if a mutation is detected that confers sensitivity to another agent, the alternative agent could be substituted into treatment regimes. Furthermore it could be used to detect disease recurrence.

6.3 A potential model for personalised medicine and stratified medicine in oncology

Personalised medicine in oncology is increasing, the “Mainstreaming cancer genetics programme” is currently piloting ways in which inherited cancer genetic testing can be expanded to many more cancer patients and is using an assay that analyses simultaneously 94 inherited cancer genes (‘Illumina TruSight Inherited Cancer Gene Panel’) (489). The future plan is to roll out genetic testing in all cancer patients. Conceivably, oncology may represent the first model of personalised genomic medicine with genomics being involved in cancer screening, prevention (including chemoprevention e.g. aspirin use in colorectal cancer patients) and treatment (67). It is highly probable that NGS technologies will enable a transition from morphology based taxonomy to a genetics based taxonomy of cancer (490). One possible model is that a familial genetic mutation could be identified from NGS of a primary tumour and paired germline DNA. Members of the proband’s family could then be screened for the genetic aberration. If prophylactic treatment is possible for example risk-reducing oophorectomy or mastectomy in BRCA mutation carriers, this could be offered to relatives who are found to be mutation carriers and the proband would receive treatment targeted to his/her cancer mutation taking into account the patient’s pharmacogenomic profile. Subsequently, the patient could receive further tailored treatment in the case of relapse after
genomic reassessment of the relapsed cancer. Similarly, as described in chapter three of this thesis, molecular taxonomy of a tumour e.g. phaeochromocytoma can direct treatment.

Precision oncology (described in figure 6.3) is where treatment decisions are based on the genetic characteristics of a tumour. The principles behind precision oncology have already been evaluated in the research setting. Plasma DNA has been sequenced from patients with breast cancer and activating mutations had been identified in $PIK3CA$ which was associated with resistance to paclitaxel. Another patient was found to have a nonsense mutation in $MED1$ which was associated with tamoxifen resistance, the patient was then switched to capecitabine and lapatanib. The patient then went on to have a splicing mutation in $GA56$ which was associated with lapatinib resistance. Thus, there is proof of principle that serial sequencing of plasma DNA can determine the aetiology of chemoresistance, potentially this can lead to more timely alteration of treatments thus reducing morbidity. Similarly, personalised medicine could be used to identify which patients would benefit from which type of adjuvant therapy. Currently, only a minority of patients benefit from adjuvant therapy (i.e. 4% of lung cancer patients) and thus the majority receive treatment with associated toxicities for no benefit. NGS technology may be able to predict which patients would benefit from treatment, thus maximising gain and reducing harm. Currently plasma free DNA represents a promising method of identifying genetic markers associated with disease recurrence or potentially progression. The proof of principle of this technique has been achieved in genetic screening for aneuploidy, where NGS has been performed on foetal DNA present in the maternal bloodstream. The potential benefits of using plasma free ctDNA have been described above.
Figure 6.3. A model for genomics driven oncology. Fresh tumour tissue from individuals with newly diagnosed cancer is evaluated using NGS technology in order to determine the tumour’s molecular taxonomy and actionable targets. Appropriate targeted treatment is instigated based on genomic data. Treatment response is determined using iterative processes such as analysis of plasma DNA in addition to use of clinical and radiological parameters. Upon relapse or disease progression, further genetic analysis is performed and new treatment decisions instigated. Then treatment response and genetic re-evaluation is required. (Adapted from Garraway.)
6.4 The future of personalised cancer treatment

It is likely that genomic analysis in cancer will become the norm in the ensuing decades. The precise technology used may well differ from the NGS technologies used in this study, as further technological advances may well enable quicker more portable testing using “bench top” NGS analysers (e.g. Oxford Nanopore) as described in section 4.7.2. However, once an individual’s cancer genome is characterised, annotation is required in order to enable treatment choices to be made. The simplest method is to categorise cancers into subtypes based on tumour characteristics. Different subtypes will require different treatments. In clinical practice, chemotherapy and targeted treatments are subject to vigorous investigation and testing prior to being instituted routinely into patient care. The same prospective evaluation should be pursued with regards to genetic testing. The organisation of cancer research within the National Cancer Research Network has enabled evaluation of genetic testing to take place under the auspices of the CRUK Stratified Medicine Pilot (493). This is collecting clinical data in addition to looking at the efficacy and cost-effectiveness of molecular evaluation of cancers. As a follow on from this study, in the phase II prospective National Lung Matrix trial, compounds in Astra Zeneca and Pfizer’s targeted treatments library will be evaluated in individuals based on the molecular characteristics of their lung cancers. These types of trial, so called ‘basket trials’ enable simultaneous evaluation of multiple drug combinations in different patients based on specific genetic profiles in their tumours. They have an adaptive design which should help provide new targeted treatments to patients and should further improve disease outcomes (41).

Recently, metastatic lung adenocarcinoma specimens were genotyped for oncogenic drivers. This revealed 64% of patients had a mutation in an oncogenic driver and survival was
statistically longer for those who received treatment directed towards the driver compared to those who did not (HR 0.69 (95%CI 0.53-0.9). (494) Disease causing genetic mutations may only occur at a low frequency making it difficult to perform randomised controlled trials in this setting. Therefore, it is key to establish integrated clinical and research networks which will enable patients to be recruited to appropriate clinical studies. Similarly, particularly in the drug discovery phase different trial endpoints such as impact on a cancer pathway should be considered instead of or in addition to traditional markers of efficacy such as PFS and OS. (483)

Personalised medicine may also have a role to play in the early diagnosis of cancers. Early diagnosis leads to cancers being detected at an earlier stage and thus being more amenable to curative treatment potentially improving survival (495). Thus, DNA testing may be able to detect early disease. DNA testing has already been successfully performed on faecal DNA for colorectal cancer associated genes. It was found to be more sensitive than conventional faecal occult blood testing. (496) A similar technique could utilise cells found in urine, providing a non-invasive monitoring mechanism for RCC focusing on renal associated genes facilitating early diagnosis. Furthermore, improvements in DNA testing technology may be able to determine recurrence in the case of patients treated with partial nephrectomy.

6.5 Ethical considerations in genome testing
If population based genomic screening strategies do become the norm in the future, which groups such as the UK Mainstreaming Cancer Genetics Programme (www.mcgprogramme.com) aim to facilitate, there are number of themes regarding genome
testing which require consideration. Firstly, as more CPG are identified it is likely that an increased proportion of the population may harbour a germline mutation in a CPG. To date, there is limited evaluation of testing strategies which in addition to an inherent cost as discussed in chapter three also have further costs in terms of screening strategies associated with their detection. Rahman noted that CPG are frequently not robustly evaluated and can be involved with over-diagnosis, misdiagnosis and a lack of efficacy. (325) Furthermore there are additional ‘costs’ to the individual with the variant who is ‘medicalized’ and has to invest emotional and financial resources into the surveillance process. Diagnosing a new variant in a proband also results in a cost to family members who may require screening and clinical management.

Currently, there are no clear guidelines regarding whom to test in the UK, there is also no definitive advice regarding what to do in the case of incidental findings or with variants of unknown significance. In a exome sequencing study of individuals with ‘benign’ conditions (largely neurodevelopmental phenotypes), 25% of those investigated obtained a new molecular diagnosis. (497). However, 30 of 250 patients had an actionable mutation in an incidental gene. The American College of Medical Genetics (ACMG) has prepared a list of fifty-six genes which should be reported to patients if a known pathogenic or potentially pathogenic mutation is detected. (498) This list includes twenty six CPG (499). The ACMG has also provided a list of gene variants which should not be disclosed. The main types of variants which should be disclosed are those which are accurate, actionable and pathological (500). However, some genetic variants cause different phenotypes (this phenomenon is called pleiotropy) some of which are actionable whilst others less so, for example, alterations in APOE influence cardiovascular risk (an actionable risk) and also
Alzheimer’s disease (unactionable) (500). At least 17% of variants display a degree of pleiotropy(500). Management of the pleiotropy in genetic variants is yet another aspect of genetic testing which will need to be clarified in the era of NGS.

Due to the amount of data available from NGS, the consent process individuals undergo for testing must also change. Currently individuals participating in the UK 100K genomes project only have genetic changes associated with their ‘reason for testing’ reported back to them. However, this maybe ethically difficult and in the US model patients undergoing testing for cancer genes using NGS may also find out about other inherited conditions (unless the patient opts out) and therefore this should be explicitly mentioned in the consent process.(67) Furthermore, although this is perhaps less relevant in the cancer setting, children may be informed of adult-onset conditions if it is deemed that the variant may impact on the child’s or parents best interests unless parents have opted out. This differs from the current doctrine of clinical genetics where traditionally children are not tested for adult onset disorders.(501) Furthermore, patients will need to consent to being contacted in the future if further genetic discoveries identify new ‘actionable’ genes.

There is also no current clear methodology for the evaluation of variants of uncertain biological significance, i.e. non hot spot mutations where it may be difficult to determine whether an alteration is associated with a functional change, this means that these changes may not be used to make clinical decisions. As NGS becomes more common there may be more such variants identified in cancer genes and this increases uncertainty for clinicians and patients alike. Strategies to aid with this uncertainty include The NIH ClinGen project which
is published on the ClinVar website (502), this is beginning to document the clinical significance of genetic variants and the Precision Medicine Initiative aims to develop a 1 million person cohort which may help clarify the significance of some variants (503).

As genetic screening in cancers becomes more ‘mainstream’ most testing models currently being evaluated involve oncologists counselling patients for genetic testing and patients seeing genetic counsellors and/or clinical geneticists only if they have a positive test. Thus, far this model has been successful in two large UK cancer centres (Edinburgh and the Royal Marsden) (203), however it is not clear how some individuals and their families will cope when they are found to harbour CPG. Furthermore, if cancers are sequenced, the associated germline DNA should also be sequenced. This may result in the identification of more incidental findings which may not be related to cancer. Thus, unexpected results could cause further distress to patients and their families. (499) Potentially, unexpected genetic results can impact on life and income insurance applications. (504) Proponents of genomic medicine suggest it will lead to cost savings as treatments will be bettered tailored however there are some upfront economic costs such as data-storage and other infrastructure costs (505).

Furthermore, the genomics revolution has identified that alterations in genes due to mutation alone are not pathogenic. Increasingly, the role of copy number changes and epigenetic silencing play important roles in cancer development. Thus, cancer genome sequencing and germline DNA sequencing may lead to false reassurance regarding the risk of cancer development. It may well be that a more complex functional genomics approach will be required in the evaluation of both inherited and acquired malignancy. Given that even with
the current amounts of data available from sequencing projects, bioinformatics support is limited, if more comprehensive genomic evaluation is performed a considerable investment must be made in order to ensure data can be appropriately interpreted. Furthermore, data storage is an issue with the quantities of data involved meaning that investment in secure storage including cloud storage is required.(506)

In the UK in 2011 there were 209 consultant clinical geneticists (507) therefore is not feasible that each cancer patient is seen by the genetics service. Thus, clear guidelines and interpretation tools are required so that clinicians can guide patients regarding risk and treatment options. Tools for management of adjuvant treatments already exist, for example the Oncotype Dx gene expression array has been approved by the National Institute for Clinical Excellence in the evaluation of whether individuals with ER positive, Her-2 negative and lymph node negative breast cancer should receive adjuvant chemotherapy.(508). Some databases have been developed by academic centres in the US in order to facilitate clinical decision making for example My Cancer Genome(509), other centres have developed ‘Sequencing Tumour Boards’ where individuals with expertise of cancer genomics, bioinformatics, pathology, clinical genetics, bioethics, clinical oncology and molecular therapeutics discuss cases and triage patients for entrance onto clinical trials.(483)

6.6 The challenges of personalised medicine
In the foreseeable future most experts believe that the multinational effort to sequence cancer samples will lead to the production of a comprehensive list of cancer causing mutations.(490) The challenges to overcome before this achieved include two main themes: (i) how to analyse the data (i.e. bioinformatics) (ii) how to validate the findings (i.e. functional annotation).
Bioinformatic analysis requires a combination of mathematical, computational and biological expertise (490). This requires a financial and time investment in order to make cancer genomic information accessible. Currently, cancer genomic information is utilised by researchers and cancer geneticists. However, genomic information will need to be accessible to individuals with less genomics training for example oncologists, other clinical specialists and pharmacists. ‘Translation’ of scientific data into ‘user-friendly’ data will require commercial input to improve interfaces so that accessing genomic data will become an easy routine means of making clinical decisions such as that seen with OncotypeDx.

The bigger challenge facing genomic medicine is to determine the functional effect of each variant. There are numerous functional assays some of which have been described in chapters four and five of this thesis. These are limited in their ability to evaluate a single cancer associated feature and although in vitro assays tend to be more predictive than in silico methods, such assays are time consuming, laborious and costly(490). An investment in resources from the major funding bodies is required to help improve systems available for functional annotation so that the functional effect of genetic variants can be determined in a timely, consistent and accurate manner. Similarly, determination of the mechanism of action a cancer causing mutation is essential in order to derive some therapeutic benefit from its discovery. The influence of a mutation on cellular function is even more complicated than determining functional significance of variants. The translation of recently identified mutations such as V600E BRAF into clinical treatments has been based on years of basic science research on the RAS signalling pathway. Therefore, although there has been a commendable and understandable drive towards translational biological research, in order to understand the significance of genetic mutations basic scientific research into how these key
genes ‘fit in’ to complex intracellular signalling networks is essential. Historically, determination of the function of genes has been non-directed, slow and scattered in time and place. The success of the human genome project and subsequent projects such as 1000 genomes, TCGA and COSMIC has been based on a coordinated, multicentre, multidisciplinary approach. In order to harness the findings of cancer genomic projects to improve clinical care, a similar approach needs to be adopted regarding functional annotation and elucidation of the mechanism of action.

Understandably, the era of genomic oncology is associated with a significant degree of optimism in clinicians, patients and even governments. However, as cancer is described as being ‘endlessly complex’(510) even when data regarding which actionable targets are available results can be disappointing. For example the MOSCATO-01 trial which used WGS and comparative genomic hybridization data to identify targeted treatments in metastatic cancer, targeted agents were associated with a 20% partial response rate and a 56% stable disease rate.(511) These results exemplify the complexity of cancer management. It is likely that only time, experience and better understanding of cancer genomics will lead to better outcomes. Some have argued that this may be achieved by analysing bigger data sets and basing clinical decision making on practice based data rather than idealised trial or ‘evidence based data’. However, this would require a significant investment in integrated IT facilities. Furthermore, there may need to be a re-evaluation of trial design from the traditional gold standard of a randomised controlled trial. These types of trial require a large investment in time and money and in one analysis 62.5% of all trials between 2005 and 2009 were negative.(512) Therefore, proof of concept studies may be more useful. These are based on precise biological understanding of a pathology, e.g. \textit{EML4-ALK} fusion in advanced
lung cancer and appropriate targeted drugs being used in this setting e.g. crizotinib. Clinical efficacy can be determined as can the influence on the target cells, if a suitable response occurs further drug development can take place, i.e. such trials provide “go/no-go” decisions. (512)

6.7 Concluding remarks
The cancer genomics revolution chronicled in part by this thesis commenced with the sequencing of the human genome sequence by a concerted international collaboration. The early success of cancer genomics has been achieved by building on basic scientific knowledge for example on the cell signalling pathways achieved by over decades e.g. funding derived from ‘Nixon’s war on cancer’ (510). This has revolutionised the clinical management of many patients with cancer for whom previously there was no effective treatment. In the next decade or so it is likely that cancer genomics will alter the way cancer is described, treated and managed. It is likely that this era will herald new drugs and treatments. However, cancer is not one disease, and the heterogeneity of cancer cells within and between individuals mean that the identification of new cancer associated genes and consequent associated treatments may not be easy. As with the sequencing of the genome, a collaborative approach in basic science, pre-clinical trials, clinical trial development and clinical practice is required. This will enable a pooling of knowledge and resources in order to interrogate data which is likely to be increasingly complex. Furthermore, improvements in data-analysis, data storage and interdisciplinary working are required. Such a concerted action may allow cancer to become a controllable chronic disease rather than a terminal condition, only time will tell whether this is achieved.
Appendix 1: Constituents of buffers/reagents used in this thesis

**PBS:** 1 tablet of PBS (Gibco) added 100ml of ddH2O. To sterilise autoclave.

**1x TE buffer:** 10ml of 1M Tris HCl pH8, 2ml of 0.5M EDTA pH8, 988ml ddH2O

(Final Concentration Tris HCl 10mM, and EDTA 1mM)

**Loading Dye:** 45ml glycerol, 34ml H2O, 1ml of 0.5M EDTA, 0.125g orange G

**Precipitation Buffer:** 1:1 mixture of 3M sodium acetate and 0.5M EDTA pH 8

**LB Broth:** 1l of ddH2O was added to 200g of LB broth powder (Sigma) this was gently agitated and then autoclaved prior to use.

**LB Agar:** LB Agar was produced by adding 16g of LB Agar powder (Sigma) to 400ml of ddH2O. This was mixed by gentle agitation and then autoclaved.

**Kanamycin:** Kanamycin (Sigma, Poole, UK) was prepared in a fume cupboard at a stock concentration of 50mg/ml, aliquotted into 1ml eppendorfs and stored at -20°C.

**RIPA Buffer:** 5ml 1M Tris pH8, 5ml 3M NaCl, 1ml 10% SDS, 200µl 0.5M EDTA 5ml 10% deoxycholate, 10ml Igepal, 73.8ml of ddH2O. For every 10 ml of RIPA buffer, one protease and phosphatase inhibitor tablet was added (Roche Applied Biosciences). The buffer was then aliquotted in 1ml eppendorfs and stored at -20°.

**Protein Loading Buffer:** 940µl of 10% SDS, 470µl 1M Tris-HCl pH 7.5, 95µl 100nM DTA, 2.45µl glycerol, 545µl ddH2O, 205µl mercaptoethanol and 0.001g of bromophenol blue. The mixture was filtered and aliquotted prior to use. Loading buffer was stored at -20°.

**PBS-Tween:** 10 PBS tablets (Oxoid Limited), 10 ml of Tween (Sigma) in 10l of ddH2O
Appendix 2: Primers used in this thesis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Exon</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDKN2B</td>
<td>1</td>
<td>AAGAGTGTGTTAAGTTTAGGTTAAGGA</td>
<td>ACATCGGCAGATCTAGGTTC</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>TGAGTTAAGCTGAAGGTGGGGTG</td>
<td>CTTACCCAATTTCCCAACCC</td>
</tr>
<tr>
<td>PTEN</td>
<td>1</td>
<td>AGAGGCATTTCCATCTGCAGA</td>
<td>ACGTTCTAAGAGAGTGACAGAAAAGGT</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>TAGTGGGAAAATCTTCTTTCTTCTACAATA</td>
<td>ATCTTTTTCTGTGGCTTAGAAGATCTTTTC</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>CCATAGAAGGGGTATTGTGTCG</td>
<td>ACTCTACCTCACTCCTAACAAGCA</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>ATTCAGGCAATGGTTTTGATGTTATGG</td>
<td>TACAGTCTATCGGGGTTAAGTTATACAA</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>ACCCAGTTACCATAAGCTTCTCTGTTTCTG</td>
<td>AGAAAACTGTTCCTCAATACATGGAGGAT</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>TGACAGTAAAAGGCACTTCGGTCC</td>
<td>CATACTACACAGTGCAACAAAGCCCCC</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>GCAACAGATAACTCGATGGCC</td>
<td>CATACTAAGCTCAACAAAGCCCCC</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>GAGGCTCATTTTAAAAAGGCCTCCTTCTTCTGTA</td>
<td>TGAGGGTAGCTTTATGCCCTCTTGTAG</td>
</tr>
<tr>
<td>CDKN2A</td>
<td>1</td>
<td>GGGTGCCACATTCGCTAAGGT</td>
<td>CTTTTTCCGGAGAAATCGAAGC</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>ACACAAGCTTCTTCTCCGTCA</td>
<td>GGCTCAGCTTCTTCGCTGGA</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>TGTCGCACTACATCTTTCGCTC</td>
<td>TCCATGCGATGAAATTGGTGTAAA</td>
</tr>
</tbody>
</table>

Table 2.7.2.2 Primer Sets Used in this Project for sequencing. Annealing temperatures used for PCR reactions were calculated using the following calculation: $64.9 + 0.41(5GC) - 600/\text{length}$. 

283
Appendix 3: PCR conditions used to amplify *CDKN2B* and *CDKN2A*

(a)

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature °</th>
<th>Time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Initial Denaturation Step</td>
<td>95</td>
<td>300</td>
</tr>
<tr>
<td>2. Denaturation</td>
<td>95</td>
<td>45</td>
</tr>
<tr>
<td>3. Annealing</td>
<td>58</td>
<td>45</td>
</tr>
<tr>
<td>Step-down decrease by 1° every cycle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. Extension</td>
<td>72</td>
<td>45</td>
</tr>
<tr>
<td>5. Cycle to 2 for 4 more times</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. Denaturation</td>
<td>95</td>
<td>45</td>
</tr>
<tr>
<td>7. Annealing</td>
<td>54</td>
<td>45</td>
</tr>
<tr>
<td>8. Extension</td>
<td>72</td>
<td>45</td>
</tr>
<tr>
<td>9. Cycle to 6 for 40 times</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10. Final Extension</td>
<td>72</td>
<td>300</td>
</tr>
<tr>
<td>11. End Reaction</td>
<td>10</td>
<td>Indefinitely</td>
</tr>
</tbody>
</table>

(b)

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature °</th>
<th>Time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Initial Denaturation Step</td>
<td>95</td>
<td>300</td>
</tr>
<tr>
<td>2. Denaturation</td>
<td>95</td>
<td>45</td>
</tr>
<tr>
<td>3. Annealing</td>
<td>62</td>
<td>45</td>
</tr>
<tr>
<td>Step-down decrease by 1° every cycle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. Extension</td>
<td>72</td>
<td>45</td>
</tr>
<tr>
<td>5. Cycle to 2 for 4 more times</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. Denaturation</td>
<td>95</td>
<td>45</td>
</tr>
<tr>
<td>7. Annealing</td>
<td>58</td>
<td>45</td>
</tr>
<tr>
<td>8. Extension</td>
<td>72</td>
<td>45</td>
</tr>
<tr>
<td>9. Cycle to 6 for 40 times</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10. Final Extension</td>
<td>72</td>
<td>300</td>
</tr>
<tr>
<td>11. End Reaction</td>
<td>10</td>
<td>Indefinitely</td>
</tr>
</tbody>
</table>

Table 2.7.2.3 PCR Conditions for amplification of (a) *CDKN2B* exon 1 and (b) *CDKN2B* exon 2. Extension time was based on the size of the PCR product.
Appendix 4 Primers used in site directed mutagenesis
2.10.3.2 Primer pairs.

<table>
<thead>
<tr>
<th>Variant</th>
<th>Forward Primer (5’ to 3’)</th>
<th>Reverse Primer (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E90X</td>
<td>CGACAGCTCCTGTAAAGCGGCG</td>
<td>CGCGCCGCTACTACGGAGGCTGT</td>
</tr>
<tr>
<td></td>
<td>CGG</td>
<td>CG</td>
</tr>
<tr>
<td>A23E</td>
<td>GAAGCCGGCGGGATACCAAAC</td>
<td>GTTGACTCCGGTTGATCCGGCCGC</td>
</tr>
<tr>
<td></td>
<td>GGAGTCGAAC</td>
<td>GGCTTC</td>
</tr>
<tr>
<td>D86N</td>
<td>CGACCAGTCGATAATGTGCACC</td>
<td>CCCGGGCGAGTTATGCACCGGT</td>
</tr>
<tr>
<td></td>
<td>GGG</td>
<td>CG</td>
</tr>
<tr>
<td>P40T</td>
<td>CCACGCGCCCGCGGCGGGGAC</td>
<td>CACTAGTCCCGCTCCGGGCGGCT</td>
</tr>
<tr>
<td></td>
<td>TAGTG</td>
<td>GG</td>
</tr>
<tr>
<td>D39N</td>
<td>GAAGCCGGCGGCGATCCCAAC</td>
<td>CTCCGGTTGGATTCCGCGCGGCTT</td>
</tr>
<tr>
<td></td>
<td>GGAG</td>
<td>C</td>
</tr>
<tr>
<td>V27A</td>
<td>CAGTGCGGGGCCGCGGATGAG</td>
<td>CAGACCCCTCATCGCGCGCGGGCCC</td>
</tr>
<tr>
<td></td>
<td>GGGTCTG</td>
<td>CCATCG</td>
</tr>
<tr>
<td>S14G</td>
<td>GGGGGCGGCGGCGGATGAGG</td>
<td>CCCTCATCGCGCGGCGGCCCC</td>
</tr>
</tbody>
</table>
### Appendix 5 PCR conditions used

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95</td>
<td>600</td>
</tr>
<tr>
<td>2</td>
<td>95</td>
<td>45</td>
</tr>
<tr>
<td>3</td>
<td>62</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>Decrease by 1 degree every cycle</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>72</td>
<td>45</td>
</tr>
<tr>
<td>5</td>
<td>Cycle to step 2 four more times</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>95</td>
<td>45</td>
</tr>
<tr>
<td>7</td>
<td>58</td>
<td>45</td>
</tr>
<tr>
<td>8</td>
<td>72</td>
<td>45</td>
</tr>
<tr>
<td>9</td>
<td>Cycle to step 6 for 40 cycles</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>72</td>
<td>forever</td>
</tr>
</tbody>
</table>

Figure 2.9.8.2 The PCR products included negative control and positive control gel. 30µl of product was mixed with 5µl of 6x loading buffer and ran out on a 3% agarose gel. The gel was then visualised using Gene SNAP vs 4 software (Synoptics Limited).
### Appendix 6 Antibodies used in this thesis

<table>
<thead>
<tr>
<th>Protein</th>
<th>Company</th>
<th>Immunoglobulin</th>
<th>Source</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>VHL</td>
<td>Cell signaling</td>
<td></td>
<td>Rabbit</td>
<td>1:1000</td>
</tr>
<tr>
<td></td>
<td>27385</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDKN2B</td>
<td>Cell signaling</td>
<td></td>
<td>Rabbit</td>
<td>1:2000</td>
</tr>
<tr>
<td></td>
<td>48225</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIF-2α</td>
<td>Abcam</td>
<td></td>
<td>Mouse</td>
<td>1:500</td>
</tr>
<tr>
<td></td>
<td>8365</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIF-1α</td>
<td>Novus BiologicaLs</td>
<td></td>
<td>Mouse</td>
<td>1:500</td>
</tr>
<tr>
<td></td>
<td>NB100-105</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclin D3</td>
<td>Abcam</td>
<td></td>
<td>Mouse</td>
<td>1:500</td>
</tr>
<tr>
<td></td>
<td>2936</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubulin</td>
<td>Abcam</td>
<td></td>
<td>Rabbit</td>
<td>1:1000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RYK</td>
<td>Abcam</td>
<td></td>
<td>Rabbit</td>
<td>1:500</td>
</tr>
<tr>
<td></td>
<td>5518</td>
<td></td>
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<td></td>
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<tr>
<td>STK-10</td>
<td>Abcam</td>
<td></td>
<td>Rabbit</td>
<td>1:1000</td>
</tr>
<tr>
<td></td>
<td>70484</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>Cell Signalling</td>
<td></td>
<td>Mouse</td>
<td>1:1000</td>
</tr>
<tr>
<td></td>
<td>2926</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 2.15.6 Antibodies directed towards different proteins used in this thesis. The concentration of the antibiotic used are described.
CHAPTER 8. REFERENCES


268. Baba M, Hong S-B, Sharma N, Warren MB, Nickerson ML, Iwamatsu A, et al. Folliculin encoded by the BHD gene interacts with a binding protein, FNIP1, and AMPK, and


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