AN INVESTIGATION OF POTENTIAL PROGNOSTIC INDICATORS AND BIOMARKERS (TRAF1, NF-κB AND CD14) IN RENAL CANCERS

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FACULTY OF MEDICINE
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AN INVESTIGATION OF POTENTIAL PROGNOSTIC INDICATORS AND BIOMARKERS (TRAF1, NF-κB AND CD14) IN RENAL CANCERS

ABSTRACT

The incidence of renal cell carcinoma (RCC) is rising annually worldwide and around 30% of patients have metastasis at diagnosis. Once metastasized, RCC is treatment resistant with poor prognosis and a 5 year survival rate of 10-20%, even with treatment of targeted therapies. Currently, there are no suitable biomarkers being used in the routine diagnosis or prognostication of RCC in the clinical setting. A better understanding of dysregulated genes or proteins in RCC helps unravel the molecular complexities which contribute to tumour growth or progression. This enables the improvement of targeted therapies and identifies potential diagnostic or prognostic markers for the monitoring of RCC progression. Therefore, the general aim was to determine the clinical characteristics and protein biomarkers that could be potential diagnostic or prognostic indicators in RCC. Currently there is no comprehensive registry on RCC patients in Malaysia, hence the first objective was to determine the clinical characteristics and factors affecting survival of RCC patients in University Malaya Medical Centre. In this cohort of patients, TNM staging and palpable abdominal mass were independent predictors for survival. The clinical data gathered was also used in subsequent chapters for prognostic analyses. The proteins of interests were TRAF1 and NF-κB subunits (p50, p52, p65 and cRel), which are linked in pathways regulating cell survival, apoptosis and proliferation. The second and third objectives were to assess the prognostic significance of TRAF1 and NF-κB as biomarkers in RCC and the roles they play in controlling cell apoptosis/proliferation. The concentration of TRAF1 in clear cell RCC (ccRCC) patients' serum and controls were determined using ELISA

analysis. TRAF1 levels were significantly higher in ccRCC serum compared to controls. TRAF1 serum concentration was higher in metastatic patients compared to localised RCC. TRAF1 and NF-κB immunopositivity was also evaluated in RCC tissue. TRAF1 expression was significantly lower in ccRCC compared to the adjacent normal tissue, and was not associated with prognostic factors. Immunopositivity of p65 was higher in RCC compared to normal kidney tissue, but p50, p52 and cRel expressions were lower in RCC tissue. In tumour tissue, higher p52 and p65 expressions were associated with a worse survival outcome, hence they could be possible prognostic indicators in RCC. RCC cell line experiments revealed that TRAF1 silencing increased pro-survival cIAP-1 and cIAP-2 protein levels while p65 silencing decreased protein concentration of proliferation proteins, cyclin D1 and IL-6. The combination of low TRAF1 and high NF-κB p65 concentration in RCC tissue might cause an imbalance in cell proliferation and apoptosis, promoting tumour growth. The final objective was the discovery of additional serum biomarker in RCC. Isobaric tags for relative and absolute quantitation (iTRAQ) analysis identified CD14 as a potential prognostic marker as serum CD14 concentration was higher in ccRCC patients compared to controls and was significantly associated with stage. Further immunohistochemistry and functional studies using cell lines would aid in understanding the role of CD14 in RCC progression. In conclusion, TRAF1 and NF-κB were dysregulated in RCC tissue, and TRAF1 and CD14 were potential serum indicators for advanced RCC.

Keywords : Renal cell carcinoma, prognostic, biomarker

ABSTRAK

Kadar kanser ginjal sel renal karsinoma (RCC) meningkat setiap tahun di seluruh dunia dan dalam sekitar 30% pesakit mengalami metastasis ketika diagnosis. Setelah merebak, RCC susah dirawati dan kadar kelangsungan hidup lima tahun hanya 10-20%, walaupun dengan rawatan terapi sasaran molekular. Pada masa ini, tidak ada biopenanda sesuai yang digunakan semasa diagnosis rutin klinikal atau untuk mengenal pasti prognosis RCC. Pemahaman yang lebih mendalam mengenai gen dan protin yang terlibat dalam pengawalan pertumbuhan sel-sel RCC akan membantu dalam peningkatan tahap rawatan terapi sasaran molekular atau mengenal pasti biopenanda baru untuk pemantauan dan rawatan pesakit RCC yang lebih berkesan. Maka, matlamat umum projek in adalah untuk mengenal pasti ciri-ciri klinikal and biopenanda yang mempengaruhi diagnosis atau prognosis pesakit RCC. Laporan registri terperinci mengenai pesakit RCC yang melaporkan maklumat seperti ciri-ciri klinikal dan kadar kematian tidak lagi wujud di Malaysia, maka objektif pertama adalah untuk menentukan ciri-ciri klinikal dan faktor-faktor yang mempengaruhi prognosis pesakit RCC di Pusat Perubatan Universiti Malaya (PPUM). Analisa statistic data pesakit menunjukkan bahawa tahap kanser (TNM) dan tumor yang dapat dirasai semasa pemeriksaan abdominal dapat meramalkan jangka hayat pesakit. Data klinikal pesakit yang dikumpul juga diperlukan dalam bab berikutnya untuk analisa prognostik. Protin yang dikaji adalah TRAF1 dan NF-κB (p50, p52, p65 and cRel), yang dikaitkan dalam pengawalan apoptosis dan proliferasi sel. Objektif kedua dan ketiga adalah untuk menilai kepentingan prognostik TRAF1 dan NF-κB sebagai biopenanda dalam RCC dan peranan protin ini dalam pengawalan apoptosis dan proliferasi sel. Konsentrasi TRAF1 di dalam serum pesakit clear cell RCC (ccRCC) dan kawalan ditentukan menggunakan kaedah ELISA. Didapati tahap TRAF1 lebih tinggi dalam serum pesakit ccRCC

berbanding kumpulan kawalan. Di kalangan pesakit RCC, konsentrasi TRAF1 lebih tinggi dalam serum pesakit metastatik berbanding pesakit tanpa metastasis. Ekspresi protin TRAF1 dan NF-κB di dalam tisu RCC dinilai dengan kaedah imunohistokima. Ekspresi TRAF1 lebih rendah di dalam tisu RCC berbanding dengan tisu ginjal tanpa kanser, dan tidak dikaitkan dengan faktor prognostik. Ekspresi p65 lebih tinggi di dalam tisu RCC berbanding tisu ginjal tanpa kanser, tetapi p50, p52 dan cRel lebih rendah di dalam tisu RCC. Ekspresi p52 dan p65 yang lebih tinggi di dalam tisu RCC dikaitkan dengan jangka hayat yang lebih pendek, maka protin ini berpotensi menjadi biopenanda prognostik untuk RCC. Eksperimen in vitro sel RCC menunjukkan bahawa protin anti-apoptosis cIAP-1 dan cIAP-2 meningkat selepas tahap TRAF1 dikurangkan dan protin proliferasi cyclin D1 dan IL-6 berkurangan selepas p65 dikurangkan. Kombinasi ekspresi TRAF1 yang rendah dan p65 yang tinggi di dalam tisu RCC mungkin menghalang apoptosis sel serta mempromosi proliferasi sel dan purtumbuhan barah. Objektif terakhir adalah usaha penemuan biopenanda baru untuk RCC menggunakan kaedah iTRAQ. CD14 diidentifikasi sebagai biopenanda prognostic yang berpotensi kerana konsentrasi CD14 lebih tinggi dalam serum pesakit ccRCC berbanding dengan kawalan dan meningkat dalam serum pesakit RCC tahap lanjut. Eksperimen ihmunohistokima dan kajian in vitro sel diperlukan untuk memahami peranan CD14 dalam RCC. Kesimpulannya, terdapat ketidak keseimbangan protin TRAF1 dan NF-κB dalam tisu RCC yang membantu pertumbuhan kanser dan TRAF1 serta CD14 berpotensi menjadi biopenanda serum untuk RCC tahap lanjut.

Kata kunci : Kanser ginjal sel renal karsinoma, prognosis, biopenanda

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I would like to dedicate this thesis to the anonymous subjects/patients/controls of this study, who selflessly donated their blood or tissue for advancement in RCC research.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage
°C	Degree celcius
18s rRNA	18s ribosomal RNA
α-SMA	Alpha smooth muscle actin
µg/ml	Micrograms per millilitre
µl	Microlitre
µm	Micrometre
AJCC	American Joint Committee on Cancer
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
ARID1A	AT-rich interactive domain-containing protein 1A
ASK1	Apoptosis signal-regulating kinase 1
ATCC	American Type Culture Collection
AUC	Area under the curve
BAP1	BRCA1 associated protein-1
Bax	BCL2 associated X protein
BCA	Bicinchoninic acid
Bcl-XL	B-cell lymphoma-extra large
BSA	Bovine serum albumin
CAIX	Carbonic anhydrase IX
ccpRCC	Clear cell papillary RCC
ccRCC	Clear cell RCC
CD14	Cluster of differentiation 14/ Monocyte differentiation antigen 14
CD40	Cluster of differentiation 40

cDNA	Complementary DNA
chRCC	Chromophobe RCC
CI	Confidence interval
cIAP-1	Cellular inhibitor of apoptosis protein 1
cIAP-2	Cellular inhibitor of apoptosis protein 2
CK8	Cytokeratin 8
cm^2	Centimetre square
CO_2	Carbon dioxide
CRP	C-reactive protein
CT	Computed tomography
DAB	Diaminobenzidine hydrochloride
DMEM	Dulbecco's Modified Eagles Medium
DNA	Deoxyribonucleic acid
DSS	Disease specific survival
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial to mesenchymal transformation
FADD	Fas-associated death domain
FAP	Fibroblast activating factor
FBS	Fetal bovine serum
FFPE	Formalin fixed paraffin embedded
FGF	Fibroblast growth factor
FH	Fumarate hydratase
g	Gram
g/L	Grams per litre
H_2O	Water
H_2O_2	Hydrogen peroxide

HGF	Hepatocyte growth factor
HIF-1α	Hypoxia inducible factor 1 alpha
HIF-2α	Hypoxia inducible factor 2 alpha
HPLC	High performance liquid chromatography
HR	Hazards ratio
HRP	Horse radish peroxidase
IFN-α	Interferon alpha
IF	Immunofluorescence
IHC	Immunohistochemistry
IKK	IκB kinase
IL-1	Interleukin 1
IL-2	Interleukin 2
IL-6	Interleukin 6
IL-8	Interleukin 8
iTRAQ	Isobaric tags for relative and absolute quantitation
IU/L	International units per litre
IκB	Inhibitor of NF-κB
kD	Kilodalton
KDM5C	Lysine (K)-specific demethylase 5C
L	Litre
LC-MS	Liquid chromatography mass spectometry
LDH	Lactate dehydrogenase
LOW	Loss of weight
LPS	Lipopolysaccharide
ml	Millilitres
mM	Millimolar

mm	Millimetre
mm^3	Millimetre cubic
mmol/L	Millimoles per litre
mRCC	Metastatic RCC
mTOR	Mammalian target of rapamycin
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NSS	Nephron sparing surgery
PBRM1	Polybromo 1
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-L1	Programmed cell death ligand-1
pRCC	Papillary RCC
PVDF	Polyvinylidene difluoride
pVHL	von Hippel Lindau protein
RCC	Renal cell carcinoma
RFA	Radiofrequency ablation
RIP1	Receptor-interacting protein 1
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
SD	Standard deviation
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis
SE	Standard error
SETD2	SET domain containing 2
siRNA	Silencing RNA
TBS	Tris buffered saline
TBST	Tris buffered saline Tween 20

TNFR1	Type 1 tumour necrosis factor receptor
TNFR2	Type 2 tumour necrosis factor receptor
TNF-α	Tumour necrosis factor alpha
TNM	Tumour node metastasis
TRADD	TNF receptor-associated death domain
TRAF1	TNF receptor associated factor 1
TRAF2	TNF receptor-associated factor 2
TRAIL-R1	TNF-related apoptosis-inducing ligand receptor 1
UMMC	University Malaya Medical Centre
USG	Ultrasonography
V	Volt
VEGF	Vascular endothelial factor
VHL	von Hippel Lindau
XIAP	X-chromosome linked IAP

LIST OF APPENDICES

CHAPTER 1 : INTRODUCTION

1.1 Background

The prevalence of kidney cancer is rising at approximately 2% per annum worldwide and the latest GLOBOCAN project which reports on cancer incidence and mortality globally placed kidney cancer as the 9th and 14th most common cancer in men and women respectively (Ferlay et al., 2013; Wu, Shu, Chow, & Gu, 2014; Znaor, Lortet-Tieulent, Laversanne, Jemal, & Bray, 2015). In Malaysia, kidney cancer was the 13th and 19th most common cancer in men and women according to the last National Cancer Registry Report 2007-2011 (National Cancer Registry [NCR], 2016). Kidney cancer was ranked as the 16th most common cause of death from cancer worldwide, with approximately 143000 deaths in 2012 (Ferlay et al., 2013; Znaor et al., 2015). Currently, as there is yet to be a comprehensive kidney cancer registry in Malaysia, the actual trend of kidney cancer and disease related mortality rate in this country is still unknown.

RCC is the most common form of kidney cancers, accounting for up to 90% of renal malignancies. RCCs are further classified into a few different subtypes based on their histopathological features. The standard treatment for localised RCC is surgical removal (nephrectomy) but approximately 30% of renal tumours are detected only after they metastasize (Yap et al., 2013). The current treatment for metastatic RCC (mRCC) is targeted therapy to inhibit tumour growth or angiogenesis directed at the vascular endothelial growth factor (VEGF) or the mammalian target of rapamycin (mTOR) pathways. Recently, immuno-targeted therapy such as programmed death-ligand 1 (PD-L1) inhibitors, have been approved for mRCC. Prognosis is poor in metastatic

cases, as the 5 year survival rate is 10-20% while in comparison, the 5 year survival rate of stages I-II RCC is 75-96% (Furniss et al., 2008; Nelson, Vogelzang, & Pal, 2013). Prognosis is still poor for mRCC even with targeted therapy, as patients eventually develop resistance to these drugs and succumb to the disease (Rini & Atkins, 2009). Additionally, the current available targeted therapy is expensive and in the long term, is financially debilitating to patients (Choueiri et al., 2012).

Therefore, there is still a necessity for better novel therapeutic targets and identification of biomarkers for early diagnosis or prognostic indicators for RCC. At present, there are no biomarkers which are used in routine clinical settings for early diagnosis or monitoring of RCC. A better understanding of genetic and proteomic dysregulations or pathway imbalance in RCC would also help in identifying the various mechanisms that may take part in tumour initiation or progression. This might aid in improving targeted therapy and patient management in the future. Among the causes of oncogenesis is the imbalance in pathways regulating apoptosis, cell survival and proliferation. Apoptosis is important in removing potentially cancerous cells while uncontrolled proliferation or resistance to apoptosis can promote tumour growth.

TNF receptor-associated factor 1 (TRAF1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) are proteins of interest in this thesis. TRAF1 takes part in the control of inflammation, apoptosis and survival pathways in the cell (S. Y. Lee & Choi, 2007; Rajandram, Bennett, Morais, Johnson, & Gobe, 2012). Preliminary studies showed that TRAF1 is dysregulated in RCC tissue (Lee & Choi, 2007; Rajandram, Bennett, Morais, et al., 2012). TRAF1 was also found to be elevated when apoptosis was induced in the RCC cell line ACHN (Rajandram, Bennett, Morais, et al., 2012). However, the exact nature of apoptotic regulation of TRAF1 is still unclear

in RCC. In haematological malignancies, TRAF1 is upregulated in B cell lymphoma, non-Hodgkin lymphoma and circulating chronic lymphocytic leukemia (Lee & Choi, 2007). In other solid tumours, the function or expression of TRAF1 is not well reported. TRAF1 can promote cell survival or death through the regulation of the transcription factor NF-κB.

NF-κB play roles in biological processes such as inflammation, cell survival, apoptosis, proliferation and cell differentiation (Hayden & Ghosh, 2012). The five subunits of NF-κB are RelA (p65), RelB, cRel, NF-κB1 (p105/p50) and NF-κB2 (p100/p52). TRAF1 has both positive and negative regulatory effects on NF-κB, depending on the cell type, ligand and receptors involved (Lee & Choi, 2007; McPherson, Snell, Mak, & Watts, 2012). Based on the preliminary findings on apoptosis in RCC, TRAF1 may be pro-apoptotic and have inhibitory effects on NF-κB. Activation of NF-κB can induce transcription genes involved in pathways implicated in cancer such as those promoting cell proliferation, survival, angiogenesis and invasiveness. The subunit p65 has been shown to be overexpressed in RCC, but other subunits are not as well reported (Kankaya, Kiremitci, Tulunay, & Baltaci, 2015; Peri, Devarajan, Yang, Knudson, & Balachandran, 2013b). The prognostic value of TRAF1 and NF-κB subunits in RCC tissue or serum is unclear at the moment.

1.2 Research Questions

These research questions were formulated to bridge the gaps of knowledge described in the introduction background section :

1. Based on the patient demographic and clinical information collected for the RCC registry, are there any factors that could predict the prognosis of this cohort of RCC patients?

2. Is there dysregulation of TRAF1 and NF-κB in Malaysian RCC patients? Could these proteins affect RCC progression and be used as prognostic markers?
3. Do TRAF1 and NF-κB regulate apoptosis and proliferation in RCC cells?
4. Are there any additional protein biomarkers in the serum of RCC patients?

1.3 Objectives

The following research objectives for this thesis were devised to answer the research questions:

1. To determine the clinical characteristics, presenting symptoms and factors affecting survival of RCC patients in University Malaya Medical Centre (UMMC)
2. To study the roles of TRAF1 and NF-κB as biomarkers and prognostic factors in RCC Formalin Fixed Paraffin Embedded (FFPE) tissue and serum samples
3. To evaluate the roles of TRAF1 and NF-κB in the regulation of proliferation and apoptosis in commercial and established Malaysian RCC cell lines
4. To discover potential prognostic markers for RCC in the serum using isobaric tags for relative and absolute quantitation (iTRAQ), a non-gel-based proteomics technique

1.4 Hypotheses

Based on these research questions and objectives, the research hypotheses (H_1) were that:

1. The clinical characteristics and presenting symptoms affect the prognosis of RCC patients treated at UMMC
2. There are differential expression levels of TRAF1 and NF-κB in RCC tissue compared to normal kidney
3. The level of TRAF1 in the serum of RCC patients are significantly different compared to healthy controls and may be a prognostic indicator for RCC
4. TRAF1 and NF-κB play a role in regulating apoptosis and proliferation in RCC cells.
5. There will be higher/lower protein levels in the serum of RCC patients compared to healthy controls and these could be potential prognostic proteins

1.5 Significance and scope of study

At present, not many studies have been conducted on RCC patients in Malaysia. There has been only one other published report on the basic clinical and demographic characteristics of RCC patients in Malaysia (Singam et al., 2010). Furthermore, there is no survival information in the Malaysian cancer registry reports. This thesis is written in the article style format and chapters are presented in the format of journal articles. Therefore, after Chapter 2 : Literature Review, Chapter 3 will concentrate on the first objective where the demographics, clinical characteristics and factors affecting survival of RCC patients in UMMC were reported. The findings from the second objective are split into two chapters (Chapters 4 and 5). The expressions and prognostic significance of TRAF1 in RCC tissue and serum samples were analysed in Chapter 4. In Chapter 5,

the expressions/localisation of NF-κB subunits and prognostic value of NF-κB staining intensity in RCC tissue were assessed. For the third objective (Chapter 6), RCC cell lines established from UMMC RCC patients and commercially available RCC cell lines were used to evaluate the effects of TRAF1 and NF-κB p65 silencing on selected apoptotic, survival and proliferation proteins. In Chapter 7, additional biomarkers were identified in serum samples collected from UMMC RCC patients via the iTRAQ method and validated using ELISA assay as the inclusion of a multi marker panel might improve the prediction value. The last chapter (Chapter 8) includes the overall summary of all findings and recommendations for future directions.

CHAPTER 2 : LITERATURE REVIEW

2.1 The kidney

The kidneys are two bean shaped organs, located at the posterior left and right of the abdominal cavity. The kidney's main function is to filter the blood in the body to remove wastes into the urine, regulate electrolytes and maintain homeostasis and fluid balance. Blood flows into the kidneys via the renal arteries, reaches the nephrons where glucose, ions, urea and water are filtered to produce urine. Each kidney contains up to a million units of nephrons. A nephron consists of a filtering tangle of blood vessels called a glomerulus enclosed by a Bowman's capsule (Reilly, Gulger, & Kriz, 2007). The filtrate enters the Bowman's capsule, passes through the proximal tubule, the loop of Henle, the distal convoluted tubule and the collecting ducts to form urine, which is transported in the ureter to the bladder (Reilly et al., 2007). The filtered blood leaves the kidney through the renal vein.

The kidney is an organ enclosed in renal capsule, which is surrounded by the perirenal fat, renal fascia and pararenal fat. Underneath the capsule is the outer layer renal cortex and inner renal medulla. The renal medulla consists of cone shaped renal pyramids and between these are the renal columns. The nephron spans the renal cortex and medulla, with the glomerulus, Bowman's capsule, proximal and distal tubules found mostly in the cortex and the loop of Henle and collecting duct in the medullary pyramid (Reilly et al., 2007). The tip of the pyramid called the papilla is connected to the minor calyx. Minor calyxes join to the major calyx which funnels into the renal pelvis and finally the ureter.

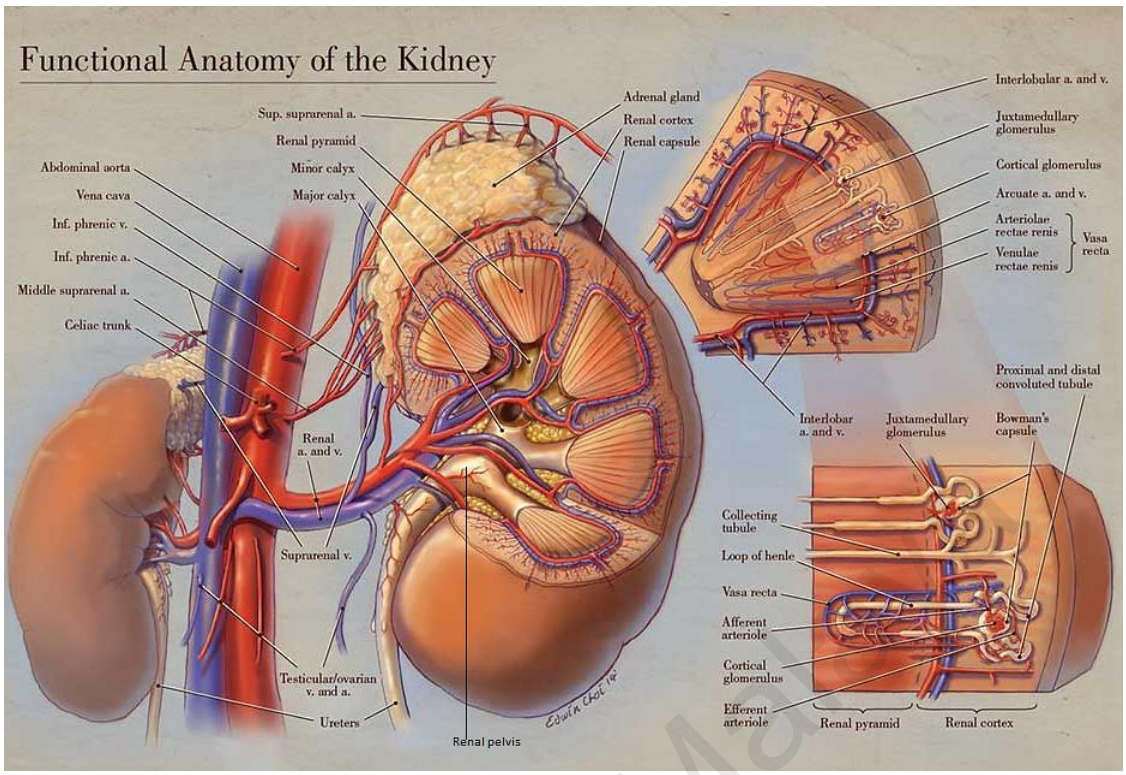


Figure 2.1. Structural anatomy of the kidney
Image taken from (Health Life Media, 2016).

2.2 Epidemiology of kidney cancer

The prevalence of kidney cancer has been rising yearly, with higher incidence rates in developed countries compared to developing ones (Znaor et al., 2015). According to the latest GLOBOCAN project which reports on cancer incidence and mortality worldwide, kidney cancer is the 9th and 14th most common cancer in men and women respectively (Ferlay et al., 2013). In Malaysia, the last National Cancer Registry Report 2007-2011 showed that kidney cancer was the 13th and 19rd most common cancer in men and women (NCR, 2016).

The incidence of renal cancer varies across continents with higher rates reported in Europe, North America, and Australia while lower in India, Japan, Africa, and China (Ferlay et al., 2010). Kidney cancer was ranked as the 16th most common cause of death from cancer worldwide, with approximately 143000 deaths in 2012 (Ferlay et al.,

2013; Znaor et al., 2015). There is no comprehensive kidney cancer registry in Malaysia at the moment, hence the current trend of kidney cancer and mortality in this country is unavailable.

RCC forms the majority of kidney cancers, accounting for 90% of renal malignancies. RCCs are malignant cells from the renal tubules. The other types of kidney cancers are Wilm's tumour, arising from the pluripotent embryonic renal cells and transitional cell carcinoma from the cells lining the renal pelvis. The incidence of renal cancer increases with age as 91% of patients are diagnosed after the age of 45 years (Choueiri & Motzer, 2017). RCC predominantly affects males with an approximately 2:1 men:women ratio (Cho, Adami, & Lindblad, 2011). Ethnicity also appears to affect the incidence and survival of RCC. Blacks have a higher incidence rate and unfavourable survival outcome compared to other races (Stafford et al., 2008). Asians however, have a lower incidence rate and better survival than other races (Stafford et al., 2008). Other risk factors for RCC include cigarette smoking, obesity, hypertension, long term haemodialysis, acquired kidney cystic disease and certain inherited genetic syndromes (Ljungberg et al., 2011).

2.3 Renal Cell Carcinoma

RCCs are classified into a few different subtypes, based on their histopathological features. The latest classification is according to the 2016 WHO classification of the tumours of the urinary system (Moch, Cubilla, Humphrey, Reuter, & Ulbright, 2016). Some RCC subtypes and their histopathologies are summarised in Table 2.1.

Table 2.1. RCC subtypes and their histopathological characteristics

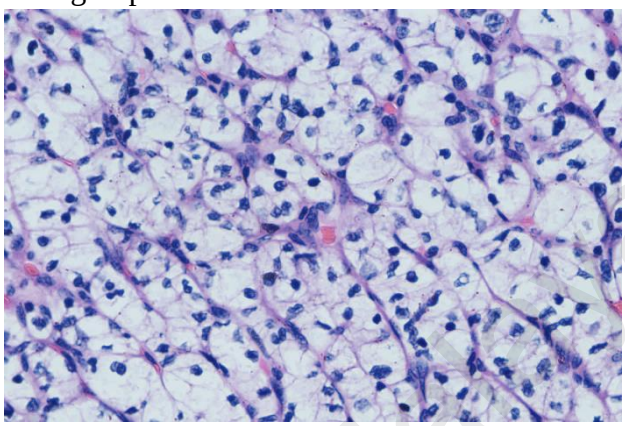
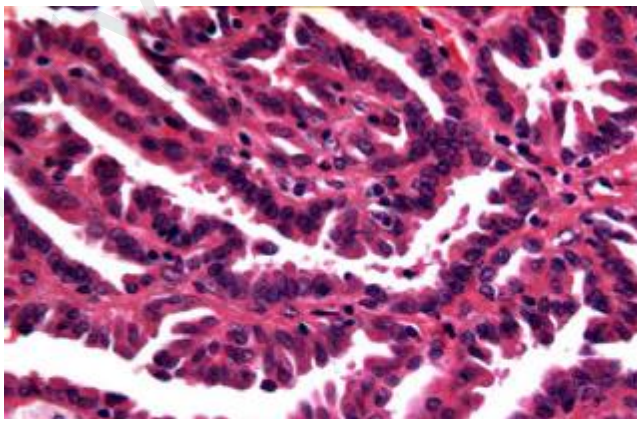
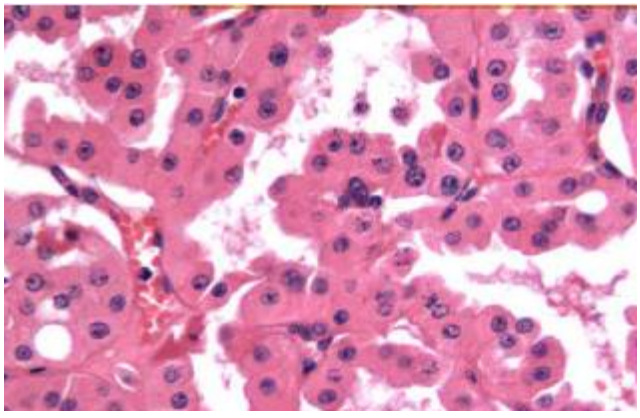
Subtype	Incidence	Histopathology
Clear Cell RCC (ccRCC)	80-90% of RCCs	Cells with abundant clear cytoplasm, a result of high glycogen and lipid content (Algaba et al., 2011). Some ccRCC may contain cells with granular eosinophilic cytoplasm. The gross appearance of the tumour is typically golden or yellow due to the high lipid content. 
Papillary RCC (pRCC)	10% of RCCs	Characteristic presence of tubulopapillary architecture. Type 1 tumours have small cells with scarce cytoplasm and small nuclei while type 2 tumours have large cells with abundant eosinophilic cytoplasm and large spherical nuclei (Delahunt & Eble, 1997). Type 2 tumours are generally more aggressive, with more advanced grade and stage than type 1 tumours (Pignot et al., 2007).  Type 1 pRCC. Source from (Algaba et al., 2011)  Type II pRCC. Taken from (Algaba et al., 2011)

Table 2.1 continued

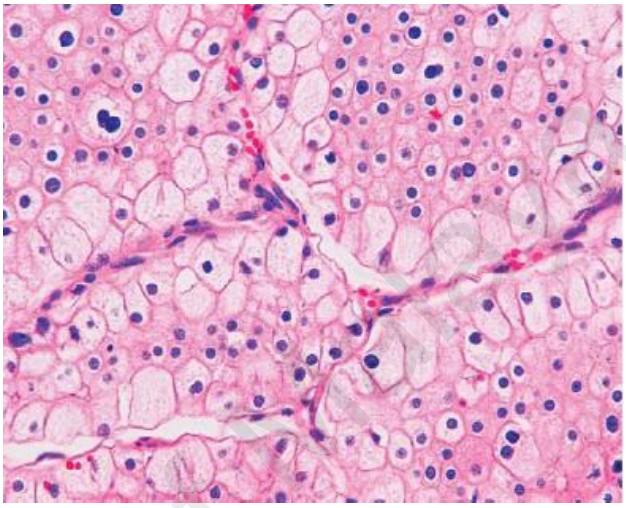
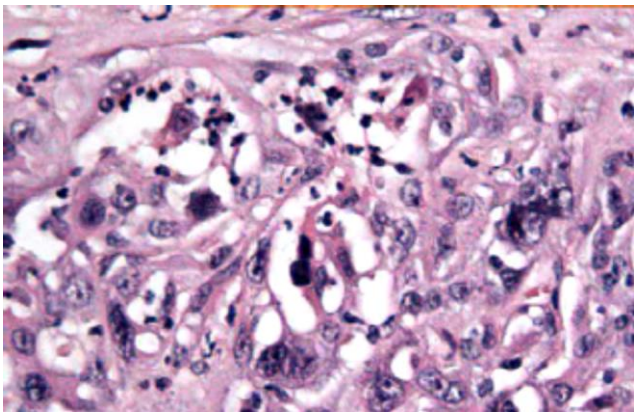
Subtype	Incidence	Histopathology
Chromophobe RCC (chRCC)	5% of RCCs	ChRCC can be grouped into the classic, eosinophilic or mixed variants. ChRCC cells are large and polygonal with distinct cell borders and irregular, wrinkled nuclei. The classic variant has pale, finely granular cytoplasm while the eosinophilic variant has granular eosinophilic cytoplasm (Amin et al., 2008). Clinical outcome is similar for the different variants (Amin et al., 2008).  An example of a mixed variant. Image taken from (Amin et al., 2008)
Collecting duct RCC (CDRCC)	0.4-1.8% of all RCCs	CDRCC cells have a tubulopapillary or hobnail structure, with eosinophilic cytoplasm and large nuclei. CDRCC is clinically aggressive with a higher metastatic rate and poorer prognosis compared to other RCC subtypes (Tokuda et al., 2006).  Image taken from (Algaba et al., 2011)

Table 2.1 continued

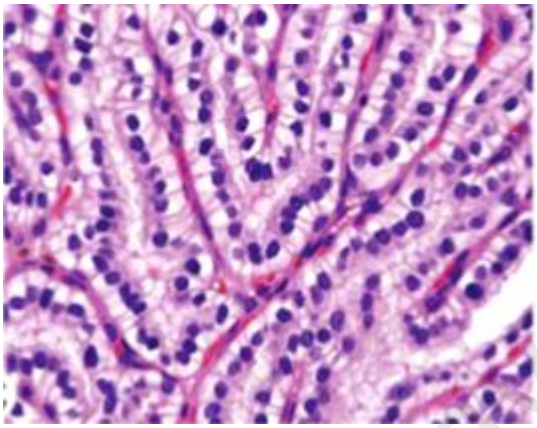
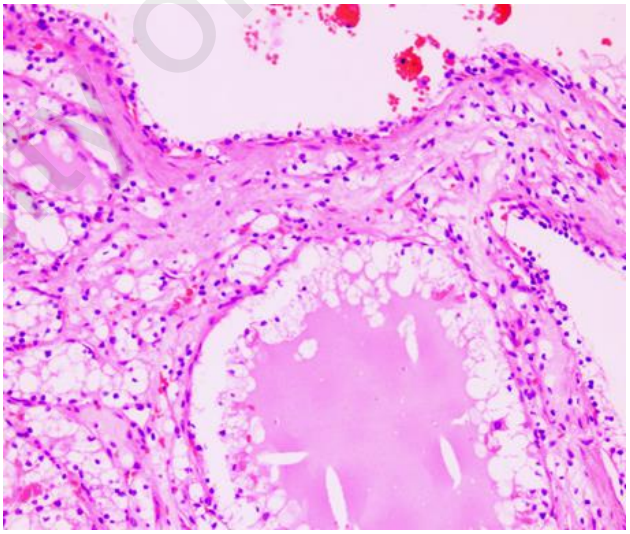
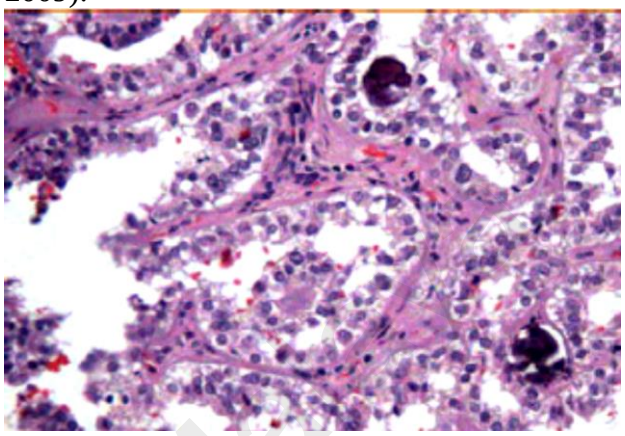
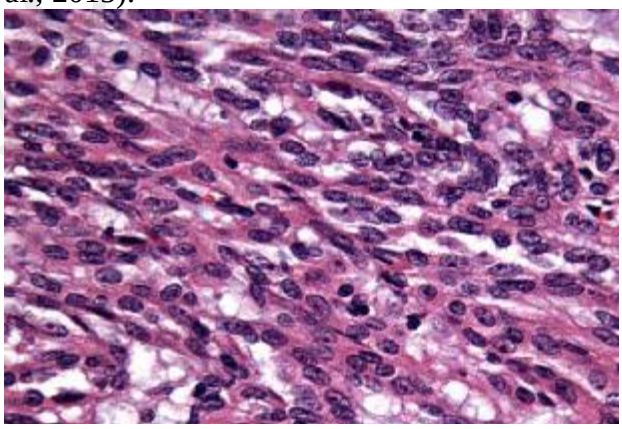
Subtype	Incidence	Histopathology
Clear Cell Papillary RCC (ccpRCC)	4% of RCCs	CcpRCC is a recently identified RCC subtype comprising of cells with clear cytoplasm and papillary architecture.  Image taken from (Zhou et al., 2014)
Multilocular cystic renal neoplasm of malignant potential	1-2%	Formerly known as multilocular cystic RCC, multilocular cystic renal neoplasm histology consists of fibrous septa with clear cells lining cystic spaces. Tumours are typically low grade with good prognosis (Hindman, Bosniak, Rosenkrantz, Lee-Felker, & Melamed, 2012). 

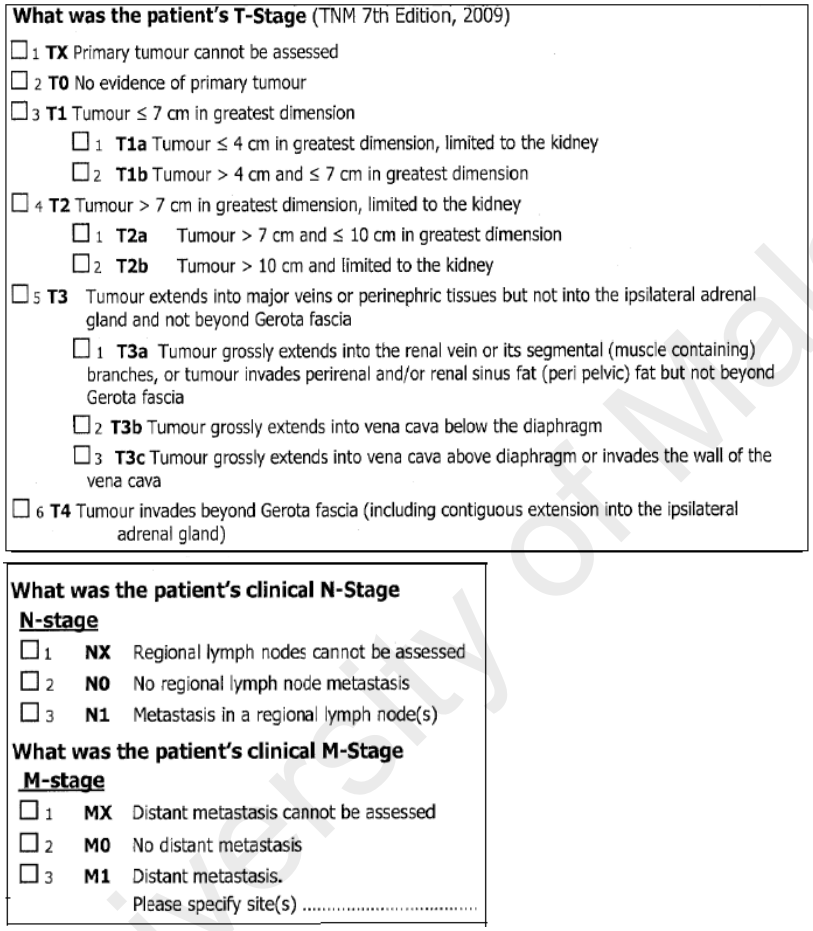
Table 2.1 continued

Subtype	Incidence	Histopathology
Microphthalmia-associated transcription (MiT) family translocation RCC	Rare but often underdiagnosed	MiT family translocation RCCs have mixed papillary or nested structure with clear of eosinophilic cytoplasm and predominantly affect children and adolescents. Clinically aggressive metastatic cases have been reported (Komai et al., 2009).  Image taken from (Algaba et al., 2011)
Mucinous tubular and spindle cell RCC	Rare	This subtype is typically low-grade, characterised by cuboidal cells organised in tubules with extracellular mucin and spindle cells (Kenney et al., 2015).  Source from (Algaba et al., 2011)

Other rare renal cell tumour types from the 2016 WHO classification of renal tumours not listed in this table are hereditary leiomyomatosis and RCC-associated RCC, renal medullary carcinoma, succinate dehydrogenase-deficient renal carcinoma, tubulocystic RCC, acquired cystic disease-associated RCC, unclassified RCC, papillary adenoma and oncocytoma (Moch et al., 2016). Papillary adenoma and oncocytoma are benign tumours (Moch et al., 2016).

2.4 Staging and grading

RCC is staged according to the tumour-node-metastasis (TNM) staging system, which depends on the size, invasion and metastatic state of the tumour (Figure 2.2). Stage grouping is shown in Table 2.2.



What was the patient's T-Stage (TNM 7th Edition, 2009)

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

What was the patient's clinical N-Stage

N-stage

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

What was the patient's clinical M-Stage

M-stage

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

Figure 2.2. TNM staging of RCC from the TNM classification of malignant tumours, 7th Edition (American Joint Committee on Cancer [AJCC], 2012)

Table 2.2. Stage grouping based on TNM criteria

Stage I	T1, N0, M0
Stage II	T2, N0, M0
Stage III	T3, N0, M0 or T1 to T3, N1, M0
Stage IV	T4, any N, M0 or Any T, Any N, M1

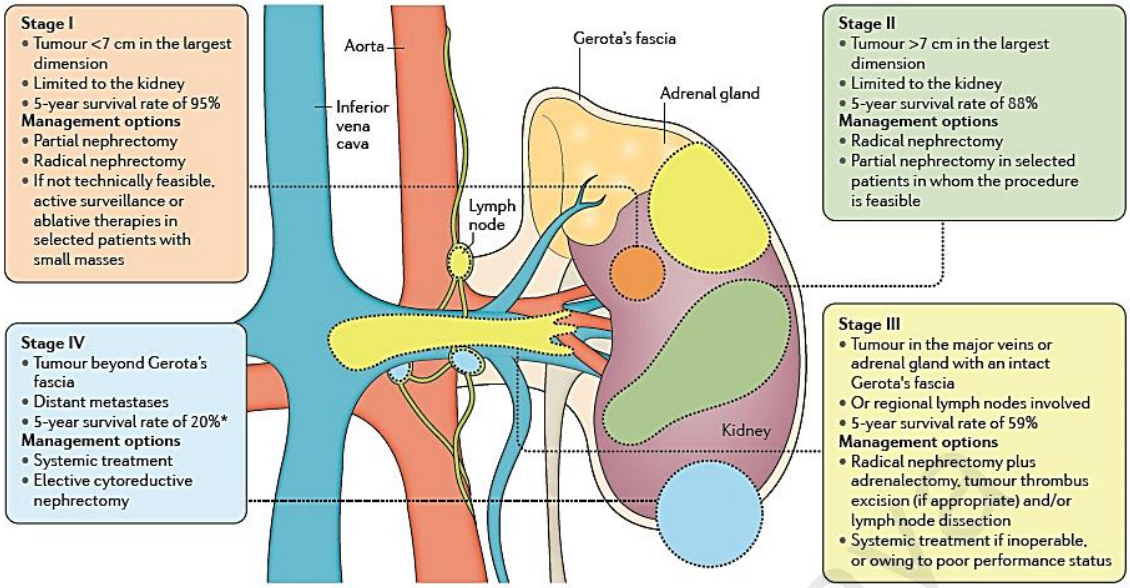


Figure 2.3. Staging of RCC affects the survival rates and management options for patients
Figure taken from (Hsieh et al., 2017).

RCC is graded 1-4 according to the nuclear characteristics. Higher stage or grade tumours are more clinically aggressive and affect the patients' prognosis. For example, prognosis is greatly reduced in metastatic cases, as the 5 year survival rate is 10-20% (Nelson et al., 2013). In comparison, the 5 year survival rate of stages I-II RCC is 75-96% (Furniss et al., 2008). Staging and grading help clinicians and patients decide on the best treatment and management plans.

Grading is conventionally based on the Fuhrman system and most recently, the International Society of Urological Pathology (ISUP) grading which has modifications from the Fuhrman grading has gained popularity (Samaratunga, Gianduzzo, & Delahunt, 2014). Fuhrman and ISUP grading systems are most frequently applicable for ccRCC and pRCC, but there is an agreement that these grading systems do not accurately represent the prognostic outcome of chRCC (Delahunt et al., 2013). Hence, grading is seldom applied for chRCC. RCC with sarcomatoid or rhabdoid transformation is not a recognised subtype of RCC as sarcomatoid or rhabdoid features

can be found in all histologic subtypes of RCC (Delahunt et al., 2013). Sarcomatoid cells are spindle shaped while rhabdoid cells have large eosinophilic cytoplasm with large irregular nuclei and prominent nucleoli. Both histological differentiation in RCC are associated with aggressive tumours and poor prognosis.

Table 2.3. Tumour grading according to the Fuhrman and ISUP systems

Grade	Fuhrman (Fuhrman, Lasky, & Limas, 1982)	ISUP (Delahunt et al., 2013)
1	Tumour cells with small (~10μm), round, uniform nuclei and inconspicuous nucleoli	Inconspicuous or absent nucleoli at 400x magnification
2	Tumour cells with larger nuclei (~15μm); irregularities in nuclei. Visible nucleoli under high power (400x)	Visible nucleoli at 400x magnification; inconspicuous/invisible at low power (100x)
3	Tumour cells with even, larger nuclei (~20μm); obvious irregular outline and prominent nucleoli at low power (100x)	Nucleoli distinctly visible at 100x magnification
4	Tumour cells with bizarre, multilobed nuclei and heavy clumps of chromatin	Rhabdoid or sarcomatoid differentiation, or tumours with giant cells or showing extreme nuclear pleomorphism with clumping of chromatin

2.5 Detection of RCC

RCCs are usually detected by ultrasonography (USG), computed tomography (CT) scans or magnetic resonance imaging (MRI). Part of the reason for the rising incidence of kidney cancers, particularly in developed nations, is due to the availability of medical imaging technologies which have improved the detection of renal tumours (Znaor, Lortet-Tieulent, Laversanne, Jemal, & Bray). As a result, the number of incidental detection of renal tumours has gradually increased over the years. These incidental/asymptomatic tumours are usually localized with lower stage and better prognosis (Sanchez-Martin, Millan-Rodriguez, Urdaneta-Pignalosa, Rubio-Briones, &

Villavicencio-Mavrich, 2008). Hence, although the incidence rate of RCC is increasing in most countries, there is a decrease in mortality from RCC in Western and Northern Europe, the USA, and Australia (Znaor et al.). Nevertheless, up to 30% of renal tumours are still detected only after they metastasize (Yap et al., 2013).

The classic triad symptoms of haematuria, flank pain and palpable abdominal mass are indicative of renal tumours. Paraneoplastic signs and symptoms, often associated with more advanced disease may also be present in localised tumours. However, many patients can be asymptomatic prior to detection. Studies have shown that symptomatic tumours, especially paraneoplastic related ones, confer an unfavourable survival prognosis (Patard et al., 2003).

2.6 Treatment or management of RCC

The standard treatment for localized RCC is partial or radical nephrectomy for the surgical removal of the tumour. Partial nephrectomy is also referred to as the nephron sparing surgery which aims to preserve kidney function and is usually performed on small renal masses (Stage I). The complete removal of the tumour greatly improves survival outcome and remains the gold standard for curative intervention of localised RCC. However, recurrences have been reported in 10-40% of localised RCC after resection (Chin, Lam, Figlin, & Belldegrun, 2006; Sorbellini et al., 2005). Small renal masses can also be treated with radiofrequency ablation (RFA) or cryosurgery which is performed for patients with one kidney or those who have comorbidities which may prevent invasive surgery. RFA utilizes high-frequency electric current to heat tissue to lethal temperatures while tumour destruction is achieved using ultra low temperatures for cryoablation.

Treatment and monitoring is more complex with higher stage tumours (Stages III-IV). Once metastasized, RCC is quite resistant to chemo and radio therapy. Treatment of mRCC in the 1980s with chemo or radiotherapy has been met with limited success (Bukowski, 1997). Immunotherapy using interferon alpha (IFN-α) and interleukin 2 (IL-2) was then discovered to be more effective in controlling the progression of mRCC. Both immunotherapy agents work by stimulating the immune system and enhancing the production of immune cells such as macrophages, monocytes, natural killer (NK) cells and T-lymphocytes which could destroy tumour cells (Bukowski, 1997). In the 1990s, immunotherapy was the standard treatment for mRCC, but patients reported significant treatment associated toxicities with the immune system being targeted as a whole (Courtney & Choueiri, 2010).

The discovery of the von Hippel-Lindau (*VHL*) gene in familial and sporadic ccRCC has revolutionised treatment for advanced RCC. Gradually, from 2005 onwards, targeted therapy directed at the vascular endothelial growth factor (VEGF) or mechanistic target of rapamycin (mTOR) pathways replaced immunotherapy. These two pathways regulate angiogenesis and proliferation in the cell. The current approved targeted therapy drugs are the tyrosine kinase inhibitors (sunitinib, sorafenib, pazopanib, axitinib and cabozantinib), monoclonal antibody to VEGF (bevacizumab) and the mTOR inhibitors (temsirolimus and everolimus) (Fishman, 2013). Recently, an immunotherapy agent targeting the immune checkpoint pathway, programmed cell death ligand-1 (PD-L1) have been approved (nivolumab) (Ghafar, Alip, Ong, Yap, & Saad, 2016). Cytoreductive nephrectomy, along with targeted therapy is recommended in mRCC patients with higher performance status to improve survival and alleviate symptoms caused by the tumour (Choueiri et al., 2011).

Even with the advent of targeted therapy, prognosis is still poor for mRCC as patients eventually develop resistance to these drugs and succumb to the disease (Rini & Atkins, 2009). The median overall survival of metastatic ccRCC patients has improved from 11 months in the immunotherapy period to 14 months in the targeted therapy era (Macleod et al., 2015). However, there is only an improvement of two months or less in the median overall survival of patients with non ccRCC and RCC with sarcomatoid features (Macleod et al., 2015). Additionally, the current available targeted therapy is costly and presents a financial burden to patients (Choueiri et al., 2012). RCC is a treatment resistant cancer and evasion of apoptosis and sustained proliferation may be part of the reason for this resistance. Hence there is a constant need for to search for better novel therapeutic targets and for biomarkers of early diagnosis or prognostic indicators for this deadly disease. In order to achieve this, a better understanding of the various molecular mechanisms contributing to the oncogenesis and progression of RCC is required.

2.7 Genetics and molecular characteristics in the pathogenesis of RCC

The majority of RCCs occur sporadically and only <3% are hereditary or familial (Yap et al., 2015). Although less frequently encountered, hereditary RCCs play a significant role in the understanding of genetic changes and pathways affecting tumour progression. The earliest gene associated with RCC is the *VHL* gene, a tumour suppressor gene located at 3p25 loci. This gene was linked to an autosomal dominant hereditary disease, the VHL syndrome, in which affected individuals develop cancers such as pheochromocytomas, hemangioblastomas and ccRCC (Latif et al., 1993). Only 1.6% of all ccRCC cases are caused by hereditary VHL syndrome but the majority of

sporadic ccRCC contain *VHL* alterations as well (Neumann et al., 1998; Yap et al., 2015). One of the important functions of the VHL protein (pVHL) is its involvement in ubiquitination and degradation of hypoxia inducible factor 1α (HIF-1α) (Kaelin Jr, 2008). HIF-1α is a transcription factor which mediates angiogenesis, glucose uptake, cell proliferation and apoptosis by regulating targets such as VEGF, platelet-derived growth factor (PDGF), transforming growth factor alpha (TGFα), C-X-C chemokine receptor type 4 (CXCR4) and NF-κB (Morais, Gobe, Johnson, & Healy, 2011; Yap et al., 2015).

HIF-1α is marked and degraded by pVHL under normoxic conditions but HIF-1α accumulates in hypoxia, inducing transcription of genes that improve cell proliferation, oxygen delivery and angiogenesis (Kaelin Jr, 2008). Aberrations of *VHL* produce non-functional pVHL which allows HIF-1α to accumulate under normoxia, promoting tumorigenesis. *VHL* aberrations are found in 57-91% of ccRCC tumours (Yap et al., 2015). Besides *VHL*, the mTOR pathway is implicated in the pathogenesis of RCC. Genes involved in the pathway, consisting of mTOR, phosphatase and tensin homolog (*PTEN*), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*), AKT serine/threonine kinase 2 (*AKT2*) and others have mutations in 26-28% of ccRCC tumours (The Cancer Genome Atlas Research Network [TCGA], 2013; Sato et al., 2013). The PI3K-AKT-mTOR pathway regulates angiogenesis, cell cycle progression and proliferation. Furthermore, mTOR activation has been shown to increase the expressions of HIF-1α and HIF-2α in RCC cells at a translational level (Battelli & Cho, 2011). Most current targeted therapy drugs for treatment of mRCC are inhibitors of the pVHL or mTOR mediated pathways.

Recently, exome sequencing studies have discovered several novel genes involved in chromatin modification which are mutated in ccRCC. The newly identified genes are polybromo-1 (*PBRM1*), AT-rich interactive domain-containing protein 1A (*ARID1A*), BRCA1 associated protein-1 (*BAP1*), SET domain containing 2 (*SETD2*), and lysine (K)-specific demethylase 5C (*KDM5C*) (Duns et al., 2012; Hakimi et al., 2013). These chromatin modification genes have been implicated in other cancers as changes in the chromatin modification proteins could lead to disruptions of transcriptional regulation and tumour formation (Yap et al., 2015).

There are more studies on the genetic and molecular alterations in ccRCC compared to other subtypes as ccRCC has the highest prevalence. In pRCC, the second most common subtype, a gene on chromosome 7 has been positively identified and linked to pRCC, which is the *MET* proto-oncogene at 7q31. The protein product of the *MET* gene is c-met, a hepatocyte growth factor receptor (HGFR). Binding of the hepatocyte growth factor (HGF), or c-met to the *MET* receptor promotes oncogenesis, including cell motility, cell differentiation, proliferation, angiogenesis and invasion (Organ, Tsao, & de Bono, 2011). Leiomyomatosis and renal-cell cancer syndrome, a hereditary condition which predisposes individuals to develop type II pRCC is caused by mutation of the fumarate hydratase (*FH*) gene. The protein fumarate hydratase (FH) is a tricarboxylic acid cycle enzyme and the deficiency of functional FH can lead to metabolic imbalance, activation of the NRF2–antioxidant response element (ARE) pathway and increased oxidative stress, thus encouraging tumour growth (TCGA, 2016). Recent exome sequencing data have identified mutations in the *BAP1*, *SETD2*, AT-rich interaction domain 2 (*ARID2)* and the Nrf2 pathway genes as driver mutations in pRCC (Kovac et al., 2015). In addition, copy number gains of chromosomes 7, 12, 16 and 17 were found as early genetic changes in pRCC (Kovac et al., 2015).

Knowledge of genetic or molecular dysregulations in RCC provides an insight into the various mechanisms that may take part in tumour initiation or progression, and possibly aid in fine tuning targeted therapy or treatment in the future. There are ten important hallmarks of cancer genesis and progression, which are sustaining proliferative signalling, evading growth suppressor, activating invasion and metastasis, replicative immortality, inducing angiogenesis, resisting apoptosis, deregulated metabolism, evading the immune system, genome instability and inflammation (Hanahan & Weinberg, 2011). RCC is known to be a treatment resistant cancer and part of the reason for this resistance is the ability to evade apoptosis and the ability to sustain proliferation. Proteins of interest in this thesis, TRAF1 and NF-κB are involved in the apoptotic and proliferative signalling of cells. Therefore, this literature review will focus more on these areas.

2.8 Apoptosis in cells

Apoptosis, derived from a Greek work which means "falling off" is a natural process of programmed cell death in a multicellular organism. The process of natural cell death regulates cell numbers and in adult tissues, cell death balances cell division (Alberts et al., 2002). Apoptosis is unlike necrosis, which occurs as a result of acute cellular injury. During necrosis, the cell swells and bursts, causing some inflammatory response as the cell contents leak into its surroundings (Alberts et al., 2002). In contrast, the cell shrinks and condenses during apoptosis. Apoptosis can be identified by morphology under the light and electron microscope. In early apoptosis, the cells look smaller as cell shrinkage occurs and the nuclei become dense and compact because of chromatin condensation (pyknosis) (Elmore, 2007). Plasma membrane blebbing occurs next and the nuclear envelope and chromosomal DNA break into fragments in a process called

karyorrhexis (Alberts et al., 2002; Elmore, 2007). However, the contents of the cell are not released into the surroundings and phagocytic cells engulf the apoptotic cell.

Apoptosis commonly occur through two evolutionarily-conserved pathways, the intrinsic (mitochondrial) or extrinsic (death receptor) pathways. The intrinsic pathway occurs when the mitochondrial permeability increases and pro-apoptotic cytochrome-c is released into the cytoplasm in response to genetic damage, hypoxia, radiation, toxins, viral infections or severe oxidative stress (Elmore, 2007; Wong, 2011). The release of cytochrome-c is regulated by the B-cell lymphoma 2 (Bcl-2) family of proteins, which are the pro-apoptotic (Bax, Bid, Bim) and the anti-apoptotic (Bcl-2, Bcl-XL) proteins. The pro-apoptotic proteins promote while the anti-apoptotic proteins prevent the release of cytochrome-c from the mitochondria. Cytochrome-c in the cytoplasm binds to Apaf-1 and pro-caspase 9 to form an apoptosome. Pro-caspase 9 is cleaved, activating the executioner caspase 3. Activated or cleaved caspase 3 sets in motion the apoptotic process such as DNA fragmentation, protein cleavage, protein cross linking and expression of phagocytic cell ligands (Elmore, 2007). Other apoptotic proteins released from the mitochondria include the apoptosis inducing factor (AIF), second mitochondria-derived activator of caspase (Smac), direct IAP binding protein with low pI (DIABLO) and Omi/high temperature requirement protein A (HtrA2) (Wong, 2011). Smac/DIABLO or Omi/HtrA2 binds to inhibitor of apoptosis proteins (IAPs) allowing the release of caspase 3 or 9 for activation (Wong, 2011).

The extrinsic pathway is mediated by death receptors such as Fas receptor, type 1 tumour necrosis factor receptor (TNFR1) and TNF-related apoptosis-inducing ligand receptor 1 (TRAIL-R1). Binding of ligands such as TNF-α or Fas ligand (FasL) to these death receptors result in the recruitment of adapter proteins such as TNF receptor-

associated death domain (TRADD) and Fas-associated death domain (FADD), forming complexes which activate pro-caspase 8 (Elmore, 2007; Wong, 2011). Activated or cleaved caspase 8 is an initiator which causes the activation of caspase 3. Cleaved caspase 8 can also facilitate the cleavage of pro-apoptotic Bid which initiates the mitochondrial mediated apoptosis (Figure 2.4). Apoptosis is important in removing potentially cancerous cells and apoptosis resistance can lead to cancer development. Moreover, uncontrolled proliferation and lack of apoptosis promotes tumour growth. Apoptosis resistance can occur through imbalance of pro and anti-apoptotic proteins, reduced caspase function or compromised death receptor signalling (Wong, 2011).

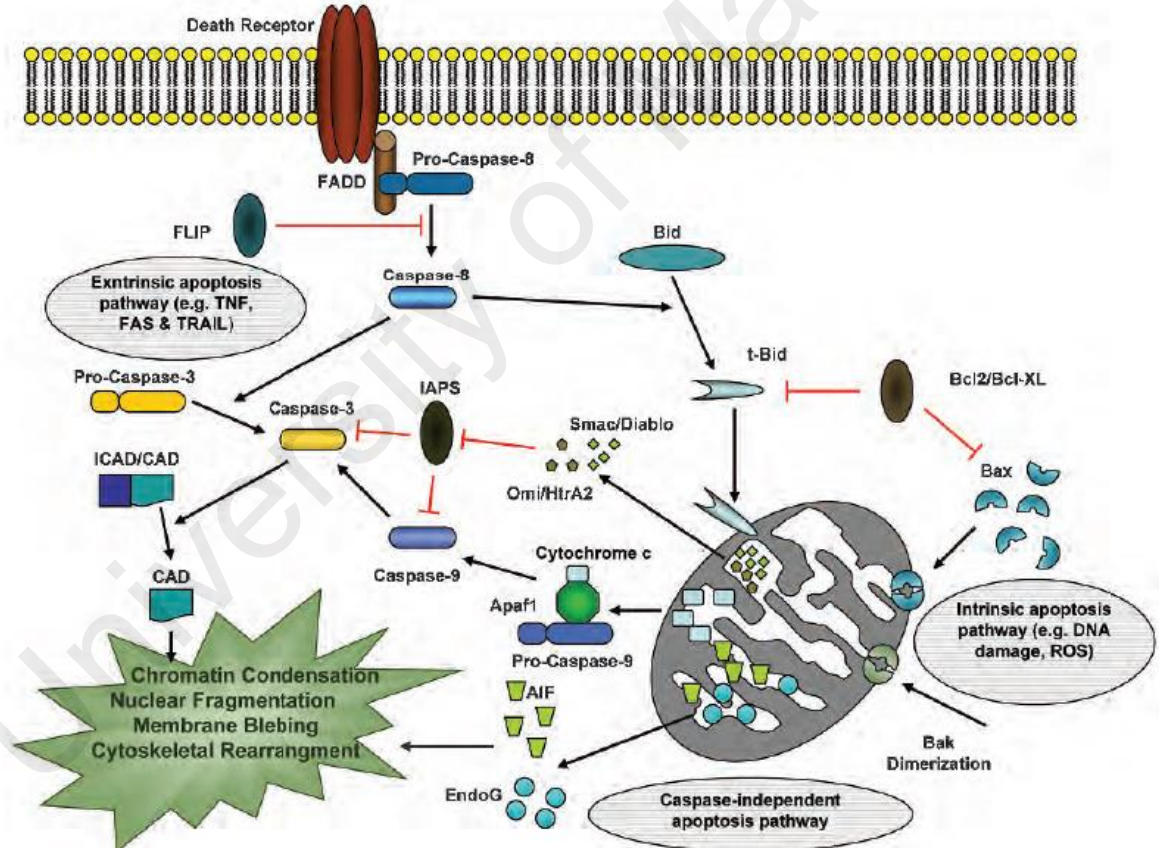


Figure 2.4. Extrinsic and intrinsic apoptotic pathways in cells
Image taken from (Ghavami et al., 2009).

2.9 Bcl-2 family of proteins

An example of the imbalance between pro and anti-apoptotic proteins leading to carcinogenesis is the dysregulation of the Bcl-2 family of proteins. The anti-apoptotic Bcl-2 is found to be upregulated in chronic lymphocytic leukaemia, mantle cell lymphoma, breast cancer and lung cancer (Kelly & Strasser, 2011).

In RCC, the association between Bcl-2 and prognosis or apoptosis is contradictory. A study by Gobe et al. (2002) noted that RCC tissue with high Bcl-2 and Bcl-XL had low apoptosis but there was no statistical correlation between Bcl-2 and apoptosis levels (Gobe, Rubin, Williams, Sawczuk, & Buttyan, 2002). Interestingly, Itoi et al. (2004) found that positive Bcl-2 expression correlated with lower stage and grade and better survival in RCC patients (Itoi, Yamana, Bilim, Takahashi, & Tomita, 2004). Immunopositivity of Bcl-2 in RCC tissue failed to show any significant association with prognosis or survival of patients in some studies (Pammer et al., 1998; Uchida et al., 2002; Vasavada, Novick, & Williams, 1998). However, one study found higher Bcl-2 expression in mRCC indicating that this protein may be involved in the progression of the disease (Lee et al., 2003). Another study found a significant association of Bax and Bcl-2 with RCC survival in the univariate analysis but neither were independent prognostic factors (Kallio et al., 2004). In RCC cell lines, inhibition of Bcl-2 expression sensitised the cells to cisplatin induced apoptosis (Kausch et al., 2005). Therefore, Bcl-2 and Bax may be involved in the apoptotic dysregulation of RCC tumour cells during oncogenesis and chemotherapy but they may not directly influence the prognosis of the disease.

2.10 Inhibitor of apoptosis proteins (IAPs)

There are a total of eight proteins in the human IAP family, which are neuronal apoptosis inhibitory protein (NAIP), cellular IAP-1 (cIAP-1), cellular IAP-2 (cIAP-2), X-chromosome liniked IAP (XIAP), survivin, apollon, melanoma IAP (ML-IAP) and IAP-like protein 2 (ILP-2). Of these, XIAP, cIAP-1, cIAP-2 and survivin are better researched and have been linked to cancers. IAPs can inhibit apoptosis in both the intrinsic and extrinsic pathways. CIAP-1 and 2 prevent apoptosis in the extrinsic pathway by ubiquitination of receptor-interacting protein 1 (RIP1). RIP1 is recruited to TRADD during TNFR1 pathway activation and the ubiquitination status of RIP1 determines whether the cell commits to apoptosis (Berthelet & Dubrez, 2013). Non ubiquitinated RIP1 will form complexes with FADD and caspases-8, activating apoptosis in response (Berthelet & Dubrez, 2013). Ubiquitination of RIP1 by cIAP-1 and 2 can also modulate cell survival via activation of the nuclear NF-κB pathway (De Almagro & Vucic, 2012). XIAP inhibits apoptosis by directly binding to caspase 3 or 9 preventing their cleavage (Berthelet & Dubrez, 2013). Although survivin inhibits apoptosis, the interaction between survivin and caspases is still unclear as reports of direct binding of survivin to caspases is contradictory (Chen, Duan, Zhang, & Zhang, 2016).

XIAP, cIAP-1, cIAP-2 and survivin have been found to be upregulated in various cancers including RCC (De Almagro & Vucic, 2012; Wong, 2011). Survivin is a potential prognostic indicator as it is an independent prognostic factor for RCC progression and survival (Byun, Yeo, Lee, & Lee, 2007; Krambeck et al., 2007; Lei, Geng, Guo-Jun, He, & Jian-Lin, 2010; Parker et al., 2006). Similarly, high XIAP expression in RCC tissue predicted a worse prognosis for patients (Mizutani et al.,

2007; Ramp et al., 2004). CIAP-1 and 2 expressions are reported to be higher in RCC tissue compared to the normal kidney and only cIAP-1 is associated with prognosis (Kempkensteffen et al., 2007). However, it is tumours with lower cIAP-1 expressions which are more aggressive and have an unfavourable outcome (Kempkensteffen et al., 2007).

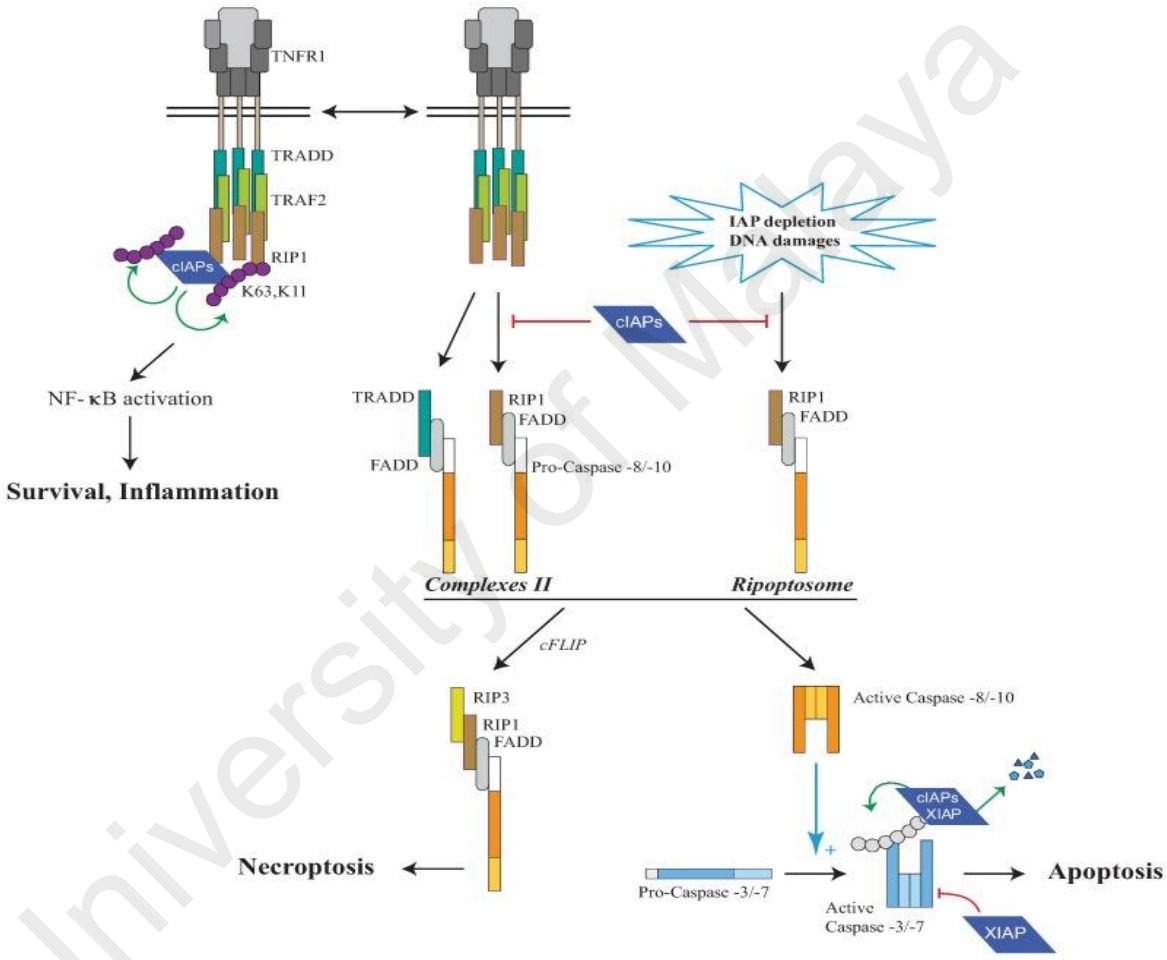


Figure 2.5. Inhibition of apoptosis via IAPs
Image taken from (Berthelet & Dubrez, 2013).

2.11 TNF receptor associated factors (TRAFs)

TRAFs are intracellular proteins which act as adaptor proteins in the formation of receptor-associated signalling complexes to mediate pathways involved in inflammatory, antiviral, survival and apoptotic responses in cells. There are seven

TRAFs identified in mammals, which are TRAF1-7. TRAFs contain a highly conserved C-terminal region called the TRAF domain (Xie, 2013). TRAF1 is unique as it lacks the N-terminal RING finger domain found in other TRAFs (Xie, 2013). TRAF2 is well described as it is involved in many TNFR related signalling complexes. One of the pathways facilitated by TRAF2 is the TNFR1 signalling cascade. Binding of TNF-α to TNFR1 initiates the recruitment of TRADD, FADD, TRAF2 and TRAF1 to the receptor, forming complexes with RIP1, cIAP-1 and 2 (Salvesen & Duckett, 2002). The function of these complexes (apoptotic or survival) depends on the ubiquitination status of RIP1 as mentioned earlier. Besides TNFR1, TRAF1 and 2 also associate with the TNFR2 and cluster of differentiation 40 (CD40) receptor pathways to regulate cell apoptosis or survival (Henkler et al., 2003; Lee & Choi, 2007).

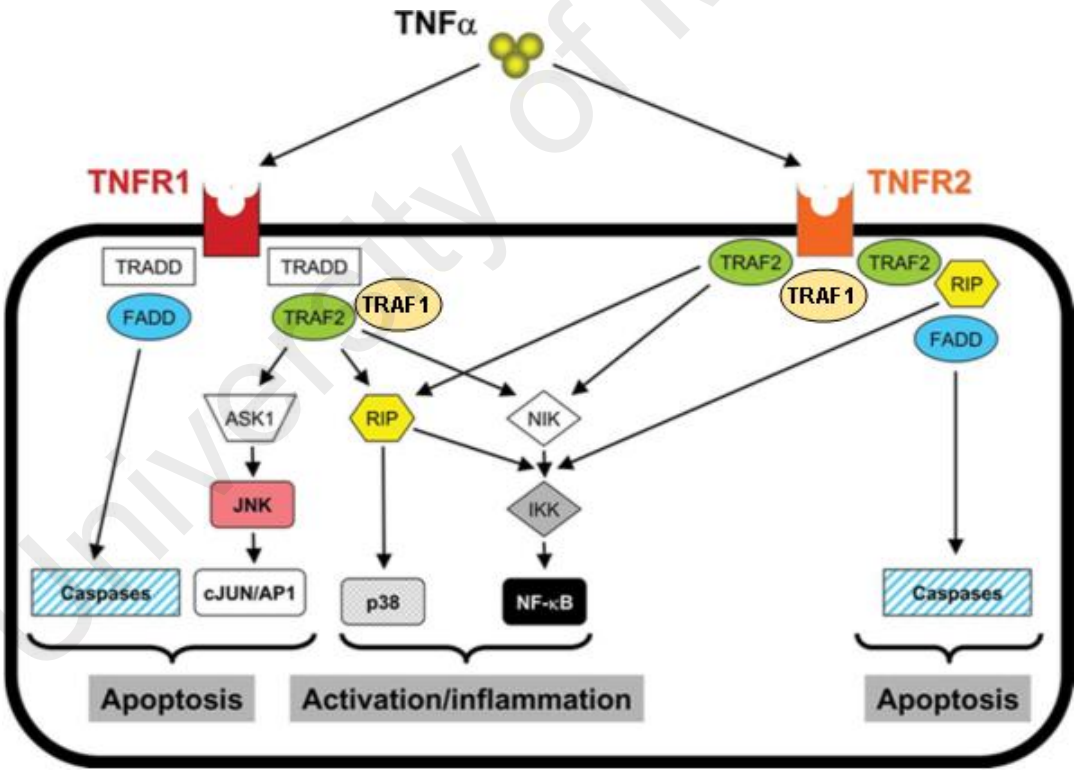


Figure 2.6. Examples of TRAF1 and TRAF2 pathways in regulating apoptosis, cell survival and inflammation
Image with modifications taken from (Russo & Polosa, 2005).

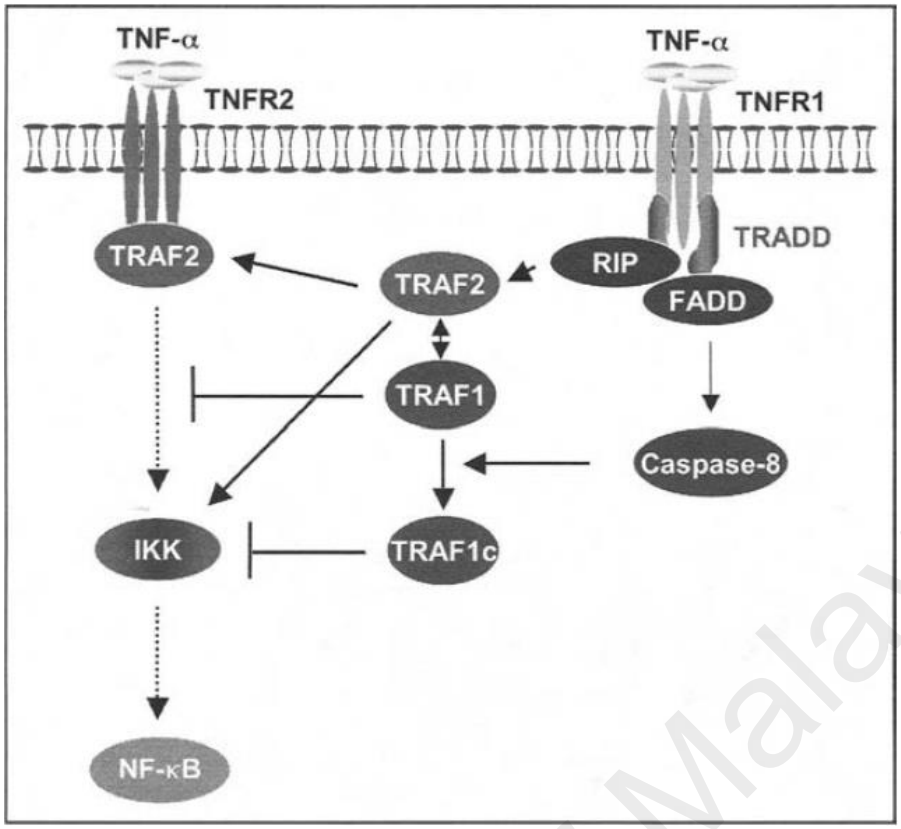


Figure 2.7. The dual roles of TRAF1 as an activator or inhibitor of NF-κB pathways. Image taken from (Lee & Choi, 2007).

TRAF1, which frequently associates with TRAF2 to exert its biological functions is not as well understood as TRAF2. TRAF1 can act as an anti or pro-apoptotic protein, depending on the cell type and cleavage by caspase 8. In its anti-apoptotic role, TRAF1 as a whole protein can interact with TRAF2, cIAP-1 and 2 to inhibit caspase 8 activation (Zapata & Reed, 2002). Furthermore, TRAF1 can induce NF-κB signalling through its interaction with TRAF2, an activator of NF-κB. However, caspase 8 can cleave TRAF1 into two fragments. The C-terminal TRAF1 fragment is pro-apoptotic as it increases TNFR1- and Fas-mediated apoptosis (Lee & Choi, 2007). The pro-apoptotic fragment is also shown to be able to prevent the binding of TRAF2 to the TNFR family, blocking the activation of NF-κB (Henkler et al., 2003; Lee & Choi, 2007). Besides that, the pro-apoptotic fragment acts as an IκB kinase (IKK) inhibitor, thus preventing activation of the NF-κB pathway (Lee & Choi, 2007). TRAF1 has also been reported to take part in apoptotic control during ischaemic events. In studies examining liver and

brain ischaemia, TRAF1 was shown to associate with apoptosis signal-regulating kinase 1 (ASK1) to activate the mitogen-activated protein kinase kinase/c-Jun N-terminal kinase (MKK/JNK) cell death pathway (Lu et al., 2013; Zhang et al., 2014). ASK1 pathway is activated as a result of oxidative stress. Hence, during liver or brain ischaemia, augmented TRAF1 levels induce the phosphorylation of ASK1 and increase cell apoptosis (Lu et al., 2013; Zhang et al., 2014).

Among the TRAFs, TRAF1 is identified as a protein of interest in RCC. In other cancers, TRAF1 was found to be upregulated in B cell lymphoma, non-Hodgkin lymphoma and circulating chronic lymphocytic leukemias (Lee & Choi, 2007). Other than RCC, the function or expression of TRAF1 in solid tumours is not well defined. Rajandram et al. (2012) showed that TRAF1 expression is dysregulated in RCC tissue from a cohort of Australian patients and is involved in apoptotic regulation in RCC cells (Rajandram, Bennett, Wang, et al., 2012). TRAF proteins have not been studied in RCC before Rajandram et al. (2009), hence this thesis would like to further elucidate the biomarker potential and functional role of TRAF1 in RCC cells (Rajandram, Pat, Li, Johnson, & Gobe, 2009).

2.12 Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)

NF-κB is involved in various biological processes such as inflammation, cell survival, apoptosis, proliferation and cell differentiation (Hayden & Ghosh, 2012). The NF-κB family of proteins consist of five subunits which are RelA (p65), RelB, cRel, NF-κB1 (p105/p50) and NF-κB2 (p100/p52). NF-κB1 and NF-κB2 are synthesized as inactive precursors and proteolytic cleavage result in the p50 and p52 active forms. NF-κB subunits form hetero or homo-dimers to bind to target genes, triggering transcription genes controlling various biological functions. In normal states, NF- κB is found in the

cytoplasm bound to inhibitory molecules known as the inhibitors of NF-κB (IκB). The different such IκB proteins identified are IκB-α, IκB-β, IκB-ε and Bcl-3 (Hayden & Ghosh, 2012). The IKK complex, which consists of two catalytic units, IKK-α and IKK-β and a regulatory unit, IKK-γ are involved in the activation of NF-κB. The binding of ligands to activating receptors can trigger the ubiquitination and phosphorylation of the IKK complex, which in turn phosphorylates and degrades the IκBs (Dellegrottaglie, Sanz, & Rajagopalan, 2006). Once activated, the NF-κB subunits translocate to the nucleus where they bind to κB elements and regulates transcription of target genes.

The activation of these dimers depends on the type of ligands and receptors involved. The two most commonly known NF-κB activation pathways are the classical and alternative pathways. In the classical pathway, the two NF-κB subunits involved are p50 and p65. The classical pathway is activated by binding of ligands such as TNF-α, CD40 ligand, interlueukin-1 (IL-1) and lipopolysaccharide (LPS) to receptors (Nishikori, 2005). This pathway is commonly implicated in cancer as the target genes are involved in regulating proliferation, survival and differentiation. Examples of genes regulated by this pathway are pro-survival genes (Bcl-2, Bcl-XL, IAPs, survivin), proliferation control (cyclins, IL-1, IL-6, c-MYC), angiogenesis (EGF, VEGF, IL-1, IL-8), metastasis (ICAM-1, VCAM-1, vimentin) and inflammation (TNF, IL-1) (Morais et al., 2011; Nishikori, 2005). In cancers, NF-κB is found to be upregulated in breast, lung, ovarian, prostate cancer and RCC, among others (Sethi, Sung, & Aggarwal, 2008).

The subunits participating in the alternative pathway are p52 and RelB. Binding to activating receptors such as B-cell-activating factor belonging to TNF family receptor

(BAFFR), lymphotoxin β-receptor (LTβR), CD40 and receptor activator for nuclear factor κB (RANK) initiate downstream signalling pathways. This sets into motion the phosphorylation of p100 by IKK-α to generate p52, which form a dimer complex with RelB. The p52/RelB dimer translocates to the nucleus where it activates signalling pathways that regulate lymphoid development, B-cell maturation and survival, dendritic cell maturation and bone metabolism (Sun, 2011). Although not as well understood, the cRel subunit is believed to play a role in proliferation, differentiation, B-cell development and cytokine production of T cells by forming a dimer complex with p50 (Visekruna, Volkov, & Steinhoff, 2012). The cRel subunit also forms dimers with p65 but other combinations can also occur (Hunter, Leslie, & Perkins, 2016).

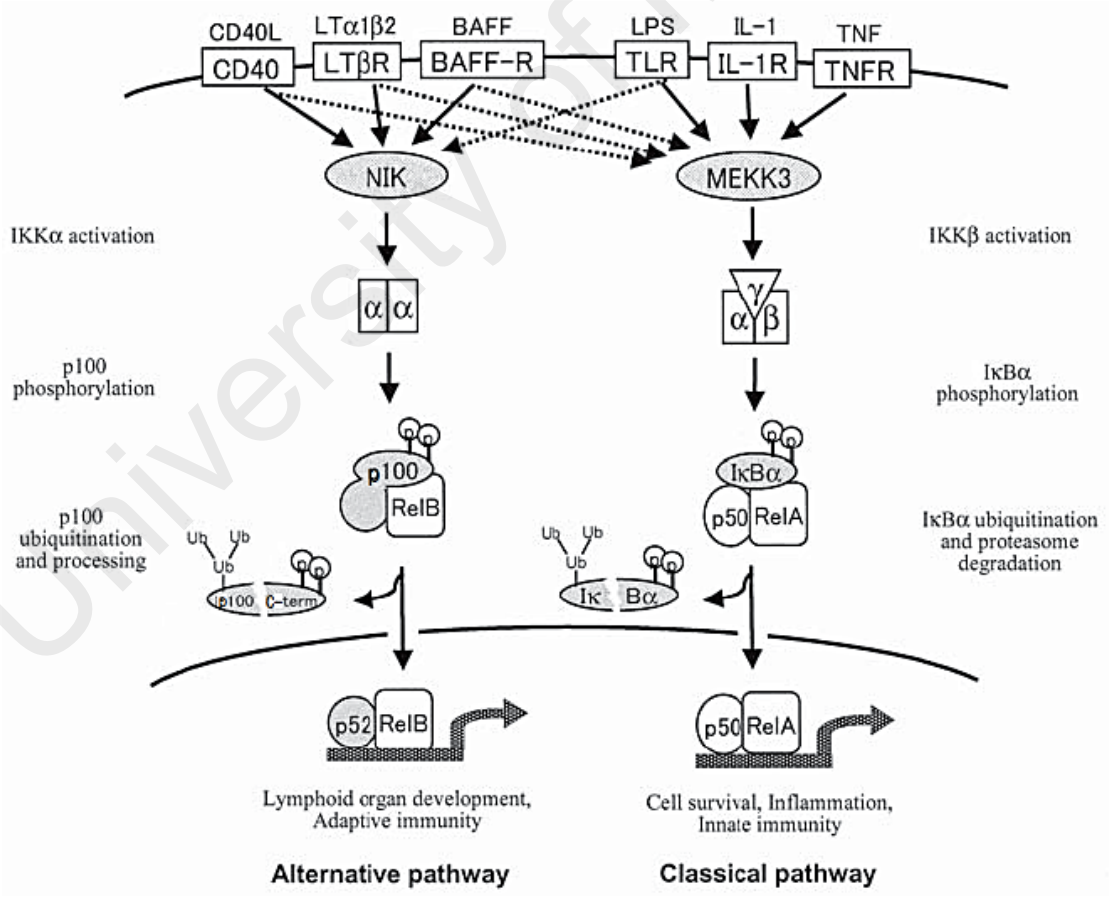


Figure 2.8. The classical and alternative pathways of the NF-κB subunits
Image taken from (Nishikori, 2005).

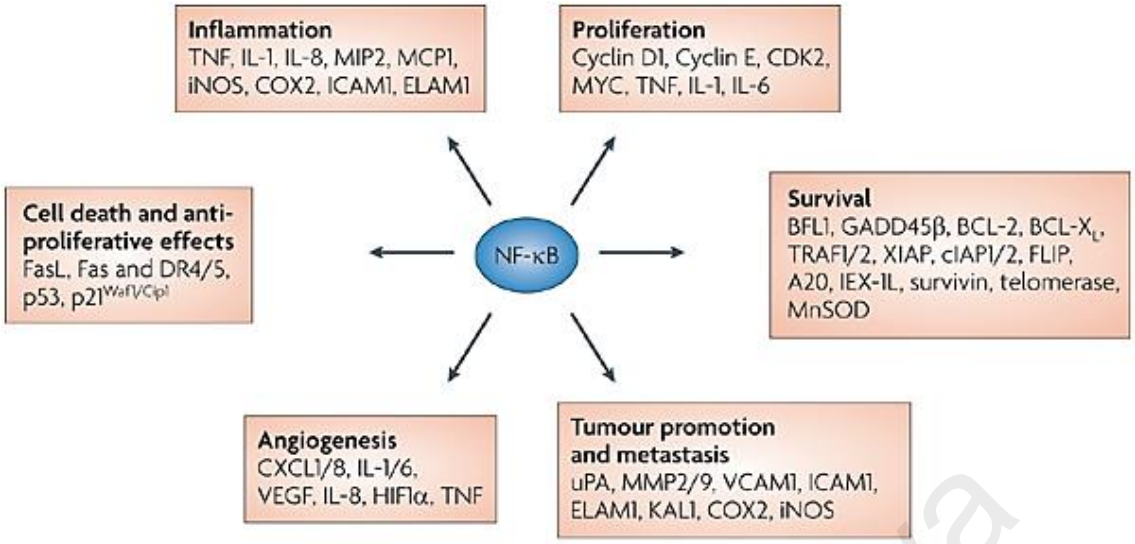


Figure 2.9. Examples of the various genes that can be activated by NF-κB pathway (Especially the classical pathway)
Image taken from (Baud & Karin, 2009).

Besides resistance to apoptosis, one of the causes of carcinogenesis is deregulation of cell proliferation. Hence, the focus of this project is on the regulation of apoptosis and proliferation by NF-κB and TRAF1.

2.13 Biomarkers and prognostic indicators in RCC

RCC is a disease with good prognosis if detected early as complete removal of the tumour can be achieved at early stages of the disease. The recurrence rate tends to be higher and occur earlier if the tumour is locally advanced (Adamy et al., 2011). Currently, there is no reliable routine diagnostic or prognostic indicator test (serum or immunohistochemistry (IHC)) in RCC, unlike in prostate and breast cancer. As mentioned earlier, metastatic RCC is resistant to chemotherapy, radiotherapy and although molecular targeted therapy has improved survival, patients develop resistance requiring second or third line therapies. The continuous research into the contribution of molecular changes to the progression of RCC has enabled new drug therapies to emerge but there is still a need to identify patients who will respond or develop resistance to the

specific therapy given. At present, monitoring of RCC is solely clinical, depending largely on routine CT or USG scans. Various predictive models have been suggested by research groups which rely on clinical characteristics of the tumour, blood test results, performance status and symptoms (Sun et al., 2011). Inclusion of circulating (serum, plasma or urine) and tissue markers into the models may help improve the diagnostic or predictive models. Furthermore, a better understanding of possible impairments in gene or protein regulation would help identify mechanisms involved in tumour formation or progression and potential drug targets for RCC. Further details on promising RCC biomarkers will be described in Chapter 7.

CHAPTER 3 : CLINICAL PROGNOSTIC FACTORS AND SURVIVAL OUTCOME OF RENAL CELL CARCINOMA PATIENTS – A MALAYSIAN SINGLE TERTIARY CENTRE ANALYSIS

3.1 Introduction

The establishment of a comprehensive cancer registry is important as it provides information on the incidence rate, demographic, disease management pattern, treatment outcome and mortality rate of cancer patients. This information provides the essential data for patient care improvement, epidemiology studies and medical research. There is a paucity of data on kidney cancer/RCC patients and RCC research in Malaysia as most research groups concentrate on more common cancers such as breast or prostate cancer. The objective of this chapter was to determine the clinical characteristics, demographics, survival rates as well as presenting signs and symptoms of RCC patients treated at University Malaya Medical Centre (UMMC). Clinicopathological and survival data obtained from this study may help shed light on the survival and prognosis of Malaysian RCC patients. Although this is a single centre registry, this would be a good starting platform to encourage multi-centre cancer databases as this will be an ongoing registry even after the completion of this PhD project. In addition, the clinical information gathered would be useful for prognostic association with proteins of interest to achieve the objectives in the following chapters.

The contents or results of this chapter have been published (Yap et al., 2013). Please refer to Appendix A for article.

3.2 Literature review

The age standardised incidence of kidney cancer in Malaysia was 2.3 per 100,000 in 2005 and 2.4 per 100,000 in the year 2006 (NCR, 2005, 2006). However, in the latest 2007-2011 National Cancer Registry report, the age standardised incidence of kidney cancer in Malaysian males was 1.7 per 100,000 (NCR, 2016). In Singapore, kidney cancer is rising and currently ranked the 9th most common cancer among males, with an age standardised incidence of 8.7 per 100,000 in the 2010-2014 Singapore Cancer Registry report (Singapore Cancer Registry [SCR], 2015). The decrease in incidence rate in Malaysia could be due to under reporting of this cancer. This limitation was mentioned in the National Cancer Registry 2007-2011. The report stated that cancer registry is relatively new in Malaysia and many years of operation is required for attaining adequate levels of data quality (NCR, 2016). Therefore, at present, it is believed that the actual incidence rate is likely to be much higher than that reported in the national cancer registry.

The improvement and wider availability of medical imaging technologies have resulted in an increased in incidence of asymptomatic localised renal tumours, especially in developed countries (Znaor et al., 2015). As mentioned in Chapter 2 : Literature Review, incidentally discovered tumours are likely to be of lower stage and grade. The classic triad of symptoms for RCC, which are haematuria, flank pain and palpable abdominal mass, are not always present. This is especially true in small renal masses. Paraneoplastic symptoms appear as the tumour progresses to more advanced stages. RCC is the most frequent urological malignancy affected by paraneoplastic syndrome (Sacco et al., 2009). These signs and symptoms can be caused by the release of tumour associated proteins from the tumour or by the inflammatory response elicited

by the tumour (Kim et al., 2003). Common paraneoplastic symptoms in RCC are anaemia, abnormal liver function, hypercalcaemia, hypoalbuminemia, weight loss, malaise, thrombocytosis, among others (Kim et al., 2003; Sacco et al., 2009). Studies have shown that symptomatic tumours, especially paraneoplastic related ones, have poor survival prognosis (Parker et al., 2006). However, most studies have analysed symptomatic tumours as a group compared to asymptomatic tumours (Lee, Katz, Fearn, & Russo, 2002; Yap et al., 2013). The prognostic influence of individual signs and symptoms was often not evaluated. To our knowledge, only Kim et al. (2003, 2004) has evaluated the presenting signs and symptoms individually and found that cachexia related symptoms (lethargy, weight loss, anorexia, hypoalbuminemia) were the strongest prognostic factors for survival (Kim et al., 2003; Kim, Han, Zisman, Figlin, & Belldegrun, 2004).

As mortality and prognostic data on renal tumours are lacking in Malaysia, this presents the first report of survival data and detailed clinical characteristics of patients with RCC in a tertiary hospital. In addition to that, the prognostic value of presenting clinical signs and symptoms were examined individually. These symptoms at presentation may offer an early prognostic insight before definitive treatment.

3.3 Methodology

3.3.1 RCC patients' database

Patients diagnosed and treated for RCC from 2003 to 2012 were identified retrospectively through the UMMC database, urological unit referrals and urology surgical operation list. Patients' clinicopathological data and demographics were

collected from UMMC online database or patients' medical record folders. Symptoms at presentation were determined by the attending physicians during pre-treatment medical history taking and physical assessment. History of frank haematuria, flank pain, fever, lethargy and loss of weight and appetite were retrieved from the medical records. The presence of palpable abdominal mass was reported during physical examination by the attending physician. Blood test results were taken from pre-treatment assessments. Elevated alkaline phosphatase (ALP) was defined as ALP higher than 136IU/L and hypoalbuminemia was defined as serum albumin lower than 35g/L. Patient with corrected calcium higher than 2.60mmol/L were considered to be hypercalcaemic. Thrombocytosis was defined as a platelet count of higher than $400x10^9$/L. Women and men with haemoglobin less than 120g/L and 130g/L respectively were considered as anaemic. Detection was taken as incidental when the patients were asymptomatic at presentation, diagnosed during investigation for an unrelated symptom or during routine health screening.

Histological type and tumour grading (Fuhrman's classification) were determined from the pathologist's report and re-confirmed by a senior pathologist. The histological type was unspecified for patients who had metastatic RCC but did not undergo surgery or biopsy. The diagnoses of mRCC in such cases were made from CT or USG scans. Some tumours had no grading for the following reasons : Fuhrman's grading was only done for ccRCC and pRCC subtypes, mRCC patients without surgery or biopsy were not graded and some biopsy samples were insufficient for proper grading. The 2009 TNM system proposed by the American Joint Committee on Cancer (AJCC) was used for pathological tumour staging (AJCC, 2012). Survival information was acquired from patients' medical records or National Registration Department, Malaysia. All data were handled with strict confidentiality.

3.3.2 Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics version 20 (IBM, USA). A p value less than 0.05 was considered statistically significant. The Cox proportional hazards regression was used to determine the prognostic factors for disease specific survival (DSS) based on the symptoms at presentation. Factors that were statistically significant in the univariate analysis were subjected to multivariate analysis, which was adjusted for stage. Survival curves and survival rates were obtained from the Kaplan-Meier and life table analyses. Survival differences between stages or groups with and without symptoms were evaluated using the log rank test.

3.4 Results

A total of 151 patients with RCC treated at UMMC were analysed. Patients' demographics and clinical characteristics are listed in Table 3.1. An average of 20 nephrectomies were performed at UMMC annually (data not shown in table). The majority of the patients were Chinese (53.6%) as the hospital is located in the proximity of housing areas populated mainly by Chinese (eg. Petaling Jaya, Kelana Jaya, Sunway, Subang Jaya). Out of this cohort of patients analysed, 76.8% were symptomatic at presentation. However, incidental detection had increased from 19.6% to 25.3% between 2003-2007 and 2008-2012 respectively. The more common signs and symptoms reported were anaemia, hypoalbuminemia, loin pain and loss of weight (Table 3.1).

Table 3.1. Patient characteristics and clinical presentations

Characteristics	Sample size
Number of patients	151
Mean age (range) years	60.7 (34-83)
Men/women (%)	66.9/33.1
Ethnicity (%) Malay Chinese Indian Others	 39 (25.8) 81 (53.6) 29 (19.2) 2 (1.3)
Tumour side (%) Left Right Bilateral	 77 (51.0) 73 (48.3) 1 (0.7)
Mean tumour size (range) cm	6.5 (1.5-17.3)
Histological type (%) Clear cell Papillary Chromophobe Multilocular cystic Unspecified	 120 (79.5) 13 (8.6) 3 (2.0) 1 (0.7) 14 (9.3)
Pathological stage (TNM,2009) (%) Stage I Stage II Stage III Stage IV	 66 (43.7) 25 (16.6) 10 (6.6) 50 (33.1)
Metastases (%) No metastasis Metastasis at presentation Metastasis post operation	 88 (58.3) 50 (33.1) 13 (8.6)
Fuhrman's grade (%) G1 G2 G3 G4 Unknown	 11 (7.3) 50 (33.1) 24 (15.9) 10 (6.6) 56 (37.1)
Presentation (%) Incidental Symptomatic	 35 (23.2) 116 (76.8)
Signs and Symptoms (%) Frank haematuria Loin pain Palpable abdominal mass	 54/151 (35.8) 60/151 (39.7) 48/151 (31.8)

Table 3.1 continued

Characteristics	**Sample size**
Loss of weight (LOW)	60/151 (39.7)
Fever	20/151 (13.2)
Lethargy	20/151 (13.2)
Anaemia	63/148 (42.6)
Hypoalbuminemia	58/144 (40.3)
Hypercalcaemia	11/100 (11.0)
Elevated ALP	27/143 (18.9)
Thrombocytosis	24/147 (16.3)
Treatment (%)	
Radical nephrectomy	104 (68.9)
Partial nephrectomy	17 (11.3)
Radiofrequency Ablation (RFA)	2 (1.3)
Cryosurgery	1 (0.7)
No surgery	27 (17.9)

The median follow up for these patients was 26 months (Range : 0.2-193.6 months). The survival rates are presented in Table 3.2. There was a total of 44 deaths from RCC in this cohort of patients, throughout the duration of the follow up period (2003-2013). TNM staging significantly affects survival ($p<0.001$), with stage 4 or metastatic patients having the worst prognosis (Figure 3.1). There was no significant association between ethnicity and stage at presentation ($p=0.971$). There was a trend towards worse survival outcome in higher Fuhrman grade tumours compared to lower grade ones, but this was not statistically significant in our case series ($p=0.088$). Patients who were symptomatic at presentation had worse survival prognosis compared to asymptomatic patients ($p = 0.009$; Hazards ratio (HR) 4.74, 95% CI 1.47-15.36). Symptomatic tumours were of higher stage compared to asymptomatic tumours ($p<0.001$). For symptomatic patients, 47.4% were stage 3 or 4 while only 14.3% of asymptomatic patients were stage 3 or 4 at diagnosis. Additionally symptomatic tumours were significantly larger ($p<0.001$), with a mean size of 7.2±3.8cm compared to incidentally detected tumours, at 4.3±2.1cm.

Table 3.2. Survival rate of UMMC RCC patients by TNM stage

	Survival rate (%)	
Stage	**1 Year**	**5 Years**
I	98	98
II	96	90
III	79	67
IV	49	13
Overall	80	69

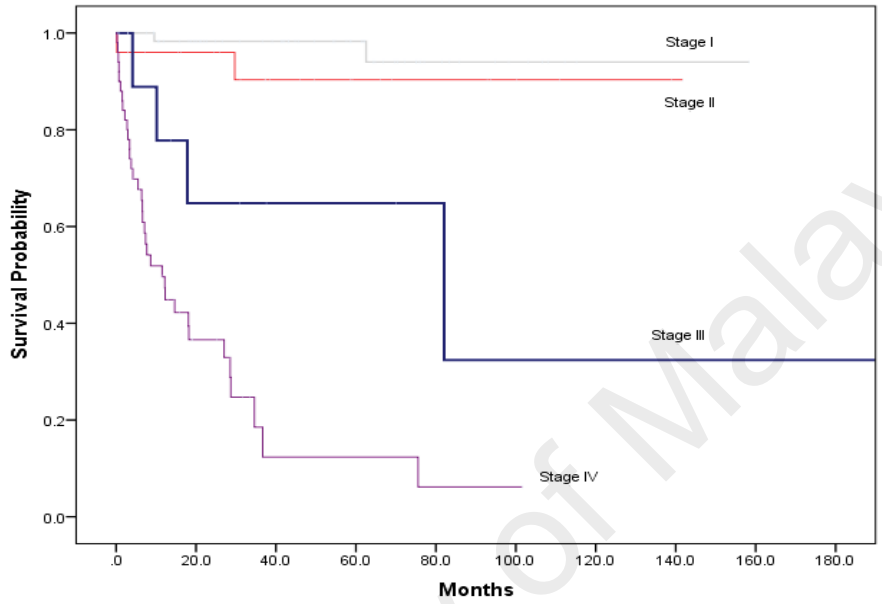


Figure 3.1. Survival of patients grouped by TNM staging
TNM staging significantly affected DSS (p<0.001).

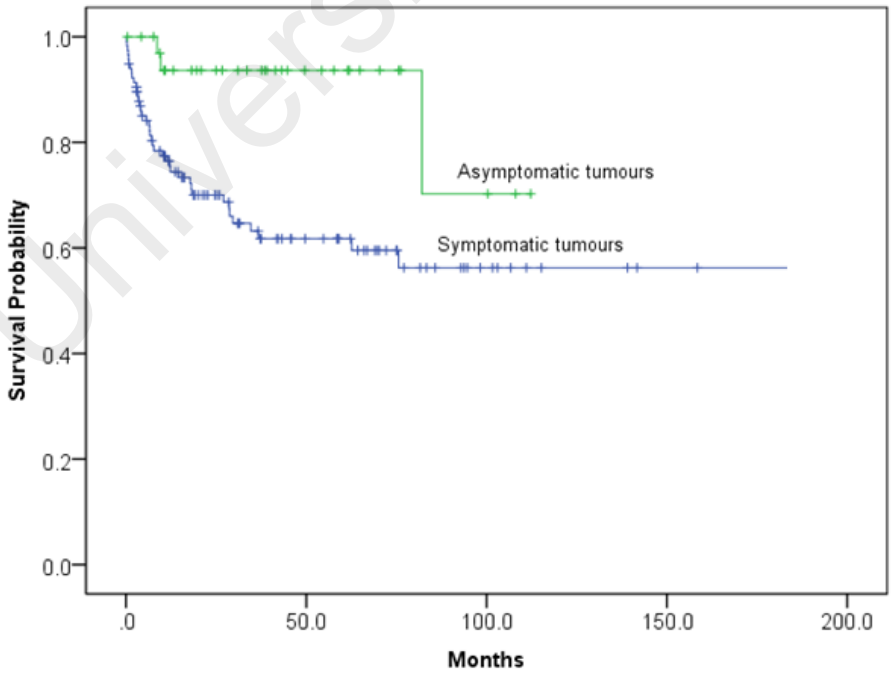


Figure 3.2. Survival of asymptomatic and symptomatic patients
Patients who were symptomatic at presentation had an unfavourable DSS outcome (Log rank p=0.004).

In a univariate Cox regression analysis to determine prognostic indicators, all factors significantly affect DSS except frank haematuria and loin pain (Table 3.3). After adjustment for stage in a multivariate analysis, only palpable abdominal mass remained statistically significant (p=0.027) while hypoalbuminemia had a near significant value (p=0.051). The mean tumour size of palpable abdominal mass, 9.5±4.3cm, was significantly larger than non-palpable mass, 5.3±2.7cm (p<0.001). Patients with palpable abdominal mass had poor survival prognosis as illustrated in Figure 3.3.

Table 3.3. Univariate and multivariate analysis of prognostic factors for DSS

	Univariate analysis			**Multivariate analysis adjusted for stage**		
	Hazard ratio	**95% CI**	**p Value**	**Hazard ratio**	**95% CI**	**p Value**
Frank hematuria	1.03	0.55-1.90	0.937	-	-	-
Loin pain	1.23	0.67-2.25	0.501	-	-	-
Abdominal mass	4.34	2.35-8.02	**<0.001**	2.77	1.12-6.74	**0.027**
LOW	2.42	1.32-4.43	**0.004**	1.82	0.76-4.63	0.169
Fever	3.69	1.84-7.39	**<0.001**	2.38	0.50-4.20	0.493
Lethargy	2.76	1.35-5.60	**0.005**	0.99	0.44-3.39	0.711
Anaemia	5.00	2.50-10.02	**<0.001**	2.95	0.62-5.30	0.282
Hypercalcaemia	7.08	3.26-15.34	**<0.001**	4.45	0.91-6.61	0.075
Hypoalbuminemia	7.74	3.69-16.28	**<0.001**	3.00	0.99-8.87	0.051
Thrombocytosis	3.33	1.71-6.49	**<0.001**	1.48	0.52-4.64	0.428
Elevated ALP	4.65	2.52-8.60	**<0.001**	1.13	0.30-2.28	0.298

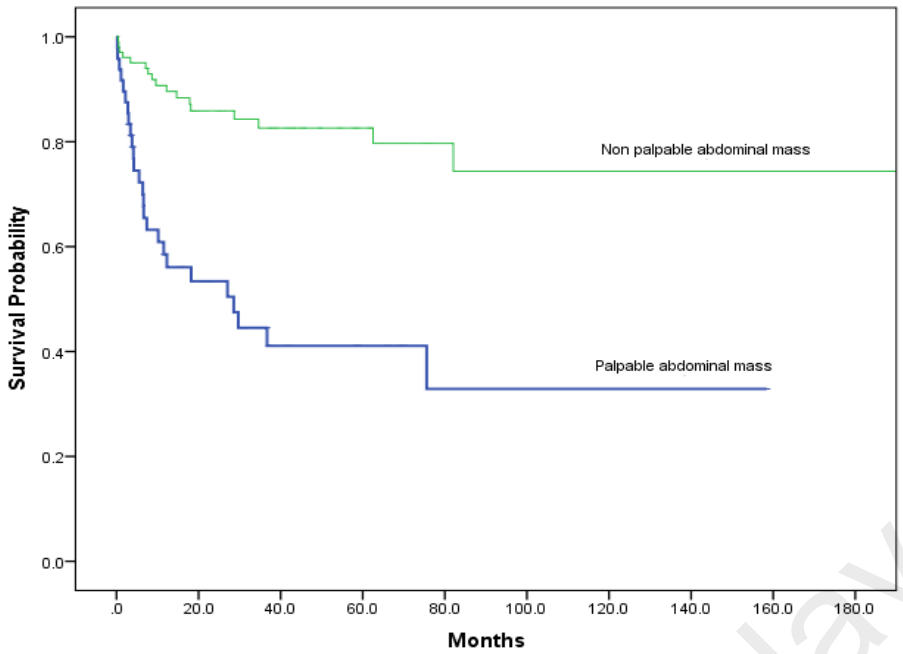


Figure 3.3. Survival of patients with palpable and non-palpable abdominal mass
DSS probability of patients with palpable abdominal mass is lower than patients with non-palpable abdominal mass (Log rank p<0.001).

3.5 Discussion

There is a lack of data on the survival rates of RCC patients in Malaysia as National Cancer Registries have only reported the incidence of kidney cancer in general. RCC is the 9th most common causes of cancer deaths in Singapore according to the Singapore cancer registry report 2010-2014 (SCR, 2015). Other South East Asian countries such as Thailand, Indonesia and Philippines also do not have sufficient information on RCC mortality (Yap et al., 2013). Therefore, this has encouraged the establishment of a more comprehensive RCC database in UMMC. The lower incidence of kidney cancer in Malaysia compared to Singapore, a country with geographical proximity and similar ethnicity groups (Malays, Chinese and Indians), is likely caused by the under reporting. There has been only one other publication on renal tumours in Malaysia which included urothelial cancers and nephroblastomas in their case series of 72 patients (Singam et al., 2010). They found that Chinese patients were presented at a later age and stage than Malay patients (Singam et al., 2010). However, there was no significant association of

race and stage in the UMMC cohort of patients. The reason might be that only RCCs were included in this case series while other kidney cancers were also analysed in the other study. A larger multi-centre sample size might give a more accurate portrayal of the differences or similarities between the different ethnic groups.

The 5 year survival rates of RCC patients in UMMC is lower than that reported in Singapore General Hospital (SGH) with 69% compared to 86% (Kanesvaran, 2009). The lower incidental detection of RCC in UMMC might have affected the survival rate, as a report of RCC cases in SGH (2001-2008) noted that 42% of tumours were detected incidentally, compared to 23.2% in UMMC (Lee, Yuen, & Sim, 2011). Based on opinions of medical personnel, it was estimated that 80-90% of RCC patients are symptomatic at presentation in Malaysia (Naito et al., 2010). The reason for the high incidence of symptomatic RCC at presentation could be multifactorial and dependent on the access to health care facilities, disease awareness or health seeking behaviour. A study exploring issues influencing health seeking behaviour in Malaysian cancer patients cited lack of belief in personal susceptibility and financial constraints among the obstacles in health screening for cancers (Farooqui et al., 2013). Furthermore, RCC symptoms such as loin pain, fever or loss of weight can mimic other diseases, hence patients may not suspect it as renal cancer. This has somewhat contributed to the late detection of RCC, and the 33.1% of metastatic cases at presentation in this case series. Hence, there is a constant need to discover and evaluate potential detection or prognostic markers for RCC.

Although incidental detection is still low in this cohort, the proportion has increased over the years. Asymptomatic renal tumours were reported to have a favourable independent effect on prognosis in two large case series from Italy and Iceland (Ficarra

et al., 2003; Palsdottir et al., 2012). This trend is similarly found in our RCC patients where symptomatic tumours predict shorter survival. Strong evidences on the prognostic value of clinical symptoms has prompted Lee et al. (2002) and Patard et al. (2003) to propose a classification system based on symptoms at presentation (Lee et al., 2002; Patard et al., 2003). Symptoms were grouped as incidental, localised or paraneoplastic. Paraneoplastic symptoms were associated with the most unfavourable prognosis followed by localised and incidental detections (Lee et al., 2002; Patard et al., 2003). Kim et al. (2003) was the first group to analyse all clinical signs and symptoms separately (Kim et al., 2003). Similarly, to determine the prognostic effect of each symptom, we analysed the presenting signs and symptoms individually. Not surprisingly, all paraneoplastic related signs and symptoms like fever, lethargy, LOW, anaemia, hypercalcaemia, thrombocytosis and elevated ALP were significantly associated with poorer survival in the univariate analysis.

Palpable abdominal mass was the sole classic triad symptom significantly associated with survival, and the only symptom which was consistently significant even after stage adjustment. This was different from the findings of Kim et al. (2003) as cachexia related symptoms were the independent factors in their case series (Kim et al., 2003). Differences in the proportions of presenting symptoms could possibly account for the different outcomes. Only 4.4% of their patients presented with palpable mass compared to 31.8% in our case series. The strong prognostic indication of palpable abdominal mass could be associated with the size of the tumour as palpable abdominal mass tumours are significantly larger compared to non-palpable tumours. Other studies have demonstrated that a larger tumour size confers a disadvantage to survival (Brookman-May et al., 2015; Suer, Baltaci, Burgu, Aydogdu, & Gogus, 2013). Symptomatic tumours were also associated with a larger mean tumour size in this analysis.

The relevance of classic triad of presenting symptoms, which usually accounts for less than a third of patients, has often been considered increasingly obsolete in detecting RCC with the improvement of imaging technologies. However, this might not be entirely true in situations where incidental detection is still low as in this study. Besides palpable abdominal mass, staging by the TNM classification remains a strong predictor for survival. Interestingly, Fuhrman grading did not reach statistical significance. Intra-observer variability might have possibly affected the results as grading was done by different pathologists (Bektas et al., 2009). Patients with metastatic disease who did not undergo surgical removal also had no consistent definitive pathological tumour grading. Sample size was a limitation in our case series as it was a single centre database. Some advanced RCC cases which were treated solely under oncological care and not referred to the urological unit might have been missed. In order to further improve the RCC registry, these patients will be included from the oncological unit records in future. Nonetheless, this is a first report which includes survival information of RCC patients and it can be taken as a reference for future databases.

3.6 Conclusion

Database collection with mortality data provides valuable information on patient outcome and this may hopefully improve on patient management. In this cohort, TNM staging and palpable abdominal mass were independent predictors for survival. Further investigation using a multicentre cohort to analyse mortality and survival rates may aid in improving the management of these patients.

CHAPTER 4 : TUMOUR NECROSIS FACTOR RECEPTOR-ASSOCIATED FACTOR 1 (TRAF 1) EXPRESSION IN RENAL CELL CARCINOMA PATIENT TISSUE AND SERUM: POTENTIAL BIOMARKER SIGNIFICANCE

4.1 Introduction

TRAF1 is an adaptor protein which plays a role in various biological functions. Impairment of TRAF1 regulation has been documented in lymphomas and myeloma, but there is limited report of its function/expression in solid tumours like RCC (Lee & Choi, 2007; Rajandram et al., 2014). Previous research from Rajandram et al. (2012) has suggested that TRAF1 is dysregulated in RCC and is involved in the control of apoptosis in RCC cells (Rajandram, Bennett, Morais, et al., 2012; Rajandram, Bennett, Wang, et al., 2012). However, the prognostic value of TRAF1 in RCC tissue and patients' serum is still unknown. The objective of this chapter was to evaluate the expression of TRAF1 in ccRCC tissue compared to normal kidney and TRAF1 in ccRCC serum compared to controls without RCC as well as the prognostic value of TRAF1 in ccRCC tissue and serum samples collected from patients treated at UMMC.

Part of the results from this chapter has been published (Rajandram et al., 2014). Please refer to Appendix B.

4.2 Literature review

TRAF1 was discovered along with TRAF2 in 1994 by Rothe and colleagues as adaptor proteins associated with the TNFR1 and TNFR2 signalling pathways (Lee & Choi, 2007). Since then, TRAFs 3-7 have been identified. TRAF1 regulates lymphocyte activation, survival and apoptosis and is found to be overexpressed in Hodgkin's lymphoma and circulating chronic lymphocytic leukemias (Guo et al., 2009; Lee & Choi, 2007). In gastric cancer cell lines, the up-regulation of TRAF1 induced by *Helicobacter pylori* infection inhibited cell apoptosis of infected cells (Wan et al., 2016). In contrast, TRAF1 level is elevated in hepatic and brain ischaemia, promoting cell death (Lu et al., 2013; Zhang et al., 2014).

There are many facets of TRAF1 in apoptotic regulation depending on the cell type, stimuli and receptor involved. TRAF1 frequently interacts with TRAF2 to exert its apoptotic or pro-survival effects via NF-κB activation. As described in Chapter 2: Literature Review (Section 2.11), binding of ligands to receptors such as TNFR1, TNFR2 or CD40 activate the TRAF1/2 mediated pathways resulting in pro-apoptosis or survival signalling cascades (Henkler et al., 2003; Lee & Choi, 2007). TNF-α, a cytokine which binds to TNFR1/2 has been shown to be involved in the signalling pathways promoting tumour progression in RCC (Chuang et al., 2008). A study by Al-Lamki et al. (2005) on TNFR reported that TNFR2 expression is increased in ccRCC tissue with higher grades, while TNFR1 remained the same (Al-Lamki et al., 2005). They found that binding of ligands to TNFR2 promotes entry into cell cycle and activate NF-κB while binding to TNFR1 promotes apoptosis (Al-Lamki et al., 2005). However, they did not study the expression levels of TRAF1 and TRAF2 nor the roles that these two proteins may play in the activation of NF-κB and apoptosis.

In RCC, TRAF1 is postulated to be involved in apoptotic regulation as a RCC cell line (ACHN) treated with radiation and IFN-α to induce apoptosis showed increase in TRAF1, 3 and 4 levels (Rajandram, Bennett, Wang, et al., 2012; Rajandram et al., 2009). TRAF1 expression is lower in RCC tissue compared to normal kidney in a cohort of Australian RCC patients (Rajandram, Bennett, Wang, et al., 2012). TRAFs 3 and 4 which were also analysed in RCC compared to normal kidney tissue did not reveal any significant difference (unpublished findings) (Rajandram, 2009). However, as the prognostic significance of TRAF1 in RCC is unclear, the association of TRAF1 with clinical features in RCC tissue and serum was determined in this study.

4.3 Methodology

Ethics approval for serum and tissue collection has been obtained from the University of Malaya Ethics Committee (Ref : 848.17).

4.3.1 Collection of tissue and serum samples

RCC FFPE (Formalin Fixed Paraffin Embedded) tissues were collected retrospectively and prospectively with the help of Pathology Department of UMMC. Inclusion criteria for tissue sample collection were patients who underwent surgery with histo-pathological diagnosis of ccRCC and the controls were the adjacent normal tissue. The tissue blocks/sections used for preparing slides were selected with the help of a senior pathologist. The grading and histology of the tissue slides were also confirmed with the help of the senior pathologist.

Blood collection was carried out for patients who have not gone for surgery or treatment for RCC. Patients who are already on treatment for RCC or have other forms of cancer were excluded. The patient cohort for tissue (retrospective and prospective samples) and serum (prospective) samples were not entirely similar as a larger sample size was used for IHC (tissue) analysis. Serum controls were collected from age/gender matched individuals who do not have medical history of cancer. These were either patients with previous abdominal imaging or renal calculi patients who were confirmed to have no renal malignancies. Written consent was obtained from all patients who gave blood samples. Patients' pathology reports and clinical data were obtained from UMMC medical reports.

Blood samples were collected in BD Vacutainer Serum Separator Tubes. Approximately 30 min after blood collection, serum was separated by centrifugation at 1000×g for 15 minutes and aliquoted into cryovials for storage at -80°C before analysis.

4.3.2 ELISA Assay (TRAF1)

TRAF1 in serum samples was detected using the human TRAF1 ELISA kit (Cusabio, Wuhan, China) according to the manufacturer's instructions. Briefly, 100µl of standards and serum samples were pipetted into a 96 well plate coated with TRAF1 antibody. After 2 hours incubation at 37°C, samples were aspirated and 100µl biotin conjugated antibody was added to each well. The plates were washed to remove unbound antibody before 100µl avidin conjugated Horseradish Peroxidase (HRP) was added to each well. The plates were subsequently washed before a substrate solution was added for colour development, which was dependent on the amount of TRAF1 bound. Colour development was stopped and reading was taken at 450nm with a reference wavelength

at 540nm using a plate reader (Multiskan Go Thermo Scientific, USA). An example of standard curve obtained can be observed in Appendix E. ANOVA and T-test were used to compare TRAF1 levels in controls vs RCC patients and RCC of different stages/grades. Receiver operating characteristic (ROC) curve analysis was performed to determine the reliability of TRAF1 as a biomarker. All statistical tests were done using SPSS Statistics v20 (IBM, USA).

4.3.3 Immunohistochemistry (TRAF1)

Formalin fixed ccRCC tissue with paired normal kidney (UMMC, 2004-2011) were immunostained with TRAF1. Heat antigen retrieval was performed in ethylenediaminetetraacetic acid/Tris buffer (1mM/0.01M, pH9.0) at 100°C for 15 minutes in a decloaking chamber (Biocare Medical, USA). Non-specific binding of peroxidase or antibody was blocked with 0.1% sodium azide in 0.3% hydrogen peroxide (H_2O_2) in Tris buffered saline (TBS), followed by 5% non-fat milk powder in TBS containing 0.05% Tween 20 and finally swine serum in 1% bovine serum albumin (BSA) in TBS. TRAF1 primary antibody (1:1000; IHC World, US Biological, USA) was diluted in 1% BSA in TBS. Positive tissue samples (human small intestine) and negative controls without primary antibody were prepared for each batch stain. The IHC procedure was performed using a Bond-Max automated immunostainer (Vision BioSystems, Australia) using the Bond Polymer Refine Detection kit (Vision Biosystems). The chromogen used was diaminobenzidine hydrochloride (DAB). Sections were counterstained with haematoxylin and then dehydrated in a series of ethanol, cleared in xylene and mounted with glass coverslips.

4.3.4 Stained slides analysis

Stained slides were scanned in an Aperio ScanScope XT slide scanning system (AperioTechnologies, USA) at 200x magnification. Digital images of the sections were viewed using Aperio ImageScope (Leica Biosystems, Germany) software. Three random fields of the same size were selected for each RCC and paired normal kidney section. Slide analysis was performed by a researcher blinded to the clinical outcome of the patients, with the help of a similarly blinded senior pathologist. Analysis was carried out using the Positive Pixel Count v9 algorithm (for total staining intensity) from the Aperio ImageScope software (Staniszewski, 2009). An example of staining intensity detection by Aperio ImageScope software is shown in Appendix F. Nuclear staining was not carried out for TRAF1 as staining was only cytoplasmic. Staining (positive pixels %) was scored according to the intensity and percentage of cells stained. The intensity output for Positive Pixel Count v9 algorithm was given as number of negative, weak positive, positive or strong positive pixels. Positive pixels (%) was calculated based on this formula :

$$\frac{\text{(Number of positive pixels + strong positive pixels)}}{\text{Total number of pixels}} \times 100 = \text{Positive pixels (\%)}$$

For survival analysis, the median positive pixel score was used to determine cut-off scores for ‘high’ or ‘low’ staining for each protein (Biswas et al., 2012).

4.3.5 Statistical analysis for TRAF1 IHC staining

Statistical analysis was performed using SPSS Statistics v20 (IBM, USA). Data analysis was carried out using T-test or ANOVA to determine the difference in positive pixels (%) or staining intensity between groups. The Cox proportional hazards regression was used to analyse TRAF1 staining intensity and survival.

4.4 Results

4.4.1 TRAF1 expression in serum (ELISA)

Age and gender matched serum samples from 49 ccRCC patients and 49 individuals without medical history of RCC or cancer were assayed for TRAF1 using ELISA kits. The demographic and clinical details of the patients are listed in Table 4.1. TRAF1 ELISA was only performed in serum of patients with ccRCC as previous IHC analysis has found no significant difference of TRAF1 expression between normal kidney and other RCC subtypes (pRCC and chRCC). Additionally, ccRCC is the most common form of RCC subtype therefore the number of serum samples available was sufficient for analysis.

Table 4.1. Demographic and clinical data for ccRCC patients and controls

Patient characteristics	RCC patients (N=49)	Controls (N=49)	p Value
Age (mean ± SD)	58.4 ± 10.1	58.9 ± 10.4	0.807
Men/Women (N)	30/19	30/19	1.000
Race (%) Malay Chinese Indian	 17 (34.7) 23 (46.9) 10 (20.4)	 23 (46.9) 16 (32.7) 10 (20.4)	 0.397
Stage (TNM) (%) I II III IV	 20 (40.8) 5 (10.2) 9 (18.4) 15 (30.6)	 - - - -	 -
Metastasis at presentation (%) No metastasis Metastasis	 34 (69.4) 15 (30.6)	 - -	 -

*p value obtained using t test and Chi-square test

TRAF1 concentration was significantly higher in the serum of ccRCC patients (193.86±28.00pg/ml) compared to the controls (112.65±16.17pg/ml) (p=0.012) (Figure 4.1). Among ccRCC patients, TRAF1 concentration in serum from metastatic cases (321.71±70.79pg/ml) was significantly higher than non-metastatic cases (137.45±19.75pg/ml) (p=0.002) (Figure 4.2). TRAF1 concentration was higher in non-metastatic ccRCC patients (137.45±19.75pg/ml) compared to controls (112.65±16.17pg/ml), but the difference was not statistically significant. There was no significant association of TRAF1 concentration in serum and tumour grade (data not shown). The area under the ROC curve (0.809) showed that elevated TRAF1 serum level is a good predictor of metastatic ccRCC compared to controls and localised ccRCC patients (Figure 4.3).

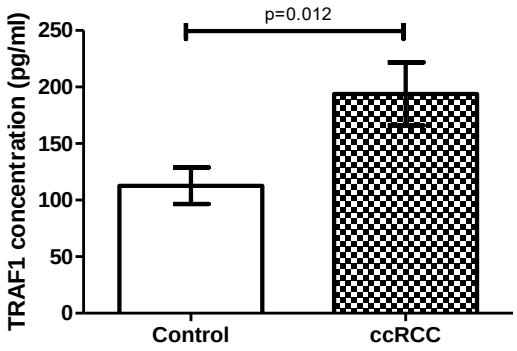


Figure 4.1. TRAF1 serum concentration in ccRCC patients and controls
TRAF1 concentration in ccRCC patients' serum was higher than the controls.

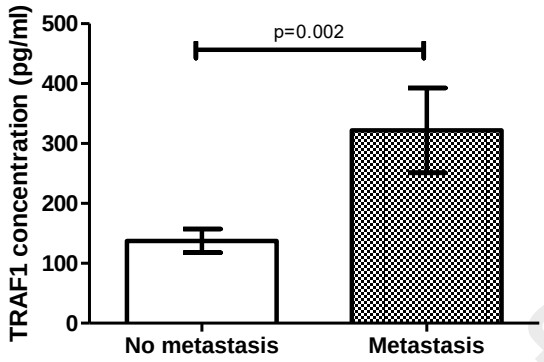


Figure 4.2. TRAF1 serum concentration in ccRCC patients with and without metastasis
CcRCC patients with metastasis at presentation (TNM stage IV) had higher TRAF1 levels compared to ccRCC patients with no metastasis (TNM stages I-III).

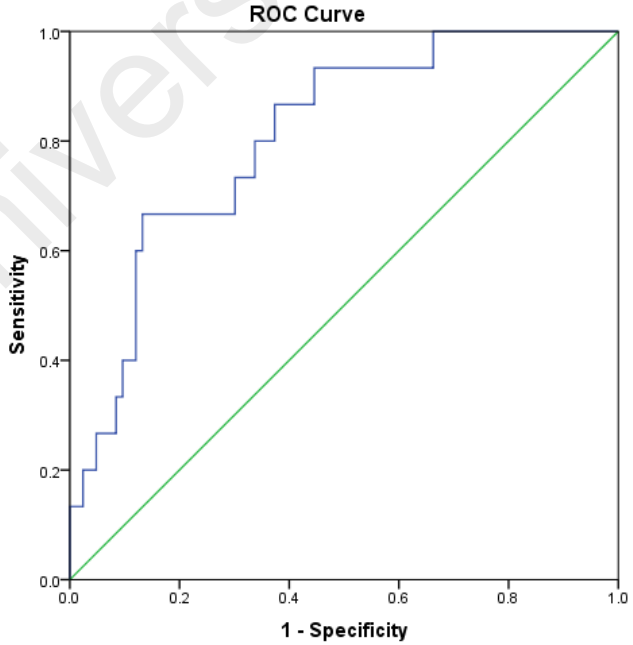


Figure 4.3. ROC curve of serum TRAF1 concentration in metastatic ccRCC compared to controls and localised ccRCC
The area under the curve (AUC) is 0.809 (95% CI 0.701-0.917).

4.4.2 TRAF1 expression in ccRCC tissue (IHC)

TRAF1 IHC was performed on ccRCC tissue slides from 61 patients. Patients' demographics and clinical details are shown in Table 4.2.

Table 4.2. Demographics and clinical data of ccRCC patients for TRAF1 IHC analysis

Characteristics	Sample size
Age (mean ± SD)	61.6 ± 10.2 years
Men/Women (N)	42/19
Race (%)	
Malay	12 (19.7)
Chinese	37 (60.7)
Indian	12 (19.7)
T Stage (%)	
1	32 (52.5)
2	20 (32.8)
3	9 (14.8)
Metastasis at presentation (%)	
No metastasis	49 (80.3)
Metastasis	12 (19.7)
Stage (TNM) (%)	
I	30 (49.2)
II	17 (27.9)
III	2 (3.3)
IV	12 (19.7)
Grade (%)	
1	8 (13.1)
2	32 (52.5)
3	16 (26.2)
4	5 (8.2)

TRAF1 was found in the cytoplasm and expression was localised to the proximal tubules in normal kidney (Figure 4.4). Quantitative analysis of the expression intensity revealed that TRAF1 overall immunopositivity was significantly higher ($p<0.001$) in normal kidney (58.31±11.95%) compared to ccRCC tissue (34.40±10.25%) (Figure 4.5). There was no significant difference in tumours grouped by stage (T stage and metastasis) and grade (Table 4.3). There was also no significant association with disease

specific survival in ccRCC patients (p=0.316). The inflammatory cells found in and around ccRCC tissue had strong TRAF1 expression (Figure 4.4 F).

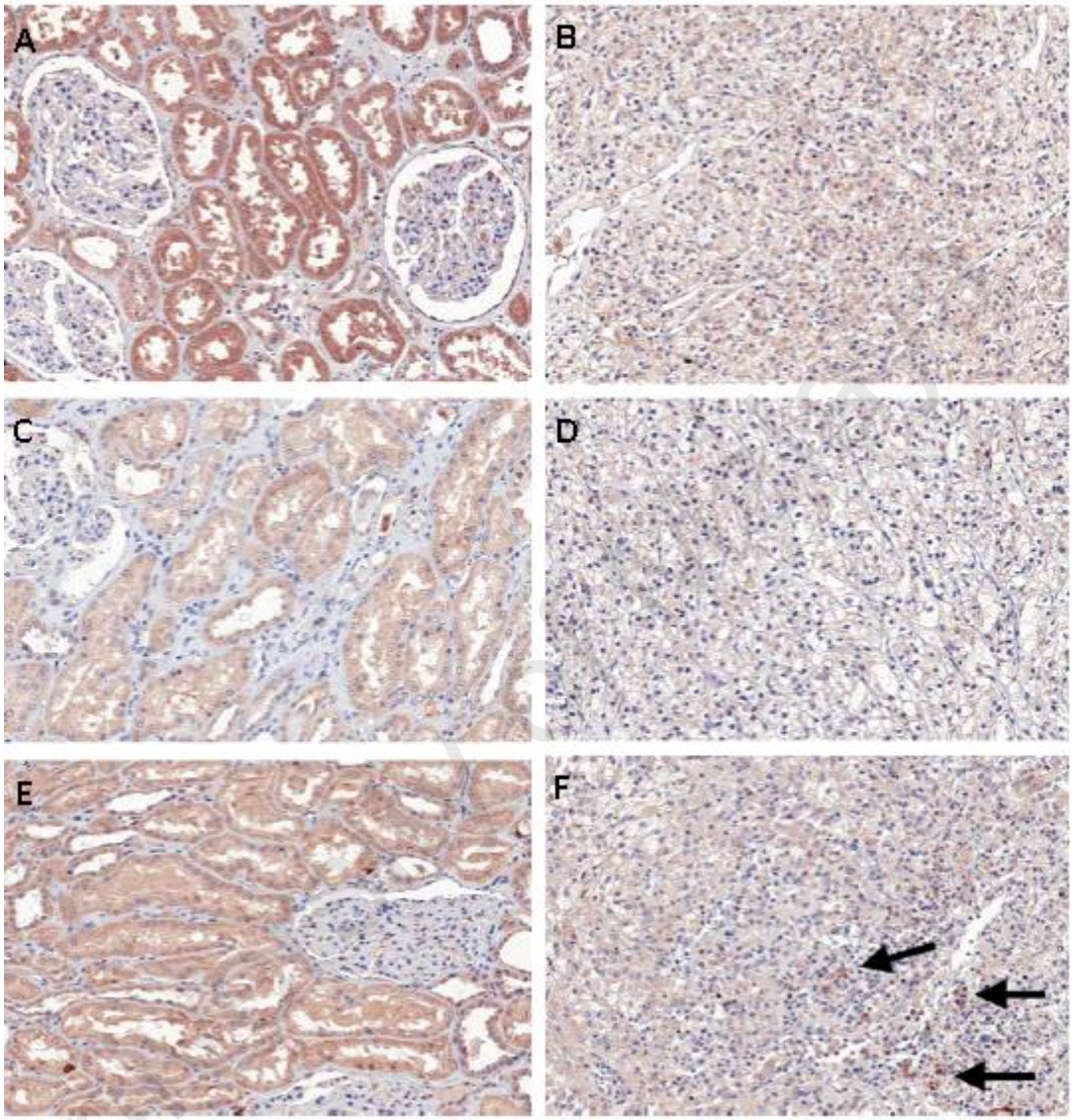


Figure 4.4. TRAF1 staining in normal kidney tissue and RCC
(A, C, D) Normal kidney and (B, D, F) ccRCC TRAF1 staining. There was lower overall expression intensity of TRAF1 in ccRCC compared with its paired normal kidney. (F) Inflammatory cells in ccRCC stained srongly for TRAF1 (arrows) (20x Aperio magnification).

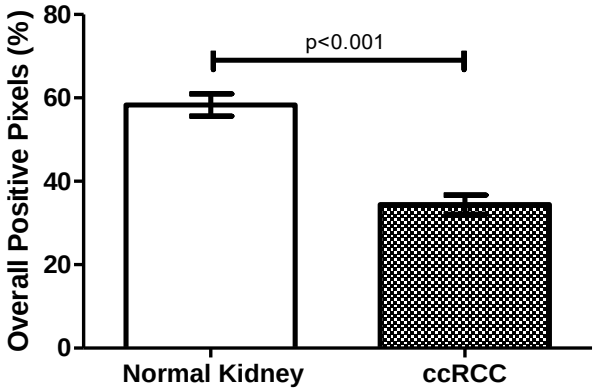


Figure 4.5. TRAF1 expression in normal kidney and ccRCC tissue
Positive pixel analysis showed higher TRAF1 expression in normal kidney compared to ccRCC tissue.

Table 4.3. Comparison of TRAF1 expression based on pathological characteristics

Characteristics	TRAF1 Positive Pixels (%)	p Value
T stage		
1	39.07±13.43	0.796
2	39.83±17.49	
3	33.29±13.27	
Metastasis at presentation		
No metastasis	38.29±14.43	0.512
Metastasis	43.08±12.32	
Grade		
1	27.91±6.59	0.455
2	38.02±9.47	
3	37.19±17.38	
4	39.48±12.39	

*p value obtained using ANOVA and t-test

4.5 Discussion

TRAF1 is present in the proximal tubular epithelium of normal kidney at significantly higher levels compared to ccRCC, as verified by the results of this study in a cohort of Malaysian RCC patients and a previous study in Australian patients (Rajandram, Bennett, Wang, et al., 2012). The decreased TRAF1 in ccRCC could be due to the low or negligible apoptosis levels seen in RCC histology (Rajandram, Bennett, Wang, et al., 2012). Interestingly, the serum levels were the inverse of the tissue expression profile. The concentration of TRAF1 in ccRCC patients overall was

significantly elevated compared to age and gender matched controls without RCC. When controls were compared with non-metastatic ccRCC patients alone, TRAF1 was higher but not statistically significant. In the ROC analysis, TRAF1 levels in serum is a good indicator of metastasis as the concentration in metastatic ccRCC serum was significantly higher compared to controls and non-metastatic cases.

The increased TRAF1 in serum could reflect active secretion of the protein from the tissue as the ccRCC develops and increased serum TRAF1 could be a potential marker of ccRCC metastasis. A possible reason is that TRAF1 could be secreted from distant metastatic sites. Several studies have shown that primary and metastatic RCC sites exhibit differences in their protein, gene or microRNA expression patterns which may affect their biological characteristics or aggressiveness (Semeniuk-Wojtas, Stec, & Szczylik, 2016). Due to the multi-faceted apoptotic/survival regulation of TRAF1, the expression might be higher in metastatic sites while lower in the primary tissue. The tissue expression of TRAF1 is not associated with stage, grade or disease specific survival in this cohort. These parameters (stage, survival) were not investigated in the Australian cohort. It is possible that TRAF1 dysregulation may contribute to the tumourigenesis of ccRCC but not play a central role in the progression of the primary tumour.

TRAF1 is also involved in the regulation of inflammatory response, often in association with NF-κB (Edilova, Abdul-Sater, Clouthier, & Watts, 2016; Rajandram et al., 2014). TRAF1, along with TRAF2, are downstream intermediaries of the TNF receptor signal transduction pathways, regulating TNF-α activation of c-Jun N-terminal kinase and NF-κB signalling pathways (Arron, Pewzner-Jung, Walsh, Kobayashi, & Choi, 2002; Rajandram et al., 2014). Additionally, expression of TRAF1 is upregulated

as a result of lymphocyte activation, and in B-cells, TRAF1 acts together with TRAF2 to enhance CD40-mediated activation signals (Lee & Choi, 2007; Xie, 2013). RCC is considered an immunogenic tumour, with tumour infiltrating lymphocytes and intratumoural neutrophils (Jensen et al., 2009; Liotta et al., 2011). Although there was lower intensity of TRAF1 in the tumour tissue, there was increased TRAF1 expression in inflammatory cells detected in ccRCC tissue. It is known that cytokines, chemokines and their receptors are sometimes secreted by the tumour (Grivennikov & Karin, 2010). Moreover, the levels of IL-6, IL-1b and TNF-α were higher in the serum of RCC patients compared to healthy controls, and RCC cells produced TNF-α (Chuang et al., 2008; Yoshida et al., 2002).

Systemic inflammatory response is elevated in metastatic RCC and could also possibly increase TRAF1 levels in metastatic ccRCC serum samples (Fox et al., 2013). This is similarly seen in elevated levels of serum C-reactive protein (CRP) which is one of the promising prognostic biomarkers of interest in RCC (Beuselinck et al., 2014; Martino et al., 2013). The limitation of this study was the sample size of the patients' serum which was collected from a single centre. Future large scale validation of TRAF1 in serum should comprise of multi centres to include patients from different countries or ethnicities.

4.6 Conclusion

TRAF1 has diverse roles in cancer cell survival, apoptosis, and inflammation, but its regulation in RCC development and progression remains unclear. In conclusion, the high serum TRAF1 may be due to active secretion from the ccRCC cells from the metastatic sites, associated with the highly expressing tumour-related inflammatory cells or as a result of systemic inflammation in metastatic disease. TRAF1 serum levels may be useful in a panel of markers to determine the progression of disease. In the tissue, TRAF1 has decreased expression in ccRCC compared with normal kidney and there is some evidence that reduced TRAF1 may have a modulatory role on apoptosis in ccRCC.

CHAPTER 5 : NF-κB SUBUNITS : IMMUNOHISTOCHEMICAL ANALYSIS AND PROGNOSTIC SIGNIFICANCE IN RENAL TUMOURS

5.1 Introduction

NF-κB proteins (p50, p52, p65, RelB and cRel) are transcription factors controlling a variety of signalling pathways and are of research interest in cancers. The impairment of pathways regulated by these proteins are associated with carcinogenesis, such cell apoptosis or survival, proliferation, inflammation, angiogenesis and epithelial to mesenchymal transformation (EMT). Additionally, NF-κB pathways are frequently linked to TRAF1 regulation. Pathway regulation was described in Chapter 2: Literature Review Section 2.11.

The aim of this chapter was to determine the expressions/localisation of NF-κB subunits and evaluate the prognostic value of NF-κB staining intensity in RCC tissue. This presents the largest sample size of RCC patients evaluated for all five subunits of NF-κB. The p50 and p65 subunits have been studied in RCC but there is a paucity of data on p52, cRel and RelB expressions in RCC tissue. A recent meta-analysis on the effect of NF-κB expression (unspecified subunit) on clinical outcomes in solid tumours analysing mainly colorectal, esophageal, gastric, ovarian and lung cancers found that NF-κB overexpression is associated with worse prognosis (overall survival and disease-free survival) (Wu. et al., 2015). The meta-analysis did not include any reports on RCC, hence the prognostic value of NF-κB in RCC is still unclear.

5.2 Literature review

The NF-κB proteins have been implicated in tumour development in breast, prostate, colorectal and renal cancers among others (Wu et al., 2015). However, the prognostic significance of NF-κB expression in these solid tumours remains contradictory. The most studied subunit in RCC is p65. The p65 subunit is overexpressed in the tumour tissue compared to normal kidney but reports on the associated with prognostic parameters is conflicting. Some studies have shown association with stage, grade or tumour invasion (Kankaya et al., 2015; Oya et al., 2003; Peri et al., 2013b) while one study found no association (Sourbier et al., 2007). An increase in p65 expression was correlated with higher VEGF expression and proliferation rate, along with reduced apoptotic activity (Djordjevic et al., 2008; Matusan-Ilijas et al., 2011). NF-κB as transcription factors can induce activation of a variety of genes promoting oncogenesis including VEGF. Moreover, Peri et al. (2013) indicated that dysfunctional pVHL, a pivotal protein in ccRCC carcinogenesis, could be responsible for alterations in p65 signalling (Peri et al., 2013b).

There has been only one study evaluating p50 immuno-staining in RCC patients' tissue and there was no correlation found with tumour stage and grade (Meteoglu, Erdogdu, Meydan, Erkus, & Barutca, 2008). Although p52 and RelB are well known for their roles in lymphoid organ development and adaptive immunity, they have also been linked with cancer development. RelB expression was increased in non-small cell lung cancer and activated in prostate cancer while aberrant activation of p52 was seen in breast and prostate cancers (Cogswell, Guttridge, Funkhouser, & Baldwin, 2000; Lessard, Begin, Gleave, Mes-Masson, & Saad, 2005; Nadiminty et al., 2010; Qin et al.,

2016) . Impairment of cRel regulation causing increased cRel activation was reported in B-cell lymphomas (Hunter et al., 2016).

Table 5.1 summarises the various studies analysing NF-κB expression in human RCC tissues. There has been no reports of p52, RelB and cRel expressions in RCC tissue at present. Hence, this would be the first study looking at the protein expressions of these three subunits in RCC patients' tissue.

Table 5.1. Available studies analysing NF-κB subunits in human RCC tissue samples

Reference	Sample size	Type of Experiment	Subunits	Findings
(Oya et al., 2003)	45 ccRCC & pRCC FFPE and fresh tissue	Electrophoretic mobility shift assay (EMSA) & immunoblot for protein expression/activation in fresh tissue (p65, p50 and IkBα) IHC (p65) in FFPE tissue	p50 & p65	EMSA : Activation of NF-κB increased with invasiveness (T stage, grade) and metastases Immunoblot : RCC with an increased NF-κB activity had a decreased IκBα expression. IHC : Nuclear localisation of p65 in RCC tissue
(Sourbier et al., 2007)	241 tissue microarray (TMA) of RCC	IHC of RCC TMA Cell line and in vivo mice experiments	p65	Higher activated p65 in ccRCC compared with other RCC types (p=0.002) and bigger tumour dimension (p= 0.02). Activated p65 not prognostic factor for patient survival. NF-κB inhibition induced cell apoptosis and reduced tumour growth

Table 5.1 continued

Reference	Sample size	Type of Experiment	Subunits	Findings
(Meteoglu et al., 2008)	40 ccRCC FFPE tissue	IHC : p50, Epidermal growth factor receptor (EGFR), p53, Bcl-2	p50	No correlation between p50 staining and age, sex, tumour size, stage and grade Significant positive correlation between the staining of p50 and VEGF (p=0.001), EGFR (p=0.004), Bcl-2 (p=0.010) and p53 (p=0.037).
(Djordjevic et al., 2008)	22 ccRCC and 9 non ccRCC	IHC : p65, VEGF and Ki67 qPCR : VEGF mRNA expression	p65	p65 higher in ccRCC than in normal renal tissue and related to higher VEGF expression (p=0.026) Cytoplasmic p65 associated with higher proliferation rate (p=0.039) and patients with localized disease (p=0.018)
(Matusan-Ilijas et al., 2011)	TMA of 87 ccRCC	IHC : Osteopontin (OPN) and p65 qPCR of OPN mRNA	p65	Association of p65 with OPN mRNA (p=0.015) and protein (p<0.001). Inverse relationship of OPN (p=0.006) with p65 (p=0.022) protein expressions and apoptotic activity of tumour cells.

Table 5.1 continued

Reference	Sample size	Type of Experiment	Subunits	Findings
(Peri, Devarajan, Yang, Knudson, & Balachandran, 2013a)	20 ccRCC tumours and 8 normal tissue Survival data for 55 patients	Meta-analysis – Published RCC studies from Gene expression Omnibus (GEO) or ArrayExpress (Expression profiles of NF-κB target genes in whole-genome transcriptomic data from 61 ccRCC and 34 normal samples) Gene expression and survival data available for 55 ccRCC patients from the TCGA (The cancer genome atlas) database IHC – p65 and Signal transducer and activator of transcription 1 (STAT1) in 20ccRCC tumours and 8 normal renal tissue	p65	Metanalysis : Up-regulation of p65 induced genes in ccRCC along with increased interferon targeted genes. Loss of *VHL* gene expression correlated with NF-κB and interferon gene signatures TCGA data : Elevated expression of NF-κB regulators/target genes (*IKBKB*, *MMP9*, *PSMB9*, and *SOD2*) significantly associated with higher relative-risk, poorer prognosis, and reduced overall patient survival IHC : Nuclear localisation of p65 in ccRCC tissue. p65 nuclear positive ccRCC samples were positive for nuclear STAT1 (IFN responsive transcription factor)
(Kankaya et al., 2015)	100 ccRCC FFPE tissue	IHC of Gelsolin, NF-κB p65, and p53	p65	Gelsolin expression associated with high grade (p=0.001), metastasis (p=0.003), late stage (p=0.008), and cancer death (p=0.001). p65 cyto-expression associated with high grade (p=0.002),

Table 5.1 continued

Reference	Sample size	Type of Experiment	Subunits	Findings
				perirenal invasion (p=0.010), local invasion (p=0.020) and late stage (p=0.003). p65 nuclear expression did not predict the prognosis of ccRCC. p53 expression correlated with high grade (p=0.045), lymphovascularinvasion (p=0.05), metastasis (p=0.001), late stage (p=0.028) and cancer death (p=0.034).

5.3 Methodology

Ethics approval for tissue collection was obtained from the University of Malaya Ethics Committee (Ref : 848.17).

5.3.1 Collection of tissue and serum samples

RCC FFPE tissues were collected retrospectively and prospectively (2003-2013) with the help of a pathologist from the Pathology Department of UMMC. Inclusion criteria for tissue sample collection were patients with pathological diagnosis of RCC and the controls were the adjacent normal tissue. The blocks/sections for preparing slides were selected with the help of a senior pathologist. Patients' demographics, clinical and pathology reports were obtained from the UMMC patients medical records. The histology and grading of selected slides were confirmed by the senior pathologist.

5.3.2 Immunohistochemistry (NF-κB Subunits)

RCC tissue slides with matched normal kidney were used for NF-κB subunits staining. These slides were selected from primary tumours of patients who underwent nephrectomy at UMMC from 2003-2013. Heat antigen retrieval was performed for all five subunits in a decloaking chamber (Biocare Medical, USA). Antigen retrieval was carried out in ethylenediaminetetraacetic acid/Tris buffer (1mM/0.01M, pH9.0) for 15 minutes at 105°C except for p65. For p65, heat retrieval was performed with citrate buffer (0.1M, pH6) at 125°C for 5 minutes. After antigen retrieval, non-specific binding of peroxidase or antibody was blocked with 3% H_2O_2 in TBS and Background Sniper (Biocare Medical, USA). Primary antibodies used were rabbit anti-human antibodies from Santa Cruz : p50 (sc-7178, dilution 1:100), p52 (sc-298, 1:100), p65 (sc-372, 1:150), RelB (sc-226, 1:100) and cRel (sc-71, 1:400). The positive control was human tonsil for all subunits and negative controls without primary antibodies were used for each batch stain.

The IHC procedures for p65 and cRel were performed on an automated slide stainer (Biocare Medical Intellipath FLX, USA) while p50, p52 and RelB were done manually. The detection kit used was MACH 1 Universal HRP-Polymer Detection (Biocare Medical, USA) with DAB as the chromogen. Sections were counterstained with haematoxylin, dehydrated in a series of ethanol, cleared in xylene and mounted with glass coverslips.

5.3.3 Stained slides analysis

Stained slides were scanned in an Aperio ScanScope XT slide scanning system (AperioTechnologies, USA) at 200x magnification. Digital images of the sections were viewed using Aperio ImageScope (Leica Biosystems, Germany) software. Three random fields of the same size were selected for each RCC and paired normal kidney section. Slide analysis was performed by a researcher blinded to the clinical outcome of the patients with the help of a similarly blinded senior pathologist. Analysis was carried out using the Positive Pixel Count v9 algorithm (for total staining intensity) and IHC Nuclear v1 algorithm (for nuclear staining) from the Aperio ImageScope software. Examples of staining intensity detection by Aperio ImageScope software are shown in Appendix F. Staining (positive pixels %) was scored according to the intensity and percentage of cells stained. The intensity output for Positive Pixel Count v9 algorithm was given as number of negative, weak positive, positive or strong positive pixels. Positive pixels (%) was calculated based on this formula :

$$\frac{\text{(Number of positive pixels + strong positive pixels)}}{\text{Total number of pixels}} \times 100 = \text{Positive pixels (\%)}$$

The output for IHC Nuclear v1 algorithm was given as percentage of pixels with 0, 1+, 2+ or 3+ staining intensity. Positive pixels (%) was calculated by adding the values for 2+ and 3+ staining. For survival analysis, the median positive pixel score was used to determine cut-off scores for 'high' or 'low' staining for each protein (Biswas et al., 2012).

5.3.4 Statistical analysis for NF-κB subunits

Statistical analysis was performed using SPSS Statistics v20 (IBM, USA). Data analysis was carried out using T-test or ANOVA to determine the difference in positive pixels (%) or staining intensity between groups. DSS curves were obtained from the Kaplan-Meier and survival differences between groups were evaluated using the log rank test. The Cox proportional hazards regression was used to analyse proteins that showed significance in the log rank test. A p value of <0.05 was statistically significant.

5.4 Results

5.4.1 NF-κB subunits (IHC)

Immunostaining of five NF-κB subunits (p50, p52, p65, cRel and RelB) was performed in RCC tissue slides from 96 patients. Immunostaining of RelB was omitted from analysis as both normal kidney and RCC tissue had negligible staining. Demographic and clinical details are shown in Table 5.2. The median follow up for the patients was 54.5 months (range 0.2 – 135 months), with 28 deaths (29.2%) at the time of analysis.

Table 5.2. Demographics and clinical characteristics of RCC patients for NF-κB immunostaining

Patient characteristics	RCC patients (N=96)
Age (mean±SD)	61.3±10.0 years
Men/Women (N)	65/31
Race (%)	
Malay	24 (25.0)
Chinese	54 (56.3)
Indian	18 (18.8)
Subtype (%)	
ccRCC	76 (79.2)
pRCC	11 (11.5)
chRCC	5 (5.2)
ccpRCC	3 (3.1)
multilocular cystic RCC	1 (1.0)
T Stage (%)	
1	53 (55.2)
2	26 (27.1)
3	14 (14.6)
4	3 (3.1)
Metastasis at presentation (%)	
No metastasis	74 (77.1)
Metastasis	22 (22.9)
Stage (TNM) (%)	
I	47 (49.0)
II	20 (20.8)
III	7 (7.3)
IV	22 (22.9)

Data presented as N (%) unless stated otherwise

5.4.2 NF-κB p50 subunit analysis (IHC)

NF-κB p50 subunit expression was cytoplasmic and nuclear. Overall and nuclear expression of p50 was higher in normal kidney compared to RCC tissue for all subtypes (Figures 5.1 and 5.2).

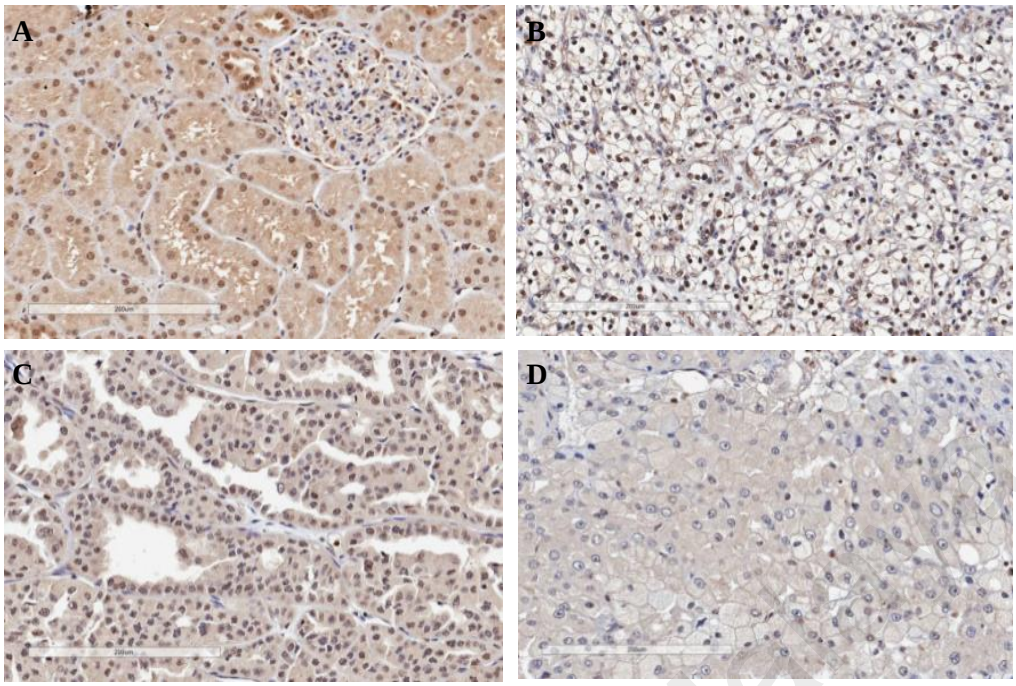


Figure 5.1. Examples of p50 immunostaining
(A) Normal kidney (B) ccRCC (C) pRCC and (D) chRCC (Aperio 20x magnification)

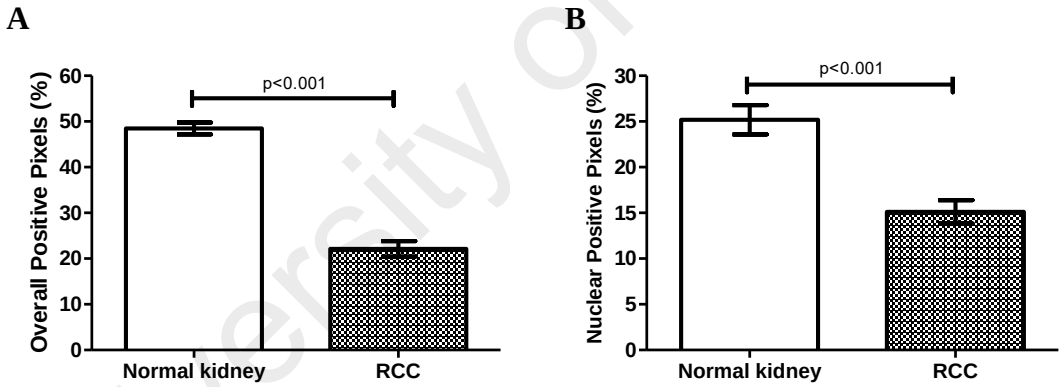


Figure 5.2. Immunopositivity of p50 in normal kidney tissue and RCC
(A) Overall and (B) nuclear expression of p50 was significantly lower in RCC compared to normal kidney tissue.

There was no significant difference in p50 nuclear overall and nuclear expression in tumours grouped by stage and grade (Table 5.3). Overall expression of p50 was higher in pRCC and ccpRCC compared to ccRCC (Figure 5.3).

Table 5.3. Comparison of p50 expression based on pathological characteristics

	Overall		Nuclear	
Characteristics	**Positive pixels (%)**	**p Value**	**Positive pixels (%)**	**p Value**
Subtypes				
ccRCC	19.60±13.45		15.57±12.27	
pRCC	35.00±24.12	0.026	8.42±8.10	0.232
chRCC	28.62±26.55		23.06±22.28	
ccpRCC	35.23±19.86		11.92±3.70	
multilocular cystic RCC	19.61		25.71	
T stage				
1	22.41±17.50		16.62±12.54	
2	18.82±14.14	0.534	11.54±10.54	0.361
3	26.43±15.83		16.70±13.40	
4	25.78±14.07		13.05±19.77	
Metastasis at presentation				
No	22.92±16.97	0.377	16.27±12.51	0.093
Yes	19.35±13.44		11.12±11.28	
TNM stage				
I	23.24±17.43		17.21±12.64	
II	18.99±14.88	0.245	12.20±10.31	0.085
III	32.02±18.04		21.65±15.73	
IV	19.35±13.44		11.12±11.28	
Grade				
1	24.53±14.12		20.89±10.00	
2	22.70±15.54	0.058	15.42±12.57	0.077
3	16.21±13.28		10.37±10.38	
4	34.41±23.00		17.14±11.34	

*p value obtained using ANOVA and t-test

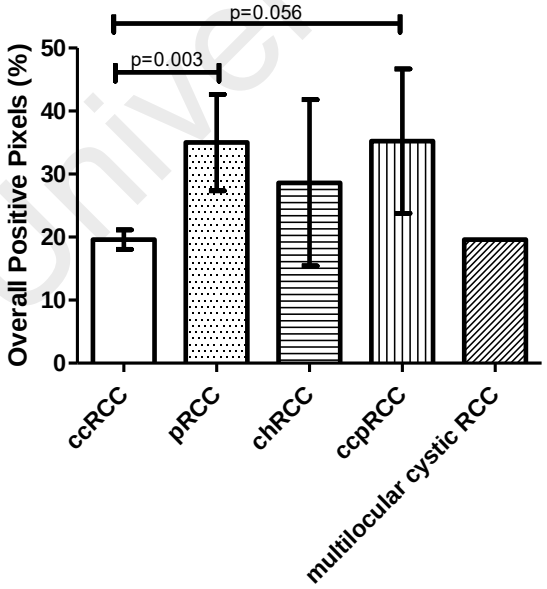


Figure 5.3. Immunopositivity of p50 in RCC subtypes
p50 overall expression was higher in pRCC and ccpRCC compared to ccRCC. The difference was near statistical significance for ccpRCC and ccRCC.

In order to perform survival analysis, median values of 17.81% (overall positive pixels) and 13.25% (nuclear positive pixels) were used as cut off points for low or high expressions. Nuclear and overall p50 expression had no statistical significance for survival. However, there was a trend of unfavourable survival outcome for patients grouped as high p50 overall expression (Figure 5.4).

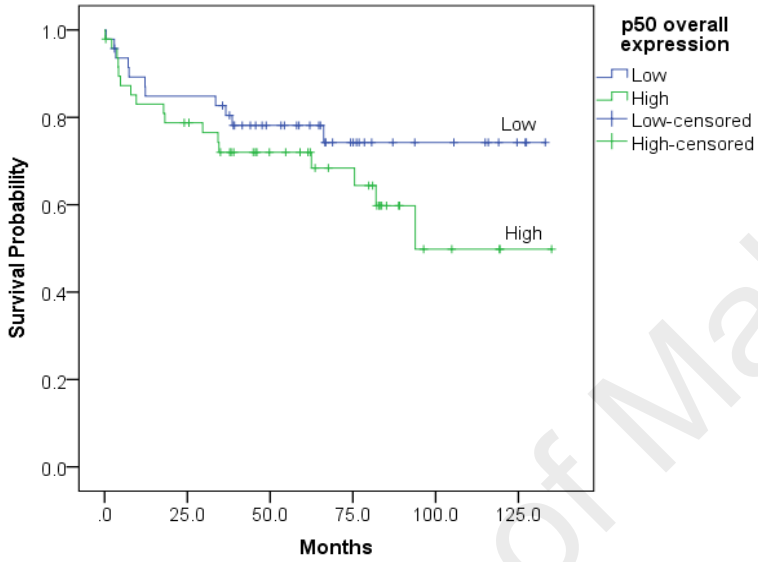


Figure 5.4. Survival of patients with low or high p50 expression in RCC tissue
RCC patients with high p50 overall expression had an unfavourable survival prognosis compared to patients with low p50 expression but the difference was not statistically significant (Log rank p = 0.219).

5.4.3 NF-κB p52 subunit analysis (IHC)

NF-κB p52 subunit expression was mainly cytoplasmic and occasionally nuclear. Overall and nuclear expression of p52 was higher in normal kidney compared to RCC tissue for all subtypes (Figures 5.5 and 5.6).

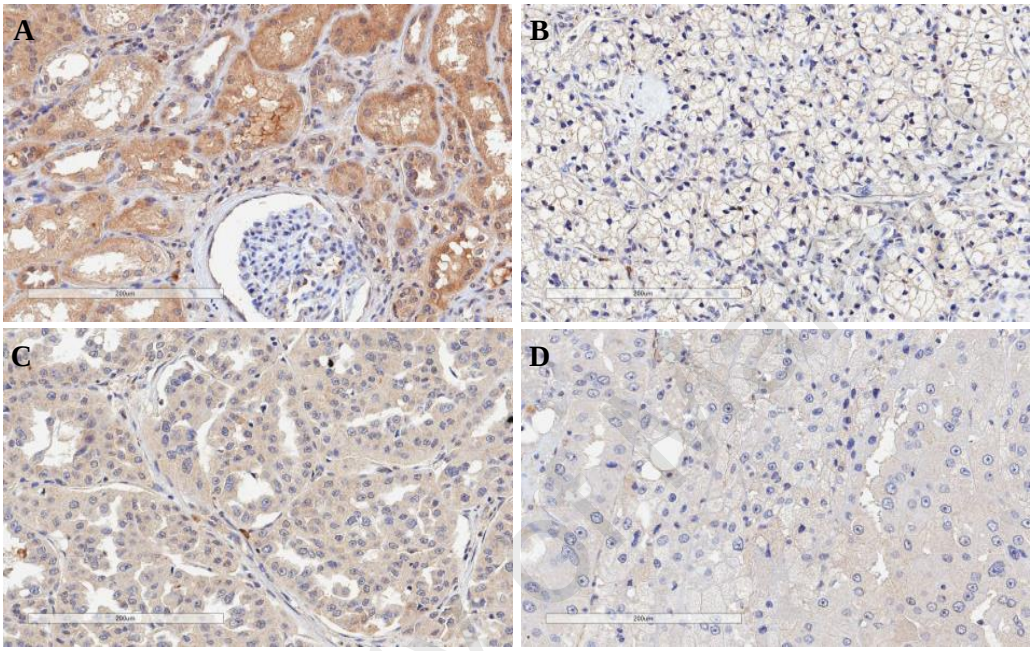


Figure 5.5. Examples of p52 immunostaining
(A) Normal kidney (B) ccRCC (C) pRCC and (D) chRCC (Aperio 20x magnification)

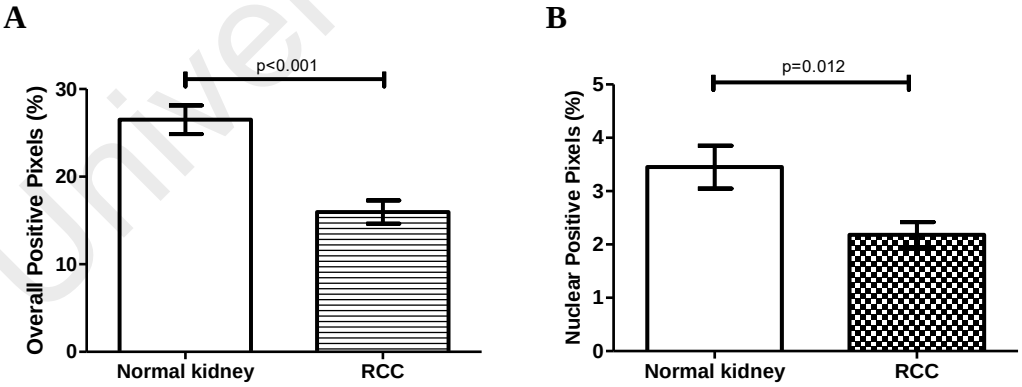


Figure 5.6. Immunopositivity of p52 in normal kidney tissue and RCC
(A) Overall and (B) nuclear p52 expression was significantly higher in normal kidney compared to RCC tissue.

There was no significant difference in p52 nuclear overall and nuclear expression in tumours grouped by subtype and stage (Table 5.4). However, there was a significant difference in overall p52 expression in the different tumour grades (Figure 5.7).

Table 5.4. Comparison of p52 expression based on pathological characteristics

	Overall		**Nuclear**	
Characteristics	**Positive pixels (%)**	**p Value**	**Positive pixels (%)**	**p Value**
Subtypes				
ccRCC	15.17±12.66	0.078	2.15±2.15	0.314
pRCC	24.97±12.83		3.10±3.64	
chRCC	9.61±6.34		0.62±0.26	
ccpRCC	8.12±7.03		1.09±0.99	
multilocular cystic RCC	23.48		3.98	
T stage				
1	15.39±11.65	0.650	2.50±2.67	0.382
2	18.22±16.61		1.54±1.08	
3	14.78±8.02		2.32±2.72	
4	8.78±2.71		1.53±1.14	
Metastasis at presentation				
No	15.22±12.00	0.240	2.09±2.25	0.093
Yes	18.84±14.90		2.49±2.59	
TNM stage				
I	13.73±9.79	0.239	2.26±2.59	0.693
II	19.11±16.47		1.65±1.15	
III	12.12±6.96		2.30±2.46	
IV	18.84±14.90		2.49±2.59	
Grade				
1	11.16±6.35	0.013	1.96±1.91	0.496
2	14.46±11.79		2.14±2.39	
3	17.28±12.86		2.14±2.29	
4	29.93±19.05		3.55±3.04	

*p value obtained using ANOVA and t-test

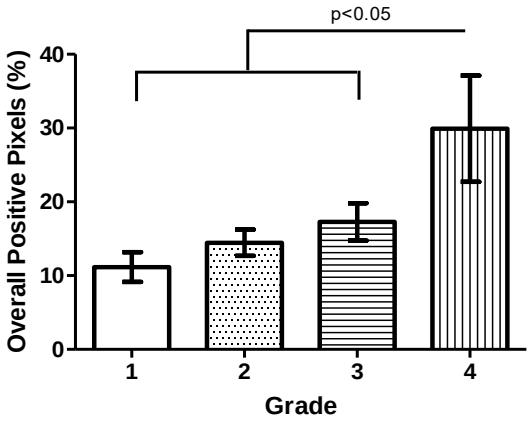


Figure 5.7. The immunopositivity of p52 according to tumour grade
There was a trend of increasing p52 immunopositivity as tumour grade increased. Grade 4 tumours had significantly higher p52 immunostaining than grades 1-3 tumours.

Median values of 13.65% (overall positive pixels) and 1.39% (nuclear positive pixels) were used as cut off points for low or high expressions to perform survival analysis. Patients grouped as p52 high overall expression was significantly associated with worse survival prognosis (Figure 5.8). Using Cox regression analysis, p52 was a significant prognostic factor in univariate (HR 2.48, 95% CI 1.10-5.60, p=0.028) and multivariate analysis adjusted for subtype, stage and grade (HR 2.59, 95% CI 1.05-6.39, p=0.039). However, nuclear p52 expression had no statistical significance for survival.

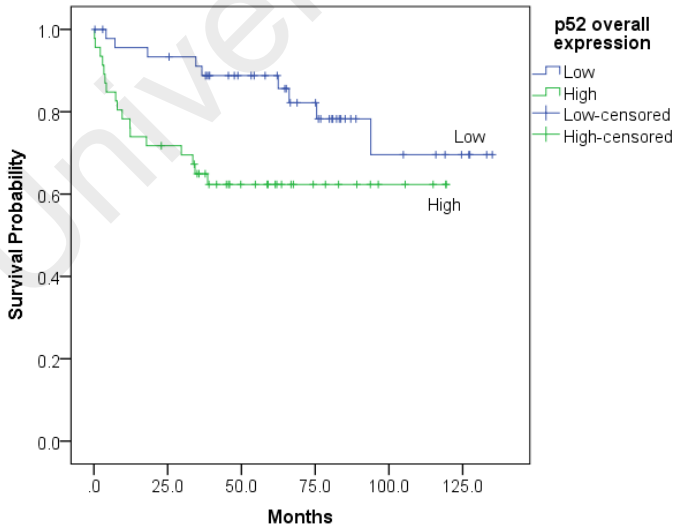


Figure 5.8. Survival of patients with low or high p52 expression in RCC tissue
RCC patients with high p52 overall expression had an unfavourable survival prognosis compared to patients with low p52 expression (Log rank p = 0.023).

5.4.4 NF-κB p65 subunit analysis (IHC)

NF-κB p65 subunit expression was cytoplasmic and nuclear. Overall and nuclear expression of p65 was higher in RCC compared to normal kidney tissue (Figures 5.9 and 5.10).

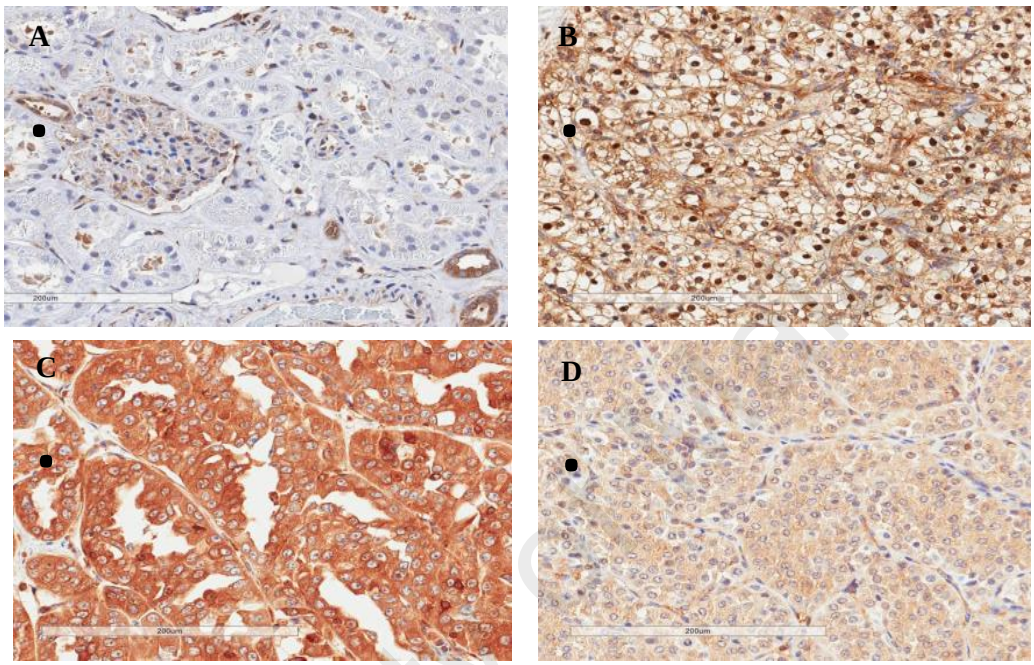


Figure 5.9. Examples of p65 immunostaining
(A) Normal kidney (B) ccRCC (C) pRCC and (D) chRCC (Aperio 20x magnification)

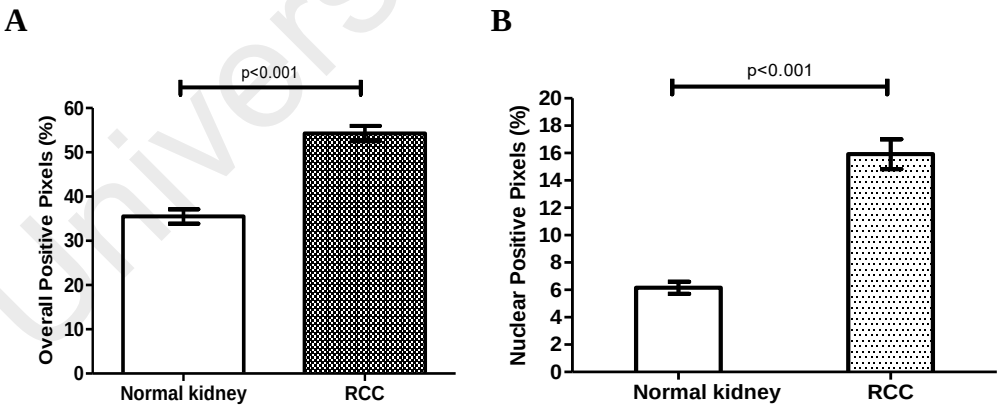


Figure 5.10. Immunopositivity of p65 in normal kidney tissue and RCC
The overall and nuclear p65 expression was significantly higher in RCC compared to normal kidney tissue.

Table 5.5 as well as Figures 5.11 and 5.12 summarize the comparison of p65 expression based on subtypes, tumour staging and Fuhrman grading.

Table 5.5. Comparison of p65 expression based on pathological characteristics

	Overall		**Nuclear**	
Characteristics	**Positive pixels (%)**	**p Value**	**Positive pixels (%)**	**p Value**
Subtypes				
ccRCC	52.40±14.50		16.97±9.73	
pRCC	70.33±16.54	0.002	10.59±11.30	0.022
chRCC	35.86±28.86		2.24±1.25	
ccpRCC	64.08±23.96		17.00±19.52	
multilocular cystic RCC	45.95		32.65	
T stage				
1	51.87±17.79		17.27±11.03	
2	53.85±12.97	0.033	12.34±7.99	0.113
3	58.49±15.72		15.77±11.05	
4	79.00±9.63		24.81±15.14	
Metastasis at presentation				
No	52.15±16.64	0.025	15.89±10.76	0.959
Yes	61.21±15.06		16.03±10.16	
TNM stage				
I	51.49±17.65		17.56±11.22	
II	52.50±13.87	0.148	13.04±8.62	0.397
III	55.38±19.14		13.33±12.50	
IV	61.21±15.06		16.03±10.16	
Grade				
1	52.67±9.43		17.88±8.38	
2	53.26±16.85	0.613	16.16±10.85	0.868
3	57.97±17.99		15.20±11.25	
4	57.37±9.12		18.01±6.17	

*p value obtained using ANOVA and t-test

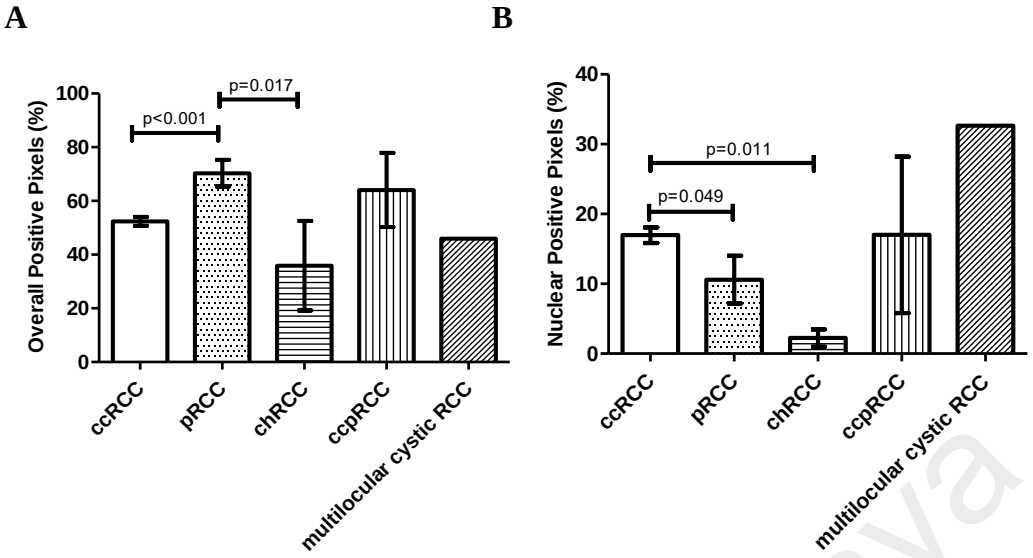


Figure 5.11. Immunopositivity of p65 in RCC subtypes
Differences in p65 (A) overall and (B) nuclear expression among RCC subtypes.

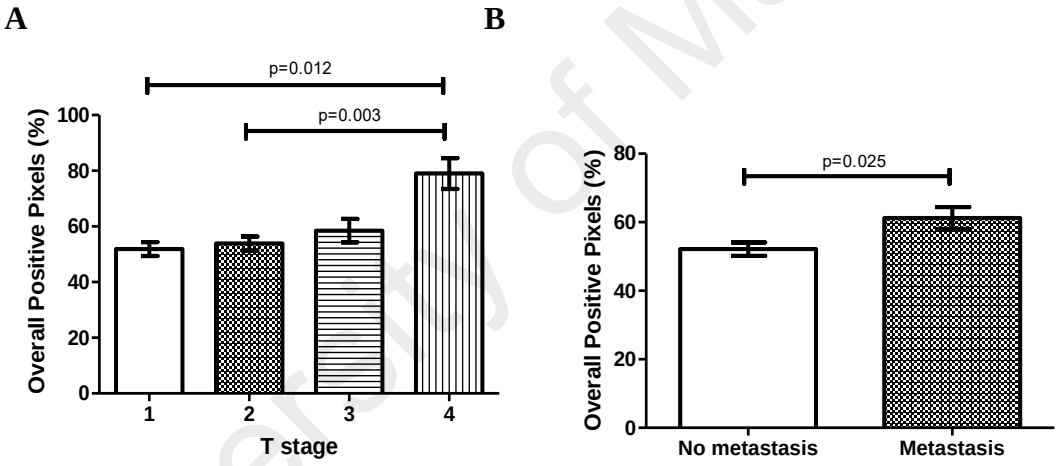


Figure 5.12. Immunopositivity of p65 in tumours of different stage and metastatic status
(A) There was a trend of increasing overall p65 expression as T stage increased. (B) Primary tumours with metastasis at presentation also had elevated overall p65 expression.

Median values of 55.95% (overall positive pixels) and 14.00% (nuclear positive pixels) were used as cut off points for low or high expressions to perform survival analysis. Patients with high p65 overall expression was significantly associated with worse survival outcome (Figure 5.13). Using Cox regression analysis, p65 was a significant prognostic factor in univariate (HR 2.30, 95% CI 1.04-5.09, p=0.040) but not

for multivariate analysis adjusted for subtype, stage and grade (HR 1.61, 95% CI 0.69-3.90, p=0.261). Nuclear p65 expression had no statistical significance for survival.

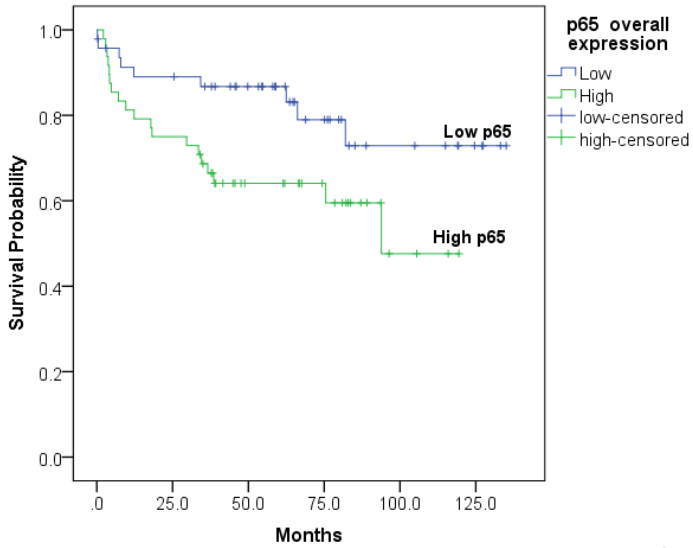


Figure 5.13. Survival of patients with low or high p65 expression in RCC tissue
RCC patients with high p65 overall expression had an unfavourable survival prognosis compared to patients with low p65 expression (Log rank p = 0.035).

5.4.5 NF-κB cRel subunit analysis (IHC)

NF-κB cRel subunit expression was mainly cytoplasmic and occasionally nuclear. Overall and nuclear expression of cRel was higher in normal kidney compared to RCC tissue for all subtypes (Figures 5.14 and 5.15).

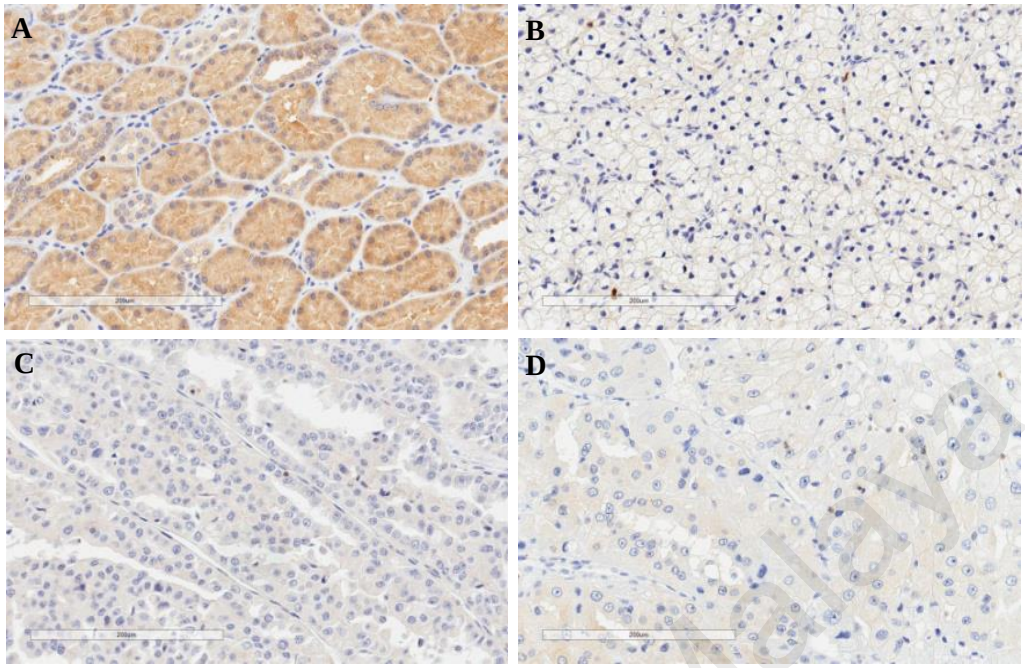


Figure 5.14. Examples of cRel immunostaining
(A) Normal kidney (B) ccRCC (C) pRCC and (D) chRCC (Aperio 20x magnification)

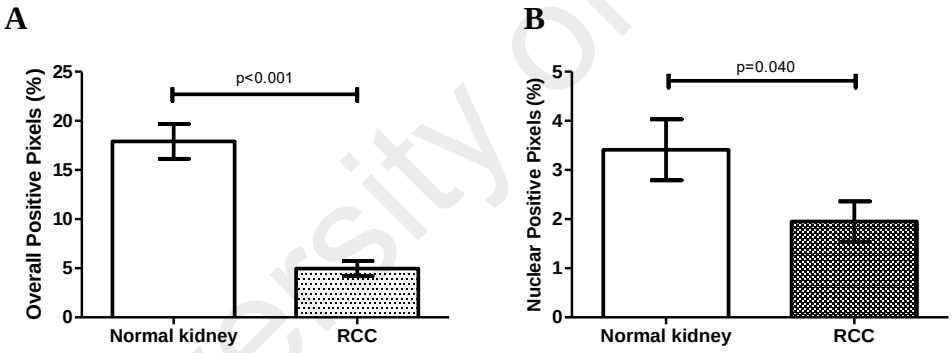


Figure 5.15. Immunopositivity of cRel in normal kidney tissue and RCC
(A) Overall and (B) nuclear cRel expression was significantly higher in normal kidney compared to RCC tissue.

Table 5.6 summarizes the comparison of cRel expression based on subtypes, tumour staging and Fuhrman grading. None of these characteristics significantly affected the immunopositivity of cRel. Additionally, cRel overall and nuclear expression had no significance on survival prognosis.

Table 5.6. Comparison of cRel expression based on pathological characteristics

	Overall		Nuclear	
Characteristics	**Positive pixels (%)**	**p Value**	**Positive pixels (%)**	**p Value**
Subtypes				
ccRCC	5.42±7.95		2.15±4.26	
pRCC	4.51±4.81	0.685	1.60±2.02	0.314
chRCC	0.50±0.25		0.60±0.59	
ccpRCC	2.45±2.07		0.43±0.08	
multilocular cystic RCC	1.70		0.43	
T stage				
1	4.42±6.41		1.52±2.19	
2	6.61±9.40	0.510	3.10±6.14	0.359
3	4.71±7.13		1.68±3.82	
4	0.24±0.20		0.17±0.04	
Metastasis at presentation				
No	4.58±6.06	0.342	1.66±3.08	0.189
Yes	6.36±11.02		2.96±5.99	
TNM stage				
I	4.59±6.62		1.34±1.53	
II	5.48±5.56	0.578	2.84±5.43	0.234
III	2.07±1.56		0.62±0.45	
IV	6.36±11.02		2.96±5.99	
Grade				
1	7.49±13.18		3.07±6.49	
2	5.34±7.01	0.305	1.53±1.98	0.207
3	3.32±5.67		1.75±4.53	
4	8.16±5.39		4.68±6.34	

*p value obtained using ANOVA and t-test

5.5 Discussion

The expression localisation and prognostic significance of NF-κB subunits (p50, p52, p65 and cRel) was evaluated in RCC tissue. Overall and nuclear expression of p65 was higher in RCC compared to normal kidney tissue, but interestingly, the opposite was seen for p50, p52 and cRel. The overall and nuclear expressions of these subunits (p50, p52 and cRel) were lower in RCC when compared to the adjacent normal kidney tissue.

In our cohort of patients, overall p65 expression was significantly associated with T stage, metastasis and survival outcome but was not an independent prognostic factor.

There was no significant prognostic association for p65 nuclear expression. Although nuclear localization indicates activation of NF-κB transcription, cytoplasmic expression may have influence on the progression of the tumour. For example, Dordevic et al. (2008) found a significant association of p65 cytoplasmic staining and proliferation rate of tumour cells (Djordjevic et al., 2008). In addition, Kankaya et al. (2015) reported a significant correlation of p65 cytoplasmic expression with high grade, advanced stage, peri-renal invasion and local invasion, but not for p65 nuclear expression (Kankaya et al., 2015). A systematic review and meta-analysis on NF-κB expression in solid tumours concluded that there was NF-κB association with worse prognostic parameters irrespective of localisation (Wu et al., 2015).

NF-κB subunits can form hetero or homo-dimers to bind to target genes, exerting positive or negative effects on the transcription of genes involved in various biological functions. The activation of these dimers depends on the type of ligands and receptors involved. The two most commonly known NF-κB activation pathways are the classical and alternative pathways. The classical pathway, involving the binding of p50 and p65 dimers to target genes regulating proliferation, survival and differentiation, is usually implicated in carcinogenesis (Nishikori, 2005). Alternative activation involves RelB and p52 dimers mainly in the regulation of lymphoid development and adaptive immunity (Nishikori, 2005). The cRel subunit play a role in proliferation, differentiation, and cytokine production of T cells (Visekruna et al., 2012). Unlike p65, RelB and cRel, the subunits p50 and p52 do not contain a transactivation domain and as a result, dimers of p50 or p52 can act as transcriptional inhibitors by competing for NF-κB binding sites of target gene promoters (Hayden & Ghosh, 2012). In order to behave as transcriptional activators, p50 and p52 bind to subunits containing the transactivation domain, p65 or RelB. Binding to Bcl-3 (B-cell lymphoma 3), which contains a transactivating domain

also enable p50 and p52 to behave as transcriptional activators (Hoesel & Schmid, 2013). Interestingly, p100, the precursor form of p52 is found to possess a death domain which may induce apoptosis (Perkins, 2003). Adding to the complexity of the regulation by the NF-κB subunits, the formation of different dimer combinations can activate different target genes. Moreover, the subunits contain sites for phosphorylation and may be subject to post-translational modifications, affecting activation and crosstalk with other signalling pathways (Hoesel & Schmid, 2013).

Further studies are required to understand the significance of lower p50, p52 and cRel expression in normal kidney compared to RCC tissue. Similar to findings by Moteoglu et al. (2008), p50 expression in our cohort of patients showed no significant prognostic association (Meteoglu et al., 2008). However, there was a trend of unfavourable survival outcome in patients with higher p50 immuno-positivity. Although the level of p52 was lower in RCC tissue compared to normal kidney, higher immuno-positivity in RCC tissue conferred a statistically significant disadvantage in survival outcome. There may be intricate interactions among the NF-κB subunits in RCC and the imbalance in expression of the subunits could possibly promote tumour formation or progression. Further functional studies in cell lines may help to unravel the complex relationship of NF-κB subunits in regulating cancer progression.

5.6 Conclusion

In conclusion, there were differential expressions of p50, p52, p65 and cRel in RCC tissue compared to the adjacent normal kidney and this may have contributed to the formation or progression of RCC. In tumour tissue, higher p52 and p65 expressions were associated with a worse survival prognosis indicating that these two subunits could be possible prognostic indicators in RCC.

CHAPTER 6 : EVALUATION OF TRAF1 AND NF-κB IN THE REGULATION OF PROLIFERATION AND APOPTOSIS IN COMMERCIAL RCC CELL LINES AND ESTABLISHED MALAYSIAN RCC CELL LINES

6.1 Introduction

TRAF1 and NF-κB subunits have been evaluated as potential detection or prognostic markers in the tissue or serum of RCC patients in Chapters 4 and 5. This aim of this chapter was to focus on the roles of TRAF1 and NF-κB p65 subunit in regulating proliferation and apoptosis using RCC cell lines. This was achieved through gene/protein expression silencing in cell line studies to evaluate the possible interaction between these two proteins in controlling apoptotic and proliferative proteins. Cell lines provide an avenue for in vitro investigation into the molecular and genetic aspects of renal carcinogenesis and pre-clinical studies for evaluating drug response or toxicity at a cellular level. At present, most commercially available renal carcinoma cell lines were established from Caucasians, such as ACHN, A-498, Caki-1, Caki-2, 769-P and 786-O which are available from American Type Culture Collection (ATCC), the most popular source of commercial cell lines. Cell lines established from other private or research laboratories which are not available commercially are not easily accessible unless a collaboration is established with the research groups.

Asians and Caucasians differ in incidence of RCC and response to targeted treatment and immunotherapy (S. H. Lee et al., 2014; Znaor et al., 2015). Hence, there might be some differences in the underlying molecular mechanisms of RCC cells in Caucasians and Asians. We have established RCC cell lines of our own as there has been an effort of blood, tissue and urine bio-banking from patients undergoing nephrectomy at UMMC since 2011. This bio-bank serves as an essential resource for this current

research as well as future research projects involving RCC. Furthermore, no other research groups in Malaysia have established RCC cell lines from their own patient cohort.

In this chapter, the gene and protein expressions of TRAF1 and p65 were knocked down in established RCC cell lines and commercial cell lines and the effects on the expressions of selected proliferation and apoptotic proteins were analysed.

6.2 Literature review

TRAF1 regulates apoptosis and proliferation mainly through NF-κB downstream pathways. TRAF1 frequently associates with TRAF2 in signalling pathways and can be pro-apoptotic or survival depending on the cell type, stimuli and receptor involved. In RCC, TRAF1 was proposed to be pro-apoptotic as there was an increase in RNA and protein levels after apoptosis was induced by IFN-α and radiation in a RCC cell line (Rajandram, Bennett, Morais, et al., 2012). The possible signalling pathways were described in Chapter 2 : Literature review (Section 2.11).

Our current study found an increase in NF-κB p65 subunit and decrease in TRAF1 protein expressions in RCC tissue compared to the adjacent normal kidney (Chapters 4 and 5). We postulate that TRAF1 may be controlling cell survival or apoptosis via activation or inhibition of p65 signalling pathways. TRAF1 has been shown to be involved in the activation or inhibition of NF-κB p65 pathways through studies conducted in other cells (T-cells, HeLa, HEK293) but not in RCC cell lines (Henkler et al., 2003; Shin, Park, Wu, & Hong, 2011). Among the genes/proteins of interest which can be activated by p65 are Bcl-XL, Bcl-2, cIAP-1, cIAP-2, cyclin D1, IL-6 and survivin. Bcl-XL, Bcl-2, cIAP-1, cIAP-2 and survivin are pro-survival proteins which

inhibit the activation of caspases and apoptotic signalling cascades. These proteins were introduced in Chapter 2 : Literature review (Sections 2.9 and 2.10).

Cyclin D1 and IL-6 can promote proliferation of cells. Cyclin D1 protein controls the checkpoint for progression from G1 to S phase of the cell cycle. Cyclin D1 levels will increase in early G1 phase and continue doing so until the G1/S transition phase where levels will decline (Alao, 2007). Overexpression of cyclin D1 in cells may speed up the passage through G1 phase in the cell cycle (Diehl, 2002). The over expression is linked to the development or progression of cancers such as breast, lung, bladder cancers and RCC (Alao, 2007; Lima et al., 2014). IL-6 is an inflammatory cytokine which can also take part in cellular signalling which promote cell survival, proliferation, angiogenesis, invasiveness and metastasis (Kumari, Dwarakanath, Das, & Bhatt, 2016). In cancers such as colorectal, breast, prostate and RCC, IL-6 was found to be elevated in the tissue or serum of cancer patients (Fu et al., 2015; Kumari et al., 2016). An increase in NF-κB p65 staining in tumour tissues might increase activation of these genes or proteins promoting tumour formation or growth.

Therefore, silencing or knock down experiments were conducted on commercial as well as cell lines established from RCC patients at UMMC to determine the effects of a reduction in TRAF1 and p65 protein levels on RCC cell proliferation or apoptosis and selected survival/apoptosis proteins of interest based on the background described.

6.3 Methodology

6.3.1 Establishment of RCC cell lines

6.3.1.1 Materials for cell line establishment

Table 6.1 lists the solutions or media which were required for the establishment of cell lines. Unless stated otherwise, high glucose Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Nacalai Tesque, Japan and cell culture materials or supplements were obtained from Gibco, USA.

Table 6.1. Solutions or media used in the establishment and maintenance of cell lines

Solution/Media	Contents
Tissue collection media	DMEM with high glucose 4.5g/L and sodium pyruvate supplemented with antibiotic-antimycotic 3X (penicillin 300units/ml, streptomycin 300µg/ml and amphotericin B 0.75µg/ml)
Phosphate buffered saline (PBS) pH 7.4	Purchased as a 10X stock solution from First Base Laboratories
Collagenase solution	Collagenase type II 1mg/ml in tissue collection media
Culture medium I	DMEM with high glucose 4.5g/L and sodium pyruvate supplemented with 10% fetal bovine serum (FBS), 1X antibiotic-antimycotic, Primocin 100µg/ml (Invivogen, USA) and Mycozap prophylactic 1X (Lonza, USA)
Culture medium II/Normal culture medium	DMEM with high glucose 4.5g/L and sodium pyruvate supplemented with 10% FBS and 1X antibiotic-antimycotic 0.25% Trypsin-EDTA solution
Cryo-media	DMEM plus 10% FBS and 10% dimethyl sulfoxide

6.3.1.2 Tissue collection

RCC tissue samples were collected from consented patients who have undergone nephrectomy for the removal of kidney tumour. Tissue collection was carried out aseptically with the help of the urologist surgeon and pathologist. The urologist surgeon confirmed the location of the RCC lesions during tissue collection and the pathologist

confirmed the pathological diagnosis after tissue processing. RCC tissue samples were taken within the tumour region, away from the tumour margin. Tumour samples were collected from the fleshy soft part of the tumour, avoiding the necrotic, fibrotic or haemorrhagic areas. Collection was carried out within an hour of the tumour or kidney removal from the patient. Figure 6.1 illustrates an example of a resected kidney with RCC from which tumour tissue was taken for cell line establishment. Each tissue sample was cut in two pieces with one part for formalin fixation (FFPE slides) and one part for cell line establishment. The RCC tissues for cell line establishment (sized 0.5-1.5cm^3) were placed in separate 50ml centrifuge tubes with 5ml of ice-cold tissue collection media.

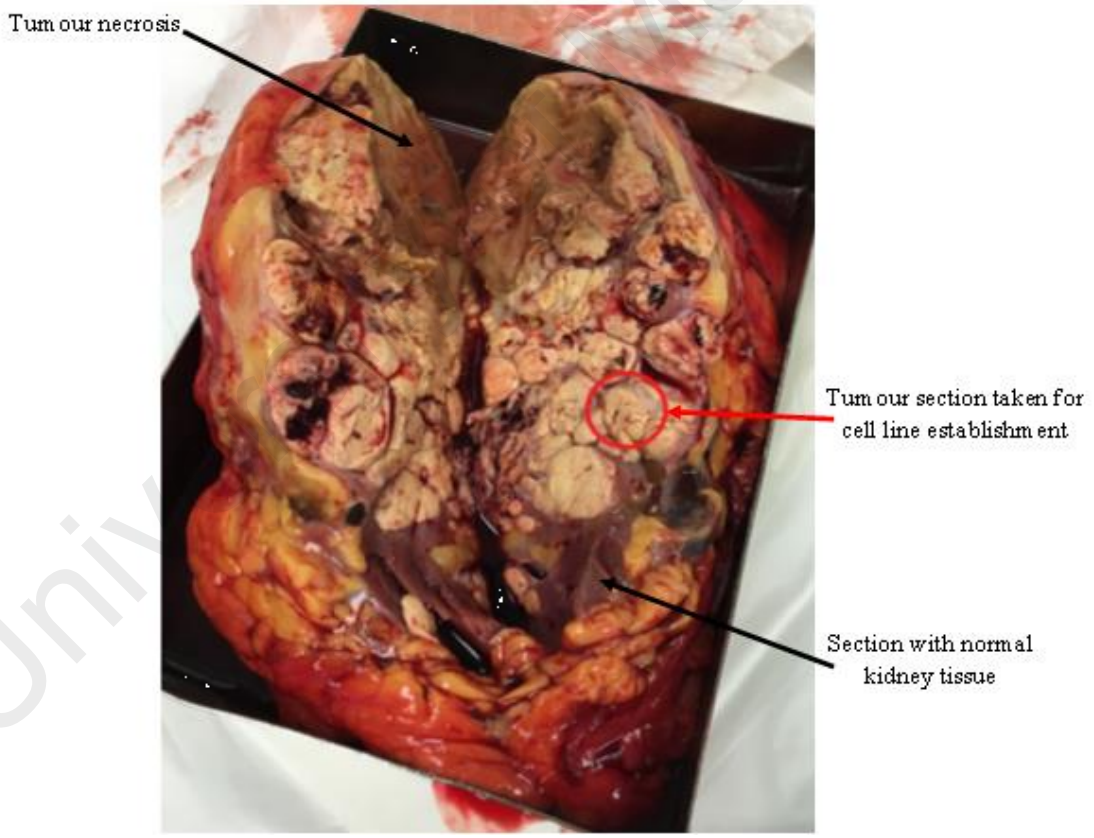


Figure 6.1. Example of tissue collection from nephrectomy sample
This image was taken before resection of tissues for cell line establishment. The tumour tissue for cell line establishment was taken from a tumour area clear of necrosis or haemorrhage. The section with normal kidney tissue was distinguishable from the tumour lesion.

6.3.1.3 Tissue Processing

Tissue samples were transported to the laboratory within an hour after collection and tissue processing was performed aseptically in the biosafety cabinet. Tissue samples were first placed on a petri dish where fat tissue and visible blood clots were dissected out. Tissue samples were then washed and vibrated with cold PBS pH7.4 in sterile 50ml tubes for 4-5 times to remove any remaining blood. Tissue samples were placed on 60mm petri dishes and minced into $1mm^3$ pieces with sterile blades. Tissue for establishment of epithelial cells was digested with collagenase next (6.3.1.4 Establishment of epithelial cell lines). However, for fibroblast cell establishment, the minced pieces were washed in PBS and placed directly in culture flasks for propagation, without collagenase digestion (6.3.1.5 Establishment of fibroblast cell lines).

6.3.1.4 Establishment of tumour epithelial cell lines

Procedures were performed at room temperature (≈27°C) unless stated otherwise. Tissue fragments were transferred to clean 50ml tubes and washed twice in fresh cold tissue collection media by centrifuging at 300g for 5 minutes. The supernatant was removed each time, and after the second wash, approximately 5ml of collagenase solution was added to each tube. The tubes containing the tissue fragments were agitated in a shaking incubator at 37°C for 45 minutes-1 hour. After enzymatic digestion, the digested tissue from each sample was sieved through a 70µm cell strainer (SPL Life Sciences, South Korea) into a clean 50ml tube to remove undigested tissue and glomeruli. The 50ml tubes containing the cells were centrifuged for 5 minutes at 300g and the supernatant was pipetted off. The cells were re-suspended in pre-warmed culture medium I and transferred to $25cm^2$ (T25) culture flasks (SPL Life Sciences,

South Korea). Typically, cells from each sample were seeded in two 25cm^2 flasks. Cell viability was not determined at this stage and seeding density was not tightly controlled. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2. After an overnight incubation (12-24 hours), the culture medium in the flasks was pipetted off along with any unattached cells and blood cells. Attached cells were gently washed once with pre-warmed wash media and replaced with fresh culture medium I. This procedure was repeated after 48 and 72 hours until the flasks were free of unattached cells and debris. Subsequently, culture medium was changed every 2-3 days until confluency. On day 7 onwards, culture medium II was used instead of culture medium I.

Yeast and bacterial contamination were determined using the Cell Culture Contamination Detection Kit (Molecular Probes, Thermo Fisher, USA) and presence of mycoplasma was detected using the MycoFluor™ Mycoplasma Detection Kit (Molecular Probes, Thermo Fisher, USA).

6.3.1.5 Establishment of cancer associated fibroblast cell lines

Fibroblast cells were grown using the tissue explant technique. Tumour tissue minced into 1mm^3 pieces were placed in a T25 culture flask and pre-warmed culture medium I was added before incubation at 37°C in a humidified atmosphere of 5% CO_2. After an overnight incubation (12-24 hours), the culture medium in the flasks was pipetted off along with any unattached cells and debris. Attached tissue pieces were gently washed once with pre-warmed wash media and replaced with fresh culture medium I. This procedure was repeated after 48 and 72 hours until the flasks were free of unattached cells and debris. On day 7 onwards, culture medium II was used instead of culture medium I. Fibroblast cells typically migrate out from the explant after 5-10 days. If

growth was slow, 5ng/ml of fibroblast growth factor (FGF) (Merck Millipore, USA) was added to the culture medium to encourage fibroblast growth.

6.3.1.6 Cell culture maintenance and subculture

Cells were grown to 80-90% confluency before passaging. Culture medium was removed and 1ml of 0.25% trypsin-EDTA was added to each T25 flask. After 5 minutes incubation at 37°C, the flasks were gently tapped to detach cells and trypsin reaction was stopped with the addition of 1ml culture medium II. Cells were pelleted by centrifuging at 200g for 5 minutes and re-suspended in culture medium II before seeding into a new T25 flask. Passaging was carried out in a ½ split ratio. For cryopreservation, cells were re-suspended in 1ml cryomedia, frozen at -80°C overnight and stored at -190°C in a liquid nitrogen tank. Cells were reactivated from cryopreservation by thawing at 37°C in a water bath and centrifuging the cells at 200g for 5 min. The cells were re-suspended in culture medium II and seeded into a T25 flask.

6.3.2 Cell characterization

6.3.2.1 Immunofluorescence (IF) staining

Cells were seeded at $1x10^5$/well in a 24 well plate. One 9mm cell culture coverslip (SPL, South Korea) was placed in each well before seeding. After 24 hours, the growth medium was removed and cover slips were washed 2x with PBS. Cells grown on coverslips were fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature (RT). The cells were then washed twice with PBS (1minute/wash) and permeabilized with 0.1% Triton X-100 in PBS for 10 minutes. Endogenous peroxidase

activity was blocked with 3% H_2O_2 in PBS for 5 minutes. After washing with PBS 3x (1minute/wash), the cells were incubated with primary antibody diluted to the optimal concentration in PBS overnight at 4°C. The dilution for pan-cytokeratin (PCK-26)(Abcam, USA) was 1:150 and alpha smooth muscle actin (α-SMA) (1A4)(Dako, USA) was 1:800. A volume of 200µl/well was required for antibody incubation. The cells were washed 3x (1minute/wash) with PBS and incubated with secondary antibody (anti-mouse/rabbit Alexa Fluor 488 from Thermo Fisher)(1:200 dilution). Cells were washed with PBS 3x and the cover slips were mounted on slides using Vectashield mounting medium with DAPI (4',6-diamidino-2-phenylindole).

Immunohistochemistry staining was also carried out using pan-cytokeratin (Abcam, USA)(1:200 dilution) and α-SMA (Dako, USA)(1:400 dilution) in FFPE ccRCC tissue collected from the operating theatre. This was done to determine the localisation and expression pattern of both antibodies in intact ccRCC tissue.

6.3.2.2 Quantitative PCR (qPCR)

The expressions of epithelial and fibroblast markers were determined in established cell lines using qPCR.

6.3.2.3 RNA extraction from cells

Cells were grown to confluency in a 60mm plate. At 80-90% confluency, the growth media was aspirated and cells were washed 2x with ice cold PBS. Then, 0.5ml Trizol was added to each petri dish. The cells were detached by repeated pipetting of Trizol on the petri dish surface before being transferred to a clean 1.5ml tube. All PCR related

pipetting from this point was performed in a designated PCR hood and procedures in tubes were performed on ice or a cooling rack. Cells were incubated in Trizol for 10 minutes and 250µl chloroform was added. The mixture was mixed by inverting the tube for 15 seconds, and left to incubate for 10 minutes. After incubation, the cell mixture was centrifuged at 16000G for 15 minutes at 4°C. Three distinctive layers will form (top-clear, middle-white, bottom-pink). The top later was removed carefully and transferred to a new 1.5ml tube where 500ul of isopropanol was added. The solution was mixed by inversion until a white precipitate can be seen and then incubated for 10 minutes. The solution was then centrifuged at 16000G for 20min (4°C) and the isopropanol was poured out gently without removing the sedimented precipitate. Without re-suspending, the RNA sediment was washed with 75% ethanol in nuclease free H_2O 3x with centrifugation (16000G for 15 minutes at 4°C), after each wash. After the last wash, the ethanol was poured out gently leaving behind the RNA precipitate to air dry in the PCR hood for 20 minutes. Twenty microlitres of nuclease free H_2O was added to the tube and the RNA was re-suspended before the A260/A280 and A260/230 was measured using Nanophotometer (Implen, USA). Acceptable A260/280 was 1.8–2.2 and A260/230 was 2.0-2.2. The concentration of RNA was calculated as ng/µl by Nanophotometer. If not immediately used, the RNA was stored at -80°C until analysis.

6.3.2.4 cDNA conversion

cDNA conversion was achieved using the RevertAid First Strand cDNA synthesis kit (Thermo Fisher, USA) according to the manufacturer's instructions. The following reagents were added to a 1.5ml PCR tube : Total RNA 1µg + Nuclease free H_2O = 11µl, random primer 1µl, 5x reaction buffer 4µl, Ribolock RNase inhibitor 1µl, 10mM dNTP

mix 2µl and RevertAid M-Mul V RT 1µl. The total reaction volume was 20µl. The tube was incubated for an hour at 42°C and reaction was terminated at 70°C for 5 minutes.

6.3.2.5 qPCR analysis

qPCR reaction was performed with the EvaGreen qPCR Mix Plus (Solis Biodyne, Estonia). The following reagents were required for one reaction or sample : 5x EvaGreen qPCR Mix Plus 4µl, forward primer (10µM) 0.5µl, reverse primer (10µM) 0.5µl, DNA template 1µl and nuclease free H_2O 14µl. Amplification was accomplished in 0.1ml PCR strip tubes using the 7500 Fast Real-Time PCR System (Applied Biosystems, USA). The run method used was as follows :

Holding Stage –		95°C, 15 minutes
Cycling –	Denaturation	95°C, 15 seconds
	Annealing	60°C, 1 minute
	Elongation	72°C, 20 seconds

Gene expression level was quantified by the comparative C_T method. The difference between the C_T values (ΔC_T) of the gene of interest and the housekeeping gene was calculated for each experimental sample. The fold-change in expression of the gene of interest between the experimental sample and reference sample was calculated from this equation :

$$2^{-\Delta\Delta CT} = 2^{(\Delta CT \text{ experimental sample} - \Delta CT \text{ reference sample})}$$

The housekeeping gene used was 18s rRNA. The expressions of epithelial markers cytokeratin 8 (CK8) and e-cadherin, as well as fibroblast markers alpha smooth muscle actin (α-SMA), fibroblast activation protein (FAP) and vimentin were evaluated. The primer sequences for the target genes are listed in Table 6.2. The primer sequences were obtained from PrimerBank, a validated public online resource for PCR primers provided by collaboration between Massachusetts General Hospital and Harvard Medical School. Vimentin was expected to be expressed in ccRCC epithelial cells as well. Gene expression of established cell lines were compared to the equivalent mRNA levels found in a representative ATCC RCC epithelial cell line (ACHN). All experiments were carried out in triplicates.

Table 6.2. Primer sequences for qPCR of epithelial and fibroblast marker expressions in cell lines

Gene	Primers	Sequence
18s rRNA	Forward	5'-CAGCCACCCGAGATTGAGCA-3'
	Reverse	5'-TAGTAGCGACGGGCGGTGTG-3'
CK8	Forward	5'-CAGAAGTCCTACAAGGTGTCCA-3'
	Reverse	5'-CTCTGGTTGACCGTAACTGCG-3'
E-cadherin	Forward	5'-CGAGAGCTACACGTTCACGG-3'
	Reverse	5'-GGGTGTCGAGGGAAAAATAGG-3'
α-SMA	Forward	5'-GTGTTGCCCCTGAAGAGCAT-3'
	Reverse	5'-GCTGGGACATTGAAAGTCTCA-3'
FAP	Forward	5'-TGAACGAGTATGTTTGCAGTGG-3'
	Reverse	5'-GGTCTTTGGACAATCCCATGT-3'
Vimentin	Forward	5'-TGCCGTTGAAGCTGCTAACTA-3'
	Reverse	5'-CCAGAGGGAGTGAATCCAGATTA-3'

6.3.3 Cell line experiments to evaluate the roles of TRAF1 and NF-κB in the regulation of proliferation and apoptosis in commercial RCC cell lines and established Malaysian RCC cell lines

6.3.3.1 ATCC (American Type Culture Collection) cell lines and own established RCC cell lines

The two ATCC cell lines used in this project were ACHN and Caki-1. ACHN was derived from the metastatic pleural effusion of a 22 year old Caucasian male diagnosed with renal cell adenocarcinoma. This cell line was used in previous experiments involving TRAF1 and showed resistance to treatment induced apoptosis. Caki-1 is a ccRCC cell line derived from the skin metastatic site of a 49 year old Caucasian male. Both are epithelial cell lines.

RCC1 and RCC2, the first two spontaneously immortalised cell lines (from cell line establishment section 6.3.1) were also used for experimentation.

6.3.3.2 Maintenance of cell lines

Cell lines were grown routinely in Dulbecco's Modified Eagle's Medium (DMEM)(#08458 Nacalai Tesque, Japan) containing the antibiotics/antimycotic penicillin (100U/mL), streptomycin (100 µg/mL) and 0.25µg/mL of amphotericin B (Gibco® Antibiotic-Antimycotic 100x, USA). The culture medium is supplemented with 10% FBS (Gibco, USA). This growth medium will be referred to as "normal culture medium". Cells were grown in T25 tissue culture flasks with 2-3mL of normal

culture medium, in a humidified tissue culture incubator at 37°C with 5% CO_2. Cells were sub-cultured at 80-90% confluency.

Sub-culturing and cryopreservation was carried using similar protocols in Section 6.3.1.6. Passaging was carried out in a 1/3 split ratio.

6.3.3.3 TRAF1 and p65 silencing experiments using siRNA

In the silencing experiments, TRAF1 and p65 proteins are knocked down using target specific 19-25 nucleotide siRNA purchased from Santa Cruz Biotechnologies, USA. The control used was a non-targeting 20-25 nucleotide siRNA (Santa Cruz Biotechnologies, USA) designed as a negative control. A separate fluorescein conjugated siRNA control was used to confirm the delivery of siRNA into cells.

6.3.3.4 siRNA transfection protocol

Cells in T25 tissue culture flasks were grown to confluency and passaged. Cells were seeded in 60mm culture dishes at $5x10^5$cells/dish for silencing experiments. Transfection was carried out after 24 hours. The transfection solution was prepared as follows : 200ul of DMEM added with 10ul lipofectamine 2000 (Thermo Scientific, USA) and 7µl siRNA (TRAF1 or p65). In the case of controls, 3µl of control siRNA was added instead. The transfection solution was incubated for 15 minutes at room temperature. Before addition of the transfection solution, culture medium was removed from the 60mm culture dishes, and the cells were washed once with 1ml DMEM (without FBS and antibiotics). The transfection solution was topped up with 800µl DMEM and added to the 60mm culture dishes. The cells were then left to incubate 5

hours at 37°C in a tissue culture incubator, then 1ml of normal culture medium containing 2 times the normal serum and antibiotics concentration was added to the cells without removing the transfection mixture and incubated for an additional 72 hours. The optimal incubation period was determined to be 72 hours from previous experiments (Rajandram, Bennett, Wang, et al., 2012). After incubation, protein or RNA extraction was carried out on the cells.

Transfection protocol was similar for the fluorescein conjugated siRNA control. Transfection was carried out as above and the cells were photographed using an inverted fluorescence microscope after 48 hours incubation.

6.3.3.5 qPCR analysis

RNA extraction, cDNA conversion and PCR protocol were performed with similar procedures as mentioned earlier (Section 6.3.2.2). Gene expressions of TRAF1 and p65 siRNA treated cells were calculated as relative expression compared to control siRNA treated cells. The genes listed in Table 6.3 were chosen based on the possibility of involvement in the TRAF1 or p65 pathways. The primer sequences were obtained from PrimerBank, a validated public online resource for PCR primers provided by collaboration between Massachusetts General Hospital and Harvard Medical School. Genes of interest were those with >1.5 and <0.5 fold change in expression compared to the control.

Table 6.3. Primer sequences for qPCR of selected genes of interest

Gene	Primers	Sequence
18s rRNA	Forward	5'-CAGCCACCCGAGATTGAGCA-3'
	Reverse	5'-TAGTAGCGACGGGCGGTGTG-3'
TRAF1	Forward	5'-TCCTGTGGAAGATCACCAATGT-3'
	Reverse	5'-GCAGGCACAACTTGTAGCC-3'
NF-κB p65	Forward	5'-GTGGGGACTACGACCTGAATG-3'
	Reverse	5'-GGGGCACGATTGTCAAAGATG-3'
Bcl-XL	Forward	5'-GAGCTGGTGGTTGACTTTCTC-3'
	Reverse	5'-TCCATCTCCGATTCAGTCCCT-3'
cIAP-1	Forward	5'-AGCACGATCTTGTCAGATTGG-3'
	Reverse	5'-GGCGGGGAAAGTTGAATATGTA-3'
cIAP-2	Forward	5'-GCCATTGACTTTTCTGTCGCC-3'
	Reverse	5'-GCAAAGCAAGCCACTCTG-3'
Cyclin D1	Forward	5'-GCTGCGAAGTGGAAACCATC-3'
	Reverse	5'-CCTCCTTCTGCACACATTTGAA-3'
IL-6	Forward	5'-CCTGAACCTTCCAAAGATGGC-3'
	Reverse	5'-TTCACCAGGCAAGTCTCCTCA-3'
Survivin	Forward	5'-AGGACCACCGCATCTCTACAT-3'
	Reverse	5'-AAGTCTGGCTCGTTCTCAGTG-3'

6.3.3.6 Protein extraction

Cells on culture dishes were washed twice with ice cold PBS before 200µl of radioimmunoprecipitation assay (RIPA) buffer (Merck Millipore, USA) with protease inhibitor cocktail (Thermo Fisher, USA) was added to each dish. Cells were scraped to one side using a cell scraper, collected in 1.5ml centrifuge tubes and left to incubate on ice for 10 minutes. Cells were then dismembranated by passing them several times through a 27-gauge needle on a 1ml sterile syringe, then centrifuging at 13000g for 20 minutes at 4°C. The supernatant was removed to a clean, labelled 1.5ml centrifuge tube and the cell pellet discarded. Protein concentration in the cell lysate was determined using the bicinchoninic acid (BCA) assay.

6.3.3.7 BCA assay

BCA assay (Pierce BCA Assay Kit) was carried out according to the manufacturer's instructions. Briefly, 10µl of BSA standards and samples were pipetted into each well of a 96-well microplate. Then, 200µl of BCA working reagent (50 parts of BCA Reagent A and 1 part of BCA Reagent B) was added to each well and the microplate was incubated at 37°C for 30 minutes. Assays were performed in triplicates. After incubation, absorbance was read at 562nm using a plate reader (Multiskan GO, Themo Scientific, USA). The standard curve is plotted using Microscoft Excel and the protein concentrations in samples were determined using the standard curve equation. An example of a standard curve is shown in Figure 6.2.

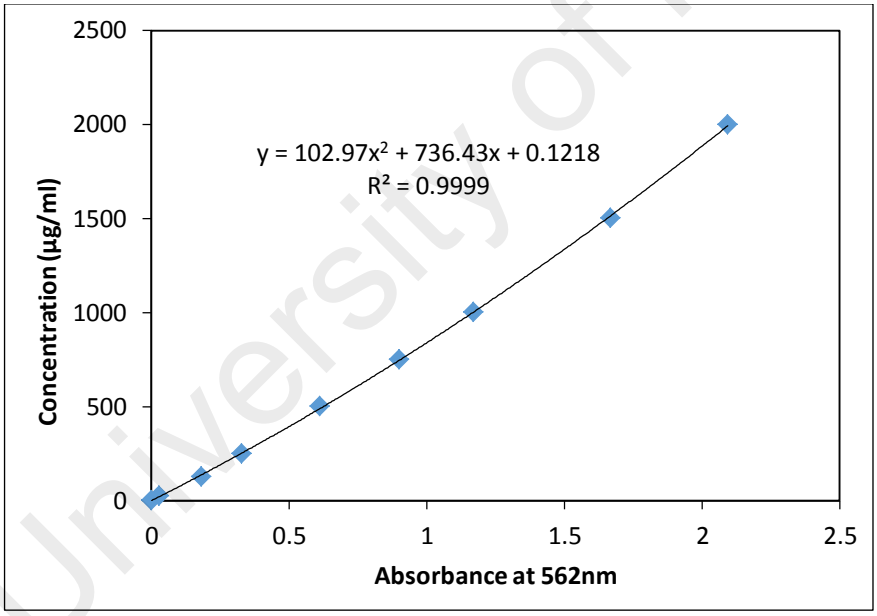


Figure 6.2. Example of a standard curve for BCA assay

6.3.3.8 Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE)

Recipes of solutions and buffers used as well as setting up of the western blotting tanks/apparatus are detailed in Appendix G and H. Gels used were 10% (for proteins 30-100kD) and 12% (for proteins <30kD) and loading volume for each sample was

30µg of protein (calculated from BCA assay). Sample preparation is detailed in Appendix H. A Western blot protein standard ladder (Precision Plus Protein™ WesternC™ Standards, Biorad, USA) was loaded for each gel at 2.5µl per well. Electrophoresis was run with the setting of 100V for 1 hour 30 minutes.

6.3.3.9 Electrophoretic transfer

Solutions and set up are detailed in Appendix H. Polyvinylidene difluoride (PVDF) membrane for transfer was activated by washing in 98% methanol for 10 minutes, then washing twice in deionised water (2 minutes/wash) and kept in transfer buffer until electrophorectic transfer. Activation of PVDF membrane was performed while running SDS-PAGE electrophoresis. Transfer was carried out at 100V for 1 hour 45 minutes.

6.3.3.10 Immunoblot protocol

The membrane was blocked using 5% milk in TBST for 1 hour with gentle shaking at room temperature. After blocking, the membrane was incubated in primary antibody diluted with 5% milk in TBST (except 3% BSA in TBST for caspase 8, cIAP-1 and IL-6) overnight with gentle rocking at 4°C. After that, the membrane was washed with TBST for three times (2 minutes each) and incubated with secondary antibody in 5% milk (in TBST) for an hour at room temperature with gentle shaking. The antibodies used and dilutions of primary/secondary antibodies are listed in Table 6.4. The membrane was then washed three times with TBST (2 minutes each) and bands were developed with the aid of Clarity Western ECL Blotting Substrate (Biorad, USA) for 5 minutes. The bands were viewed using ChemiDoc XRS+ (Biorad, USA) gel imaging system. Actin was used as the loading control for each well. Membranes containing

target proteins with bands near to or overlapping 43kD (actin) were stripped with Restore Western Blot stripping buffer (Thermo Scientific, USA) for 5 minutes at 37°C. The membranes were then re-probed with actin. Bands were analysed using Image Lab 4.5 software from Biorad and normalised against the actin loading controls. The blots were performed in triplicates and calculated as mean ± standard error of relative expression ratio compared to controls.

Table 6.4. Antibodies used and their dilutions

Primary antibody	**Company and reference no.**	**Dilution**
Actin (goat polyclonal)	Santa Cruz Biotechnologies, sc-1616	1 in 1000
Bcl-2 (rabbit polyclonal)	Santa Cruz Biotechnologies, sc-492	1 in 200
Bax (rabbit polyclonal)	Santa Cruz Biotechnologies, sc-526	1 in 200
Caspase 3 (rabbit polyclonal)	Santa Cruz Biotechnologies, sc-7148	1 in 300
Caspase 8 (mouse monoclonal)	Cell Signalling Technology, #9746	1 in 500
cIAP-1 (rabbit monoclonal)	Abcam, ab108361	1 in 500
cIAP-2 (rabbit polyclonal)	Santa Cruz Biotechnologies, sc-7944	1 in 150
Cyclin D1 (mouse monoclonal)	Santa Cruz Biotechnologies, sc-8396	1 in 200
IL-6 (rabbit monoclonal)	Cell Signalling Technology, #12153	1 in 500
NF-κB cRel (rabbit polyclonal)	Santa Cruz Biotechnologies, sc-71	1 in 100
NF-κB p50 (rabbit polyclonal)	Santa Cruz Biotechnologies, sc-7178	1 in 400
NF-κB p52 (rabbit polyclonal)	Santa Cruz Biotechnologies, sc-298	1 in 400
NF-κB p65 (rabbit polyclonal)	Santa Cruz Biotechnologies, sc-372	1 in 1000
TRAF1 (rabbit polyclonal)	Santa Cruz Biotechnologies, sc-7186	1 in 200
TRAF2 (rabbit polyclonal)	Santa Cruz Biotechnologies, sc-7187	1 in 200
Secondary antibody	**Company and reference no.**	**Dilution**
Bovine anti-mouse IgG-HRP	Santa Cruz Biotechnologies, sc-2371	1 in 2000
Goat anti-rabbit IgG-HRP	Santa Cruz Biotechnologies, sc-2004	1 in 2000
Rabbit anti-goat IgG-HRP	Santa Cruz Biotechnologies, sc-2786	1 in 2000

6.3.4 Statistical analysis

Statistical analysis was carried out using T-test from the SPSS v20 (IBM, USA) software. A p value of <0.05 was statistically significant.

6.4 Results

6.4.1 Establishment of Malaysian RCC cell lines

Tumour tissue collection and epithelial cell line establishment protocol was optimized after 12 trials. After optimization, 11 cell lines spontaneously immortalised out of 51 trials. Therefore, the tumour epithelial cell spontaneous immortalisation rate was 21.6% with the optimized protocol. Cells were considered immortalised if they could be passaged beyond the 10^{th} passage. Most cells senesced and stopped proliferating after 3-5 passages. Out of the spontaneously immortalised cell lines, 7 were ccRCC variants, 2 were ccRCC with sarcomatoid transformation, one was mostly undifferentiated RCC with some papillary features and one was Ewing's sarcoma. Each cell line had distinctive morphology ranging from polygonal epithelial to spindle shaped and elongated. Nine immortalised cell lines were from patients with stage T2 tumours and above, whereas two were stage T1b with metastasis. In total, 6/11 (54.5%) immortalised cell lines were from patients having metastasis at presentation. The cell lines were from tumours with grade 2 and above except for two with grade 1 and metastasis (both T1b tumours).

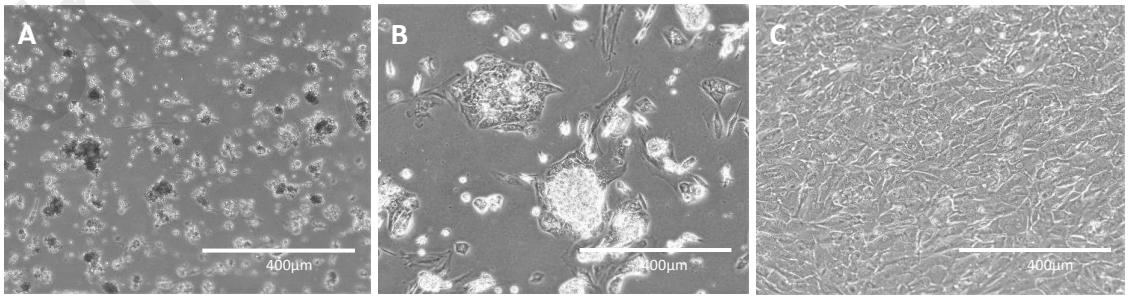


Figure 6.3. The sequence of a typical RCC epithelial cell line during establishment
(A) Day 1 – Viable cells attach to the flask surface. (B) Day 4 – Attached cells start to flatten out and proliferate from the cell clumps. (C) Cells reach confluency in 7 to 15 days. All images were taken at 100x magnification.

Out of 11 trials of growing RCC cancer associated fibroblast cells, none has immortalised at the moment. Compared to RCC epithelial cells, RCC fibroblasts were harder to grow and contamination of fibroblast cells in RCC epithelial lines was rarely an issue. Morphologically, RCC epithelial and fibroblast cells were distinguishable (Figure 6.4).

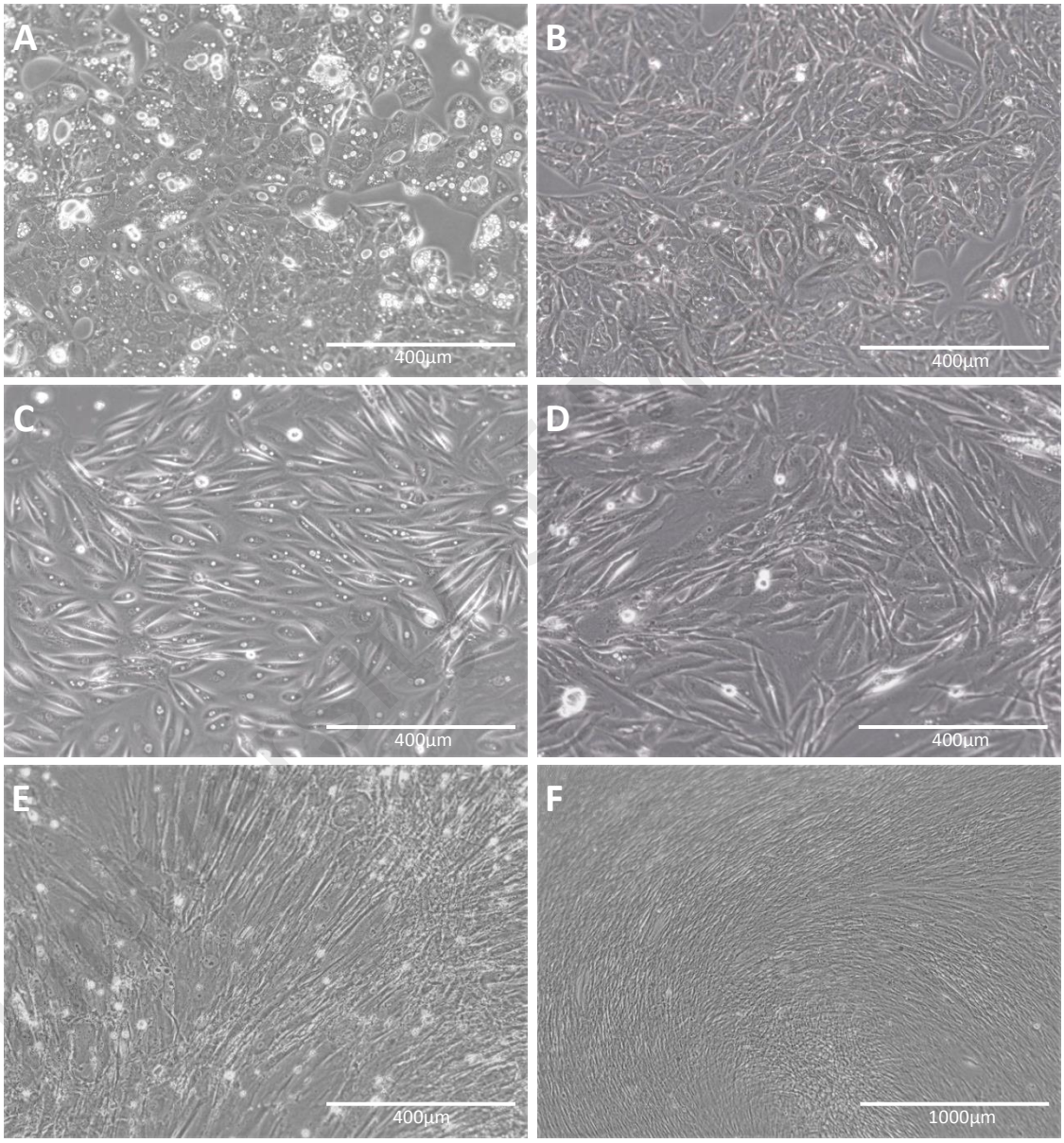


Figure 6.4. Examples of RCC epithelial and fibroblast cell lines
(A) ccRCC cells at confluency with lipid/glycogen globules. (B) Polygonal cells of ccRCC cells at confluency. C. Multilocular cystic RCC cells. (D) Spindle shaped ccRCC with sarcomatoid transformation. (E) Long spindly elongated cells of ccRCC fibroblast. (F) Fibroblast cells have a swirling fingerprint like pattern at confluency. All images were taken at 100x magnification except for F at 40x magnification.

RCC epithelial cell lines stained positive for epithelial marker pan-cytokeratin and negative for fibroblast marker, α-SMA (Figure 6.5).

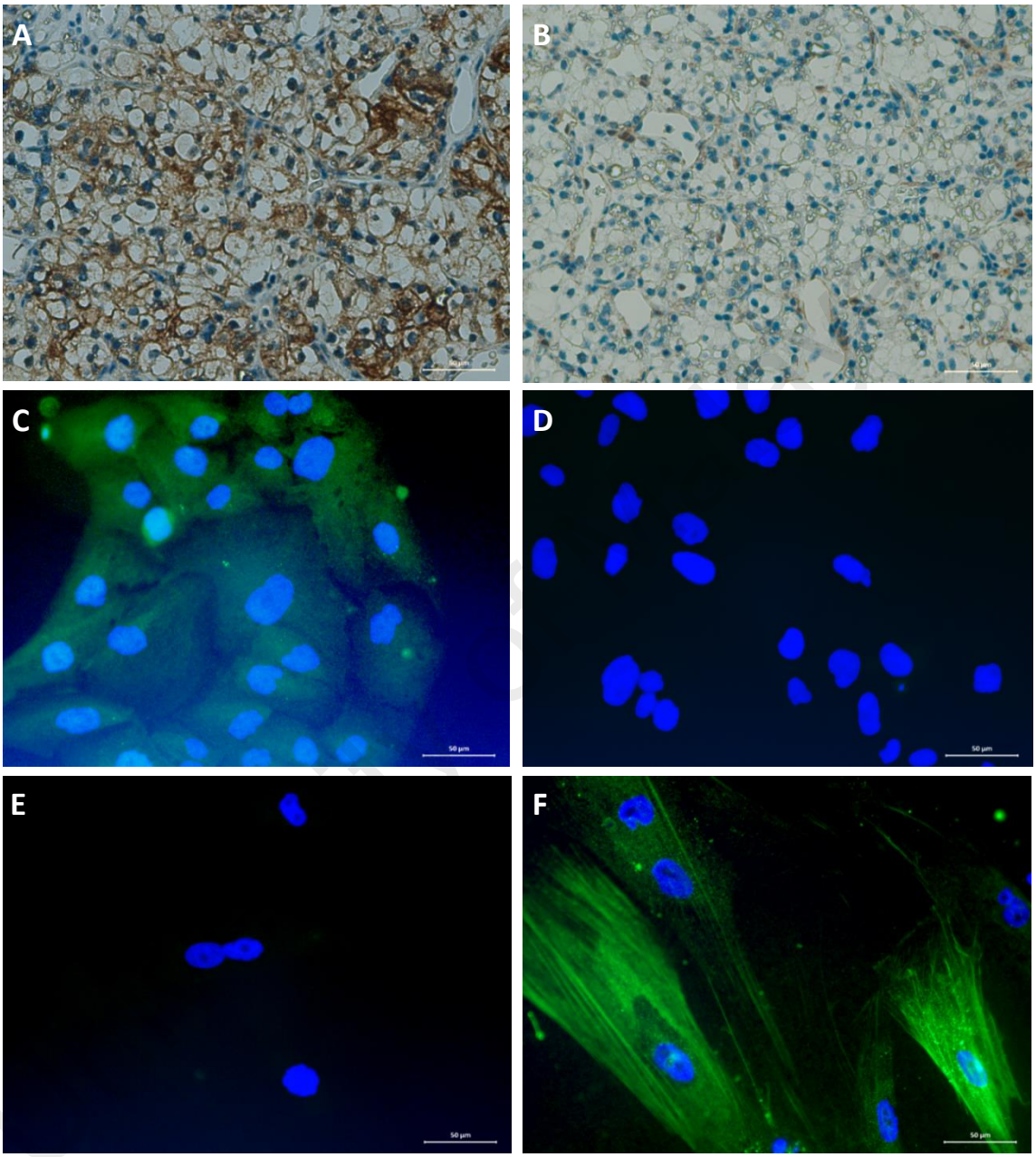


Figure 6.5. IHC and IF of pan-cytokeratin and α-SMA in RCC tissue and cells
(A) Positive IHC staining of pan-cytokeratin in ccRCC tissue. (B) Negative/weak staining of α-SMA in ccRCC tissue. (C) IF staining of pan-cytokeratin and (D) negative IF staining of α-SMA in established epithelial ccRCC cell line. (E) Negative IF staining of pan-cytokeratin and (F) positive staining of α-SMA in fibroblast ccRCC cell line. All taken at 400x magnification.

Quantitative PCR analysis revealed higher epithelial marker expressions in these epithelial cell lines compared to fibroblast cells (Figure 6.6). RCC1 and RCC2 showed higher epithelial marker (CK8 and E-cadherin) and lower fibroblast marker (α-SMA and FAP) expressions compared to Fibroblast 1 and 2. All cell lines had mixed vimentin (fibroblast marker) expression as ccRCC epithelial cells are known to express this protein.

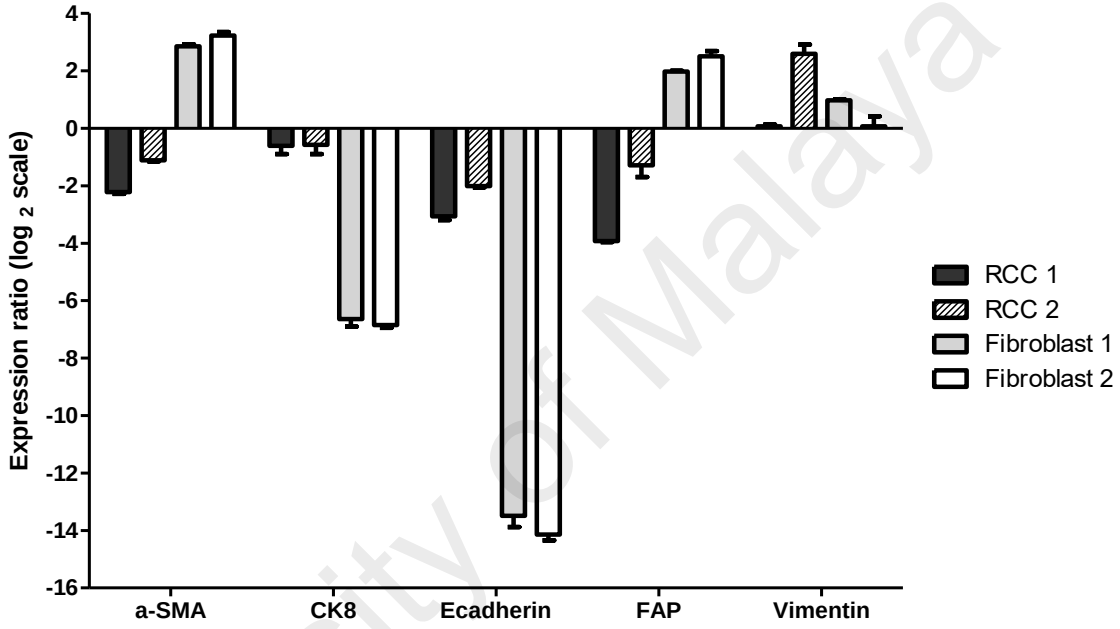


Figure 6.6. Epithelial and fibroblast marker expressions of ccRCC epithelial cell lines (RCC1 and RCC2) and ccRCC fibroblast cells (Fibroblast 1 and 2)
Data shown are representative of triplicate experiments. The expression ratio of the genes in the newly established cell lines were compared to the equivalent mRNA levels found in a representative ATCC epithelial cell line (ACHN).

The first two spontaneously immortalised cell lines (RCC1 and RCC2) were used in silencing experiments along with commercially available ATCC cell lines ACHN and Caki-1. RCC1 is a ccRCC cell line established from a local Chinese woman who was diagnosed with metastasis to the lungs. She had a grade 3 tumour which was 20cm in its greatest dimension. The cell line was established from the primary tumour. RCC2 was established from a Malay man with a localised stage T2a (10cm) and grade 3 tumour. Both were able to grow up to over 100 passages. However, only passages 10-30 were

used in experiments. Similarly, for ACHN and Caki-1, only passages below 30 were used for experiments.

6.4.2 Baseline protein levels of TRAF1 and NF-κB p65 in selected cell lines

Caki-1, RCC1 and RCC2 had low TRAF1 protein expression compared to ACHN (Figure 6.7). It was sometimes difficult to detect the TRAF1 band in these three cell lines and a higher concentration of TRAF1 (1 in100 dilution) caused the appearance of higher background signals. Hence, only ACHN cell line was used for TRAF1 siRNA experiments. Protein expression of p65 was lowest in ACHN and highest in RCC2. The band for p65 was strong in all cell lines tested using 1:1000 dilution. Therefore, p65 siRNA experiments were performed in all four cell lines. The subunit p65 was chosen for silencing experiments as it is postulated to be involved in the regulation of cell proliferation or apoptosis along with TRAF1.

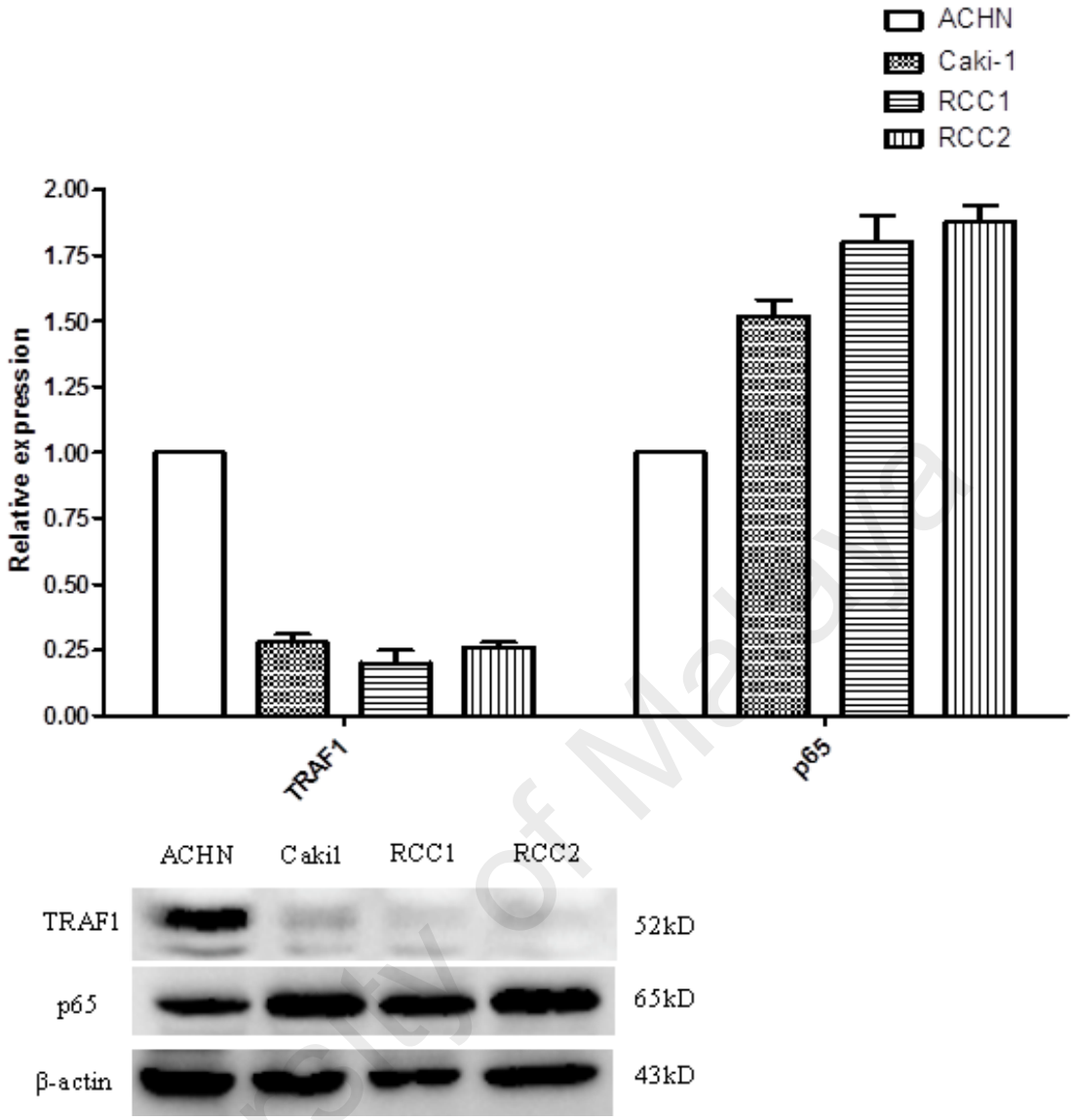


Figure 6.7. Western blotting of baseline TRAF1 and p65 protein expressions in selected cell lines

6.4.3 Evaluating the roles of TRAF1 and NF-κB p65 in the regulation of proliferation and apoptosis using RCC cell lines

6.4.3.1 Delivery of RNA into cells using lipofectamine

The fluorescein tagged control RNA was successfully delivered or transfected into ACHN cells using Lipofectamine 2000 as shown in Figure 6.8.

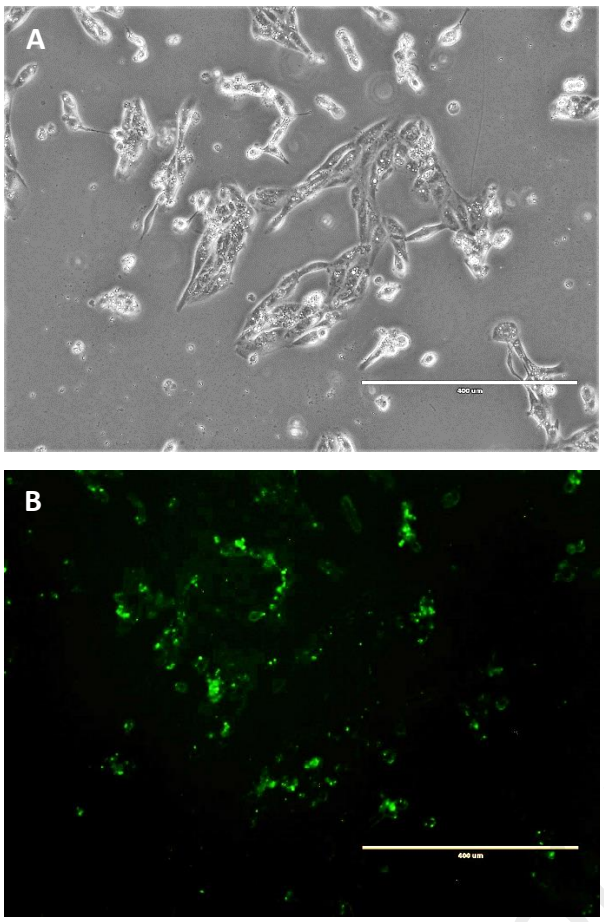


Figure 6.8. Image of ACHN cells after transfection with fluorescein tagged control RNA
Images taken using (A) transmitted light and (B) fluorescence (GFP filter) inverted microscope (100x magnification)

6.4.3.2 Knockdown of TRAF1 and NF-κB p65 in ACHN using siRNA compared to controls

Knockdown of TRAF1 protein expression (<0.5 fold) was seen in ACHN cells treated with TRAF1 siRNA compared to the different controls (UC, LC, SC) (Figure 6.9). There was also a decrease in TRAF1 expression for p65 siRNA treated cells. Knockdown of p65 protein expression (<0.5 fold) was also seen in ACHN cells treated with p65 siRNA compared to the different controls (UC, LC, SC) and TRAF1 siRNA. There was no knockdown of TRAF1 or p65 protein expressions in the control siRNA treated cells, hence this was used as the control for subsequent silencing experiments.

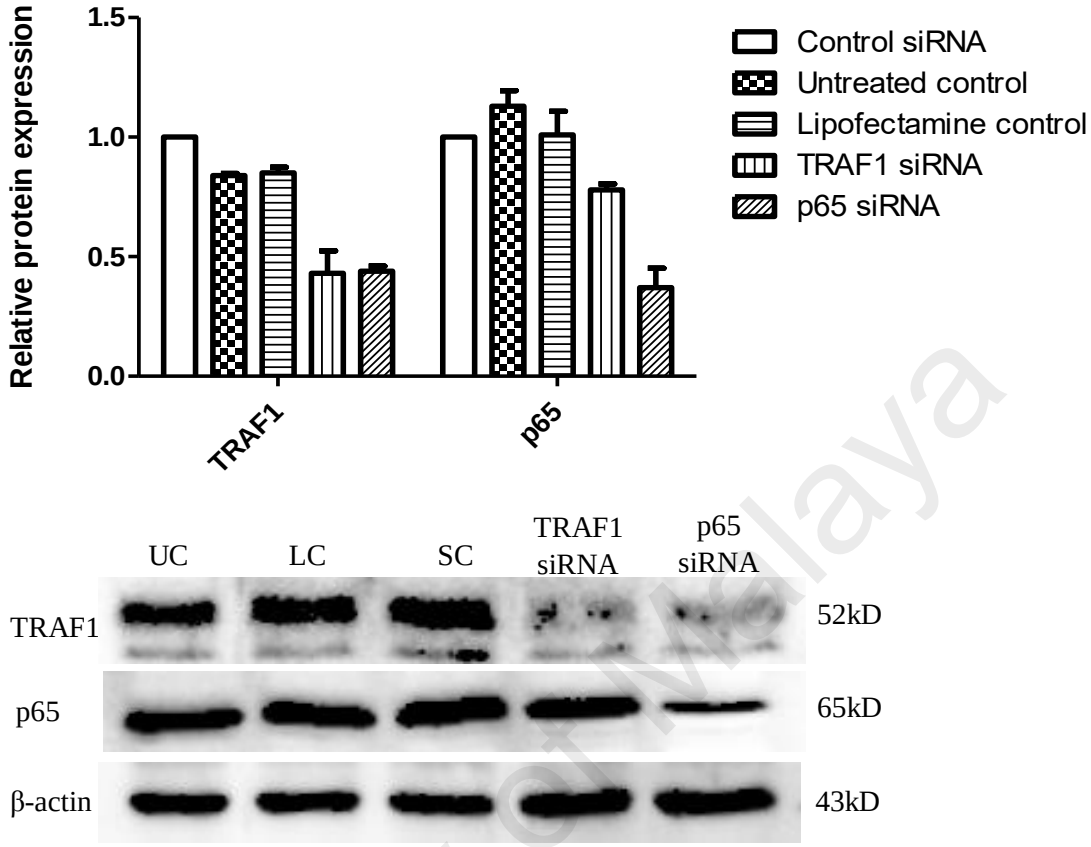


Figure 6.9. Western blot to compare knock down of TRAF1 and p65 protein expressions in ACHN cell line
The different treatments were : Untreated control (UC), Lipofectamine only control (LC), Control siRNA + lipofectamine (SC), TRAF1 siRNA + lipofectamine and p65 siRNA + lipofectamine.

6.4.3.3 Potential target genes for NF-κB p65

Quantitative PCR was carried out in ACHN and Caki-1 cell lines after silencing experiments (TRAF1, p65) to determine additional target genes/proteins of p65. Western blotting of apoptotic/survival proteins was performed for Bax, Bcl-2, caspase 3, caspase 8 and TRAF2. Bax, Bcl-2, caspase 3 and caspase 8 are proteins commonly evaluated in cell apoptosis/survival signalling and TRAF2 is intricately involved in the regulation along with TRAF1. In order to better understand TRAF1 and p65 in regulating apoptosis and proliferation, additional p65 target genes/proteins were

evaluated using qPCR in ACHN and Caki-1 cell lines. Genes with changes in expression <0.5 or >1.5 fold after TRAF1 or p65 silencing were then chosen to be evaluated for protein expression using western blotting.

ACHN had an approximately 36% knockdown of TRAF1 gene expression after transfection with TRAF1 siRNA and a 46% knockdown of p65 gene expression after transfection with p65 siRNA (Figure 6.10). Genes with a >1.5 fold change in expression were cIAP-1 and cIAP-2 after TRAF1 siRNA transfection. Genes with <0.5 fold down-regulation after p65 siRNA transfection were cyclin D1 and IL-6.

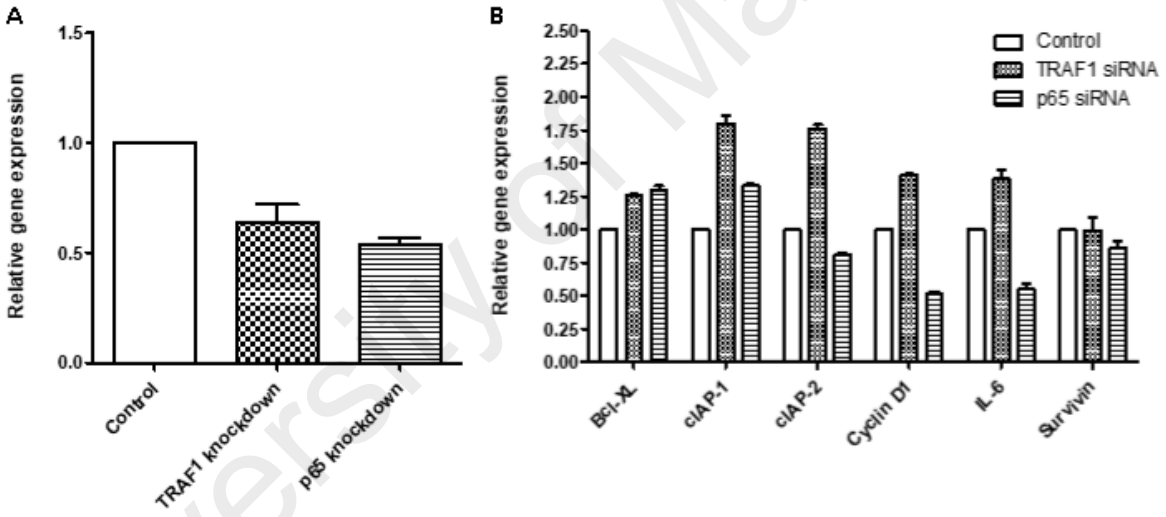


Figure 6.10. TRAF1 and p65 silencing in ACHN cells
(A) TRAF1 and p65 gene expressions were reduced after silencing. (B) Gene expressions of selected p65 target genes after TRAF1 and p65 silencing.

Caki-1 had an approximately 39% knockdown of p65 gene expression after transfection with p65 siRNA. There were no genes with >1.5 fold change in expression (Figure 6.11). Genes with <0.5 fold down-regulation after p65 siRNA transfection were cyclin D1 and IL-6. Based on these results, additional proteins that were selected for western blotting were cIAP-1, cIAP-2, cyclin D1 and IL-6

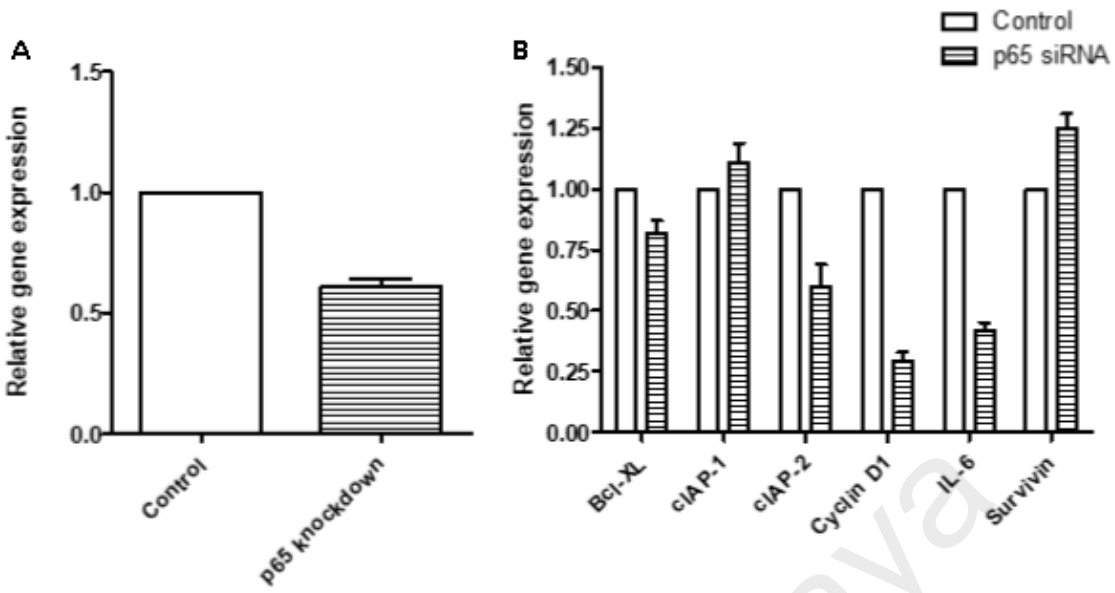


Figure 6.11. p65 silencing in Caki-1 cells
(A) p65 gene expression was reduced after silencing. (B) Gene expressions of selected p65 target genes after p65 silencing.

6.4.3.4 Silencing experiments in ACHN cell line

Transfection of TRAF1 siRNA in ACHN cells reduced the TRAF1 protein expression to 0.50±0.05 fold compared to the control siRNA treated cells (Figure 6.12 and 6.13). Bax was also significantly decreased by 0.52±0.08 fold in expression compared to the control after transfection with TRAF1 siRNA. Proteins with significant increase in expression after transfection with TRAF1 siRNA were cIAP-1 (1.23±0.04 fold) and cIAP-2 (1.41±0.07 fold). There was no significant change in other proteins after TRAF1 silencing.

Transfection of p65 siRNA in ACHN cells reduced the p65 protein expression to 0.51±0.07 fold compared to the control siRNA treated cells (Figures 6.12 and 6.13). TRAF1 and Bax protein expression were also reduced to 0.53±0.06 and 0.44±0.06 fold respectively. Other proteins with significant decrease in expression (compared to control) after transfection with p65 siRNA were cyclin D1 (0.45±0.08 fold) and IL-6

(0.49±0.08 fold). There was a slight but non-significant increase in caspase 8 expression after p65 silencing. No cleavage/activation of caspase 3 and caspase 8 was observed. Cleavage or activation of caspase 8 would produce bands at 43kD and 18kD positions, while activation of caspase 3 would produce a cleavage protein band at 20kD position. The antibodies for caspases 3 and 8 were confirmed to be able to detect cleavage protein bands (43kD for caspase 8 and 20kD for caspase 3) using a positive control of epitoside treated Jurkat cell lysate purchased from Cell Signalling (Results not shown). This positive control was used as epitoside treated Jurkat cells undergo apoptosis and activation of caspases 3 and 8.

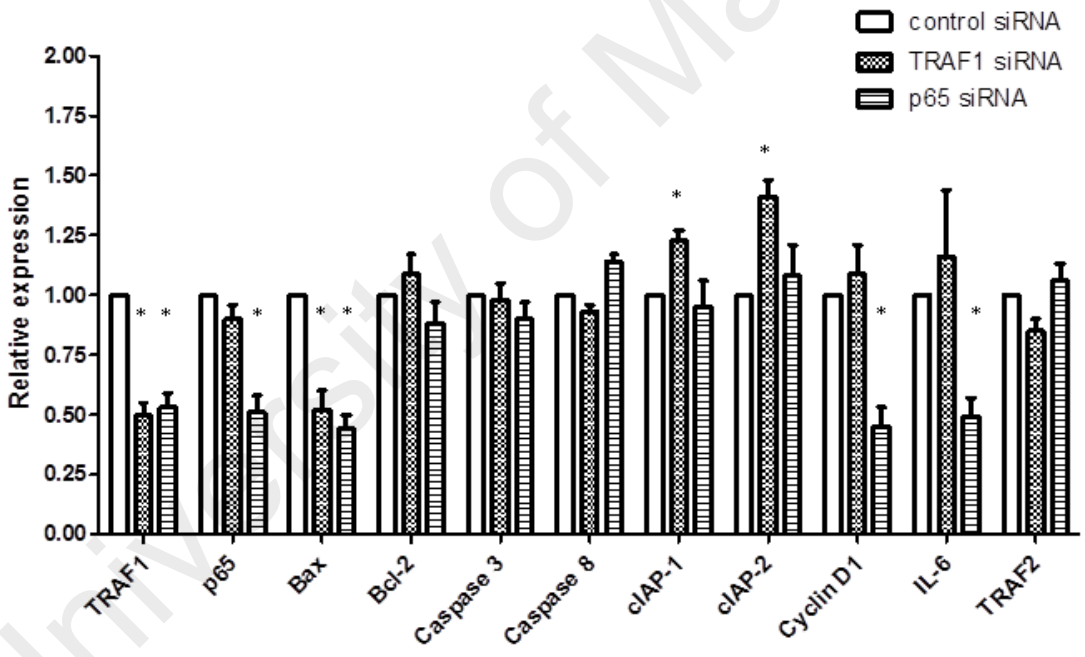


Figure 6.12. Relative protein expressions of TRAF1 and p65 siRNA transfected ACHN cells compared to control based on western blotting band intensity
*Indicates statistical significance $p<0.05$ compared to the control. Experiments were performed in triplicates.

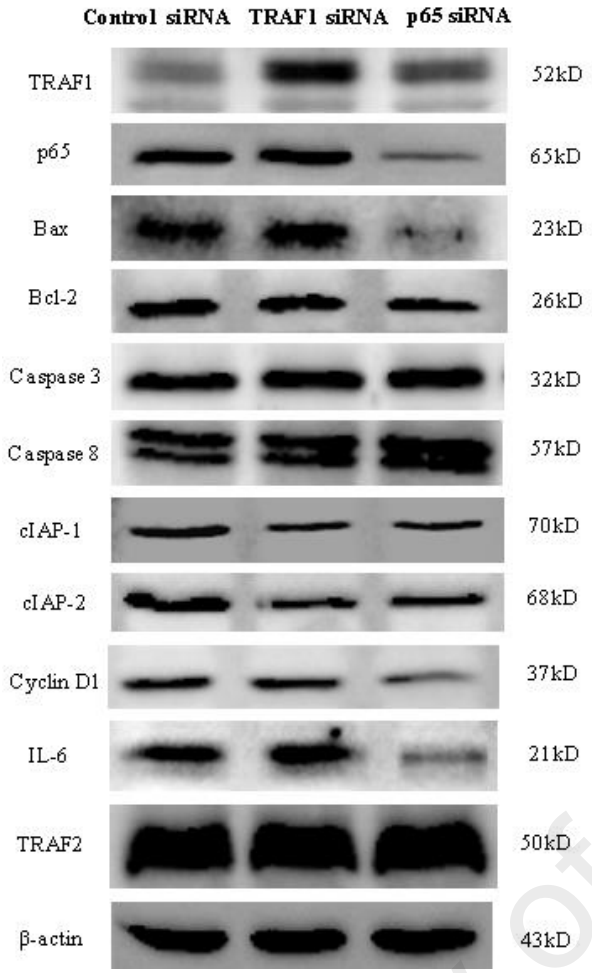


Figure 6.13. Western blot analysis and protein bands of TRAF1 and p65 siRNA transfected ACHN cells compared to control based on band intensity

6.4.3.5 Silencing experiments in Caki-1 cell line

Transfection of p65 siRNA in Caki-1 cells reduced the p65 protein expression to 0.51±0.05 fold compared to the control siRNA treated cells (Figures 6.14 and 6.15). Proteins with significant decrease in expression (compared to control) after transfection with p65 siRNA were : cIAP-1 (0.80±0.02 fold), cyclin D1 (0.51±0.04 fold) and IL-6 (0.23±0.02 fold). Other proteins did not show any significant change in expression after p65 silencing. There was a non-statistically significant decrease (0.66±0.11 fold) in Bax expression after p65 silencing. No cleavage/activation of caspase 3 and caspase 8 was observed.

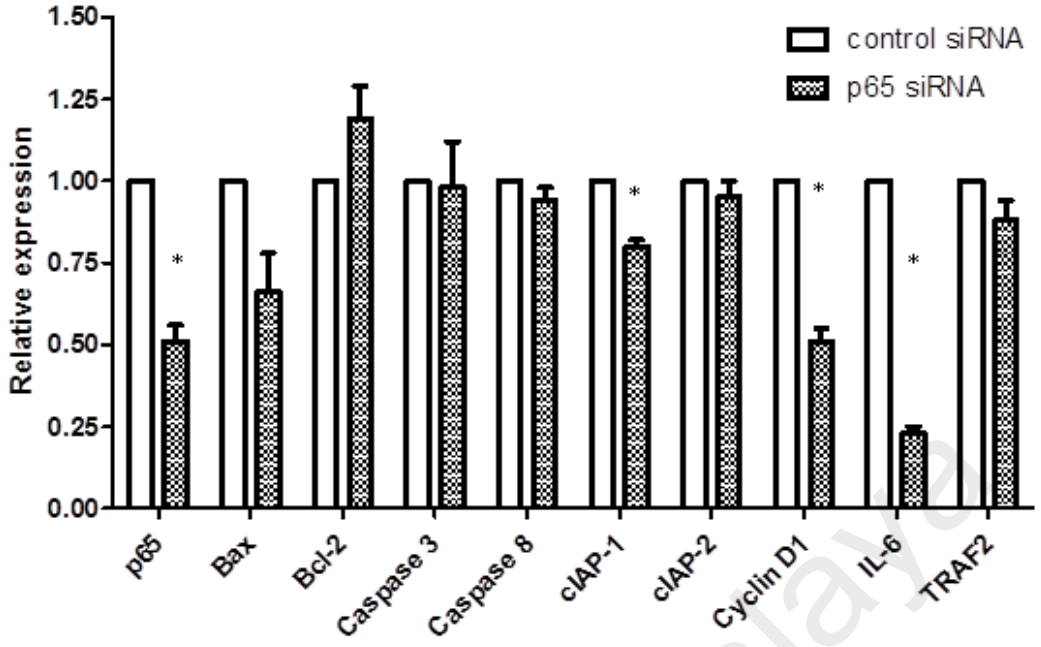


Figure 6.14. Relative protein expressions of p65 siRNA transfected Caki-1 cells compared to control based on western blotting band intensity

*Indicates statistical significance $p<0.05$ compared to the control. Experiments were performed in triplicates.

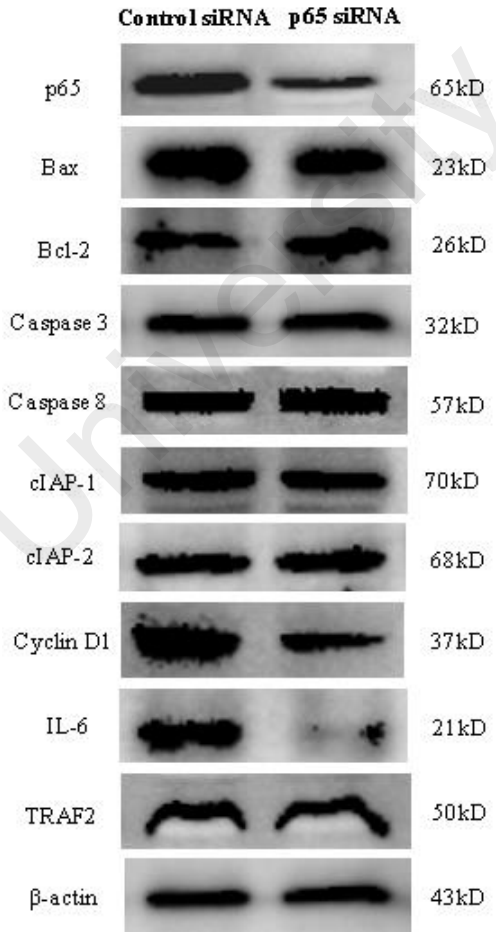


Figure 6.15. Western blot analysis and protein bands of p65 siRNA transfected Caki-1 cells compared to control based on band intensity

6.4.3.6 Silencing experiments in RCC1 cell line

Transfection of p65 siRNA in RCC1 cells reduced the p65 protein expression to 0.48±0.04 fold compared to the control siRNA treated cells (Figure 6.16 and 6.17). Proteins with significant decrease in expression (compared to control) after transfection with p65 siRNA were : cyclin D1 (0.31±0.06 fold) and IL-6 (0.37±0.10 fold). Other proteins did not show any significant change in expression after p65 silencing. There was a slight but non-statistically significant increase (1.20±0.10 fold) in caspase 8 expression after p65 silencing. No cleavage/activation caspase 3 and caspase 8 bands was observed.

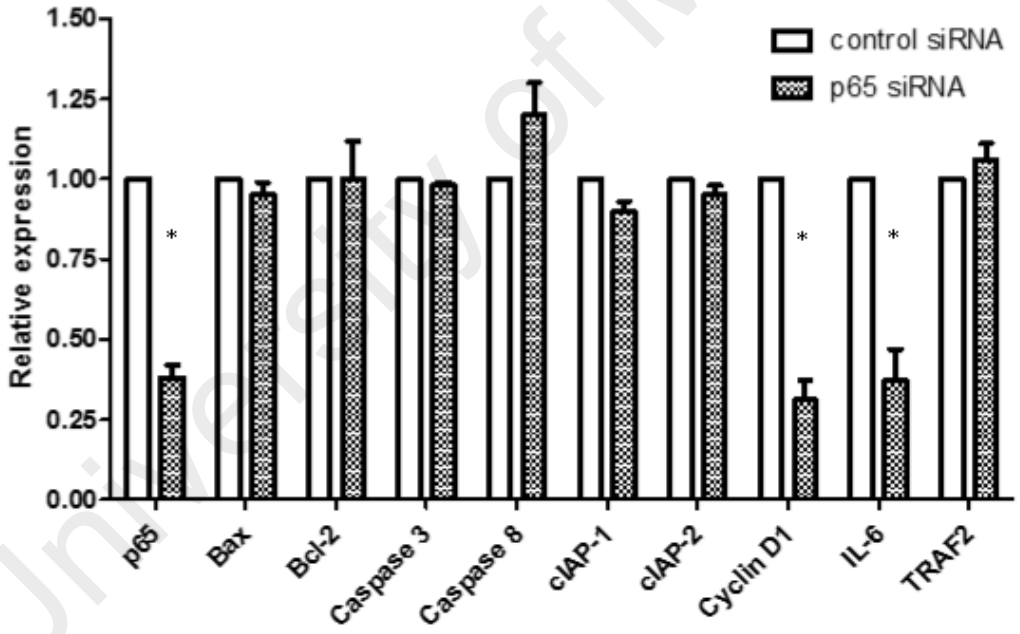


Figure 6.16. Relative protein expressions of p65 siRNA transfected RCC1 cells compared to control based on western blotting band intensity
*Indicates statistical significance $p<0.05$ compared to the control. Experiments were performed in triplicates.

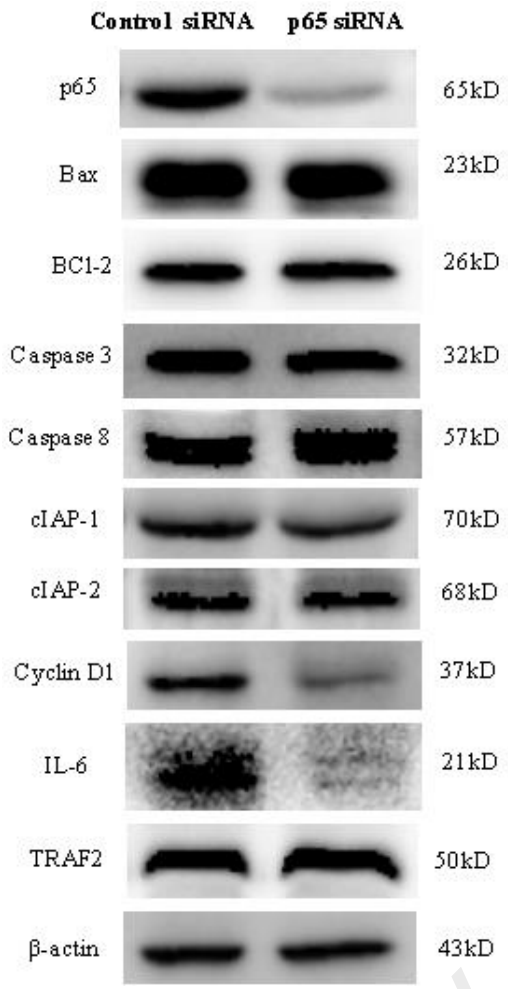


Figure 6.17. Western blot analysis and protein bands of p65 siRNA transfected RCC1 cells compared to control based on band intensity

6.4.3.7 Silencing experiments in RCC2 cell line

Silencing of p65 using p65 siRNA in RCC1 cells reduced the p65 protein expression to 0.56±0.02 fold compared to the control siRNA treated cells (Figure 6.18). Proteins with significant decrease in expression (compared to control) after transfection with p65 siRNA were : Bax (0.74±0.06), cyclin D1 (0.65±0.01 fold) and IL-6 (0.55±0.06 fold). Caspase 8 was increased by 1.18±0.02 fold in p65 siRNA treated cells. However, no cleavage/activation of caspase 3 and caspase 8 proteins was observed. Other proteins did not show any significant change in expression after p65 silencing.

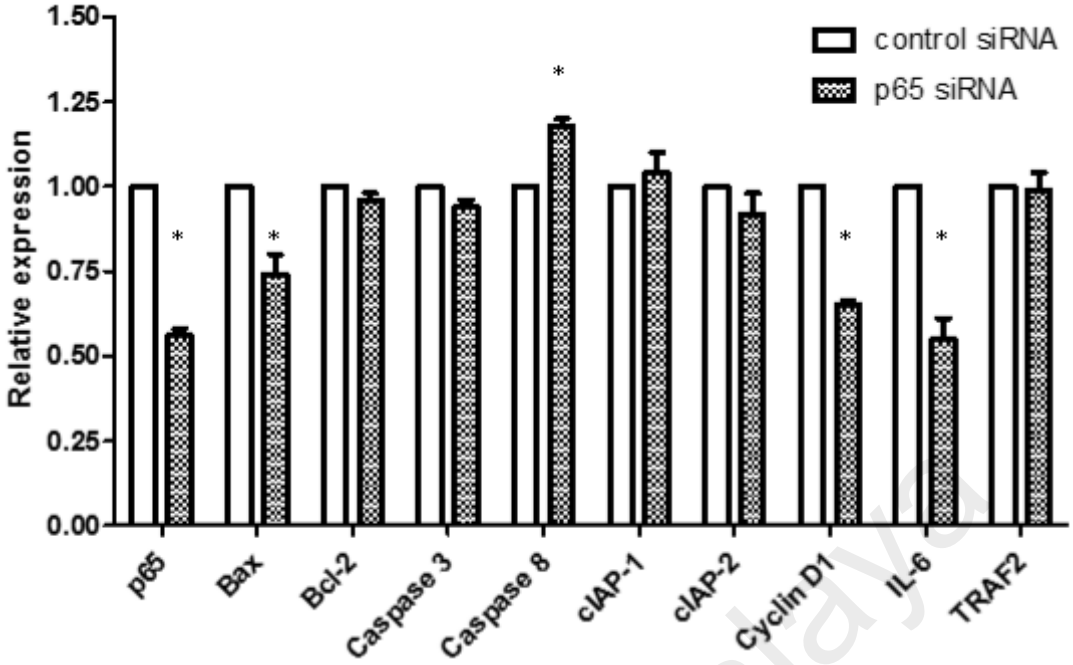


Figure 6.18. Relative protein expressions of p65 siRNA transfected RCC2 cells compared to control based on western blotting band intensity

*Indicates statistical significance $p<0.05$ compared to the control. Experiments were performed in triplicates.

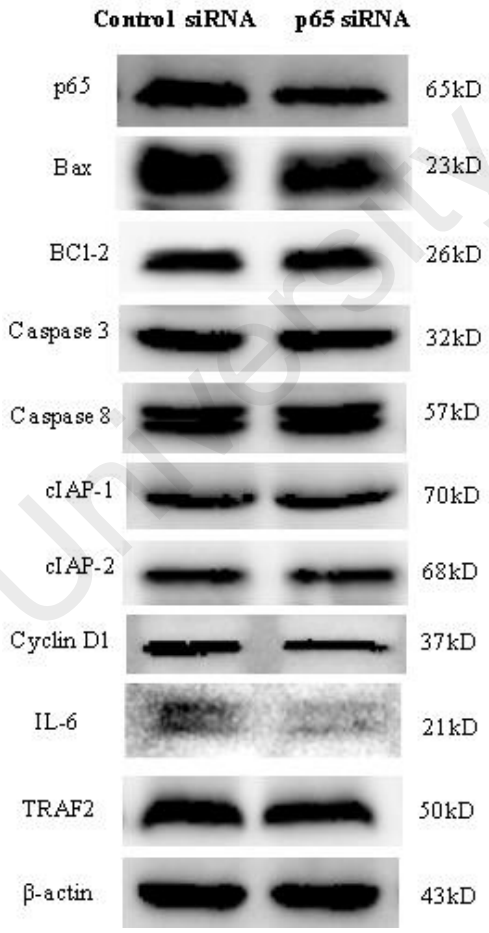


Figure 6.19. Western blot analysis and protein bands of p65 siRNA transfected RCC2 cells compared to control based on band intensity

6.5 Discussion

Epithelial RCC cell lines were established from tumour tissue of RCC patients with a spontaneous immortalisation rate of 21.6%. This was slightly higher than the 12.7% rate obtained by Ebert et al. (1990), which reported the spontaneous immortalisation rate of RCC cells (Ebert, Bander, Finstad, Ramsawak, & Old, 1990). Immortalised cell lines were from tumours with more clinically aggressive characteristics such as larger tumours (stage T2 and above), higher grade (grade 2 and above) or has metastasized. Established cell lines were confirmed to be epithelial cells with epithelial markers such as pan-cytokeratin, CK8 and e-cadherin. Surprisingly, contamination with fibroblast cells was not an issue as kidney tumour associated fibroblast cells were harder and slower to grow than the epithelial cells. The first two spontaneously immortalised cell lines as well as commercial ACHN and Caki-1 were use in silencing experiments to determine the effects of TRAF1 and p65 knock down in a few selected proliferation, apoptotic and survival proteins.

Knock down of TRAF1 in ACHN cells increased cIAP-1 and cIAP-2 gene and protein expressions. This would confer a protective effect from apoptotic events in the cell as both IAPs are pro-survival proteins. Furthermore, a previous study by Rajandram et al. (2012) showed that TRAF1 inhibition decreased sensitivity to apoptosis induced by radiation and IFN-α in ACHN cells (Rajandram, Bennett, Wang, et al., 2012). TRAF1 may have induced c-IAP transcription via activation of p65, as cIAP-1 and 2 are among the proteins regulated by p65 (Fan et al., 2005). In another study by Kempkensteffen et al. (2007), both cIAP-1 and 2 gene expressions were shown to be elevated in RCC tissue, but lower cIAP-1 expression in tumour tissues predicted a faster time to recurrence and worse disease specific survival (Kempkensteffen et al., 2007). However, the reason for this is unclear as the authors only presented a preliminary study

on cIAP gene expressions in RCC tissue. In p65 siRNA transfected ACHN cells, TRAF1 protein expression was also decreased as it is a one of the target genes/proteins regulated by p65, a transcription factor (Lee & Choi, 2007). The interaction of TRAF1 and p65 may serve as a regulatory feedback loop and the appropriate levels of these two proteins may be important to maintain a balanced rate of cell survival or apoptosis.

Knock down of p65 in all four RCC cell lines (ACHN, Caki-1, RCC1, RCC2) consistently decreased the protein levels of cyclin D1 and IL-6. Cyclin D1 regulates proliferation by promoting progression of cells in the G1 phase of the cell cycle and was found to be increased in RCC tissue compared to normal kidney, but tumours with higher cyclin D1 expression was associated with better prognosis compared to tumours with low cyclin D1 (Lima et al., 2014). It was suggested that cyclin D1 may be involved in the early stages of tumour formation but not in the progression of the tumour as other compensatory inactivating mutations in cyclin D1/CDK4 inhibitors may occur (Lima et al., 2014). IL-6 promotes proliferation of cells by activating epithelial growth factor, hepatocyte growth factor and other growth factors (Guo, Xu, Lu, Duan, & Zhang, 2012). In RCC cell lines AHCN and 769-P, IL-6 was found to induce proliferation through the signal transducer and activator of transcription 3 (STAT3) pathway (Horiguchi, Oya, Marumo, & Murai, 2002). STAT3 is a transcription factor which can activate various genes including those regulating cell proliferation and survival. High IL-6 levels were reported to be independent prognostic factors conferring worse outcomes in localised RCC tissue and in mRCC serum (Fu et al., 2015; Negrier et al., 2004). Interestingly, Bax was also decreased in p65 silenced ACHN and RCC2 cell lines. Li et al. (2013) reported that TNF-α treatment in alveolar epithelial cells induced p65 and Bax transcription and p65 silencing decreased Bax levels (L. Li et al., 2013). However, p65 activation could also suppress Bax transcription (Cianfrocca et al., 2008).

Therefore, the effect of p65 on Bax could be dependent on the cell type, stimuli or receptor involved. Bcl-2 expression was not much affected by p65 silencing here. It may be likely that p65 silencing alone does not significantly affect Bcl-2 expression as p50 homodimers can also regulate Bcl-2 transcription (Kurland et al., 2001; Luna-López et al., 2013).

Reduction in NF-κB activation or expression may reduce tumour aggressiveness as studies demonstrating inhibition of NF-κB activity decreased tumour growth in mice models. Renal tumour size was smaller in mice treated with ammonium pyrrolidinedithiocarbamate (PDTC), a NF-κB inhibitor as cell proliferation was significantly reduced (Morais, Healy, Johnson, & Gobe, 2010). PDTC did not specifically target a subunit as the authors noted a significant reduction in p50, p52, c-Rel and RelB subunits (Morais et al., 2010). In a separate study, BAY 11-7085, another NF-κB inhibitor increased RCC cell apoptotic levels and reduced tumour size in RCC xenograft nude mice treated with the inhibitor (Sourbier et al., 2007).

In this experiment, TRAF1 and p65 siRNA transfected cells as well as controls had minimal apoptotic levels hence there was no significant difference in apoptosis between controls and knock-down treatments. This was also shown in the western blot where there was no activation of caspases 3 and 8. In contrast, proliferation or cell viability of cells after p65 knock-down decreased significantly compared to the controls. This confirmed that p65 silencing reduced proliferation and proliferation proteins of RCC cells in these experiments.

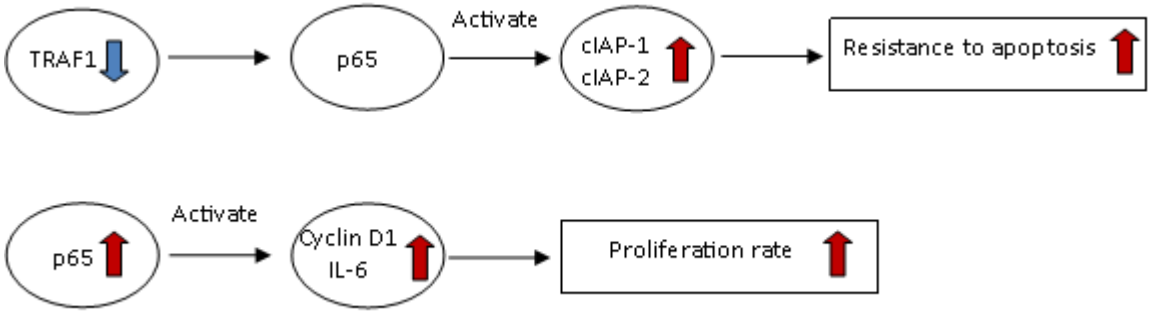


Figure 6.20. Decreased TRAF1 and increased p65 expressions in RCC tissue can cause an imbalance in cell apoptosis or proliferation

6.6 Conclusion

TRAF1 silencing in RCC cell line (ACHN) increased pro-survival cIAP-1 and cIAP-2 protein levels while p65 silencing in RCC cell lines decreased protein concentration of proliferation proteins, cyclin D1 and IL-6. The combination of low TRAF1 and high NF-κB p65 concentration in RCC tissue might create a loss of balance between cell proliferation and apoptosis and induce tumour growth.

CHAPTER 7 : DISCOVERY OF POTENTIAL BIOMARKERS IN RCC SERUM BY iTRAQ ANALYSIS

7.1 Introduction

As emphasized in Chapter 2 : Literature Review (Section 2.13), the discovery of reliable novel biomarkers could increase early diagnostic rates, aid in prognostication and provide a better understanding of tumour biology and factors affecting aggressiveness. Previous chapters have explored the roles of TRAF1 and NF-κB in RCC tissue, serum or cell lines. High TRAF1 protein concentration in serum may potentially indicate presence of advanced or metastatic disease and higher NF-κB subunits p52 and p65 expression in RCC tissue confer an unfavourable clinical outcome (Chapters 4 and 5).

In order to identify additional protein markers, biomarker discovery can be achieved through the iTRAQ method. iTRAQ is a relatively new non-gel-based technique which enables quantification of proteins from different sources in a single experiment. Peptides from individual samples are labelled with iTRAQ reagents, pooled together, fractionated using liquid chromatography (LC) and identified using mass spectrometry (MS). iTRAQ reagent labelling enables the differentiation of samples when reporter ions, each of which has a different mass (E.g. 114.1, 115.1, 116.1 or 117.1 Da) are released during mass spectrometry. Proteins/peptides are identified based on their fragmentation patterns and mass. Control and diseased samples can be analysed simultaneously, saving time and reducing inter-run variation. Relative quantification can then be carried out to determine up or down regulated proteins in diseased samples compared to the controls. The newly identified proteins of interests can be validated

using ELISA in serum or IHC in tissue. The iTRAQ technique has been used for screening of serum biomarkers in different diseases and cancers such as brain injury, oral cancer, prostate cancer, ovarian cancer and RCC (Boylan, Andersen, Anderson, Higgins, & Skubitz, 2010; Hergenroeder et al., 2008; Rehman et al., 2012; Zhang et al., 2008; Y. Zhang, Cai, Yu, & Li, 2015). In RCC, the two known iTRAQ reported analysis in serum was mostly concentrated on early detection and not prognostication (L. Zhang et al., 2015; Y. Zhang et al., 2015).

The aim of this chapter was to discover new potential detection or prognostic markers in the serum of RCC patients using the iTRAQ method.

7.2 Literature review

Identification of suitable biomarkers in RCC would be helpful for early detection, patient management or surveillance post-surgery and monitoring treatment effectiveness. Additionally, these biomarkers could allow for a better understanding in the mechanisms of tumour formation, disease progression and drug resistance. The discovery of *VHL* mutation in RCC has changed the treatment strategy for RCC in the targeted therapy era that the obvious choices for biomarkers would be proteins directly affected by pVHL malfunction. Interestingly, the *VHL* mutational status in the primary tumour itself has contradictory prognostic outcome in RCC patients (Yap et al., 2015). It is likely that the mutation is an early event in tumour formation and may not play a significant role in tumour progression (Yap et al., 2015). On the other hand, VEGF levels in serum, plasma or tissue showed correlation with the tumour size, stage, grade and survival of ccRCC patients (Ngo, Wood, & Karam, 2014; M. Sun et al., 2011).

Contradictory survival results were also found for HIF-1α levels in tissue (Ngo et al., 2014).

Another well studied potential biomarker in RCC is carbonic anhydrase IX (CAIX), which is one of the proteins regulated by VHL and HIF-1α. CAIX is elevated in response to hypoxia and functions to maintain the pH homeostasis in favour of tumour growth and survival (McDonald, Winum, Supuran, & Dedhar, 2012). High CAIX in ccRCC tissue is an independent prognostic factor for favourable survival outcome (Sun et al., 2011). In contrast, serum CAIX is higher in ccRCC than other RCC subtypes and is associated with stage and metastasis but is not necessarily correlated with tissue CAIX expression (Takacova et al., 2013; Zhou, Ireland, Rayman, Finke, & Zhou, 2010). Other promising RCC biomarkers which are well researched are C-reactive protein (CRP), lactate dehydrogenase (LDH) and survivin. CRP can increase in response to systemic inflammation, while LDH levels are elevated during hypoxia and survivin protects against apoptosis. Serum C-reactive protein is an independent prognostic factor in RCC patients and response rate predictor in RCC patients treated with sunitinib (Beuselinck et al., 2014; Martino et al., 2013). A meta-analysis on LDH in RCC showed that LDH serum levels are associated with worse prognosis in RCC patients (Shen et al., 2016). High LDH levels in the serum is also able to predict survival benefit in RCC patients treated with temsirolimus (Armstrong, George, & Halabi, 2012). Survivin expression in RCC tissue is positively associated with poor survival, higher stage, grade and metastasis (Xie et al., 2016).

Recently, a novel early detection marker in RCC serum, heat shock cognate 71kDa protein (HSC71) was discovered by Zhang et al. using iTRAQ technique (Y. Zhang et al., 2015). The have performed preliminary validation in ELISA but the function of

HSC71 in RCC is still unclear (Y. Zhang et al., 2015). Although these biomarkers showed promise, none are currently in use for routine clinical settings yet, hence the search for and validation of reliable biomarkers for RCC continues.

7.3 Methodology

7.3.1 Patients and controls

Serum samples were taken from our bio-bank of RCC samples stored at -80°C. Ethical approval was acquired from UMMC ethical committee (Ref : 848.17). Blood collection and serum processing were as mentioned earlier (Methododology for Chapter 4, Section 4.3.1). Serum from ccRCC patients with pathological diagnosis who have not undergone surgery or treatment and controls with no known medical history of cancer (renal calculi patients or individuals with abdominal imaging) were used for analysis. The histology and grading of the ccRCC cases were re-confirmed by a senior pathologist. For the discovery phase (iTRAQ), serum was divided into three groups ; localised ccRCC, metastatic ccRCC and controls (N=6/group). Patients and controls were matched as closely as possible according to age and gender (Table 7.1).

Table 7.1. Patients and controls for iTRAQ analysis

Group	Subject	Gender	Age	Stage	Grade
Controls	1	F	49		
	2	M	53		
	3	M	57		
	4	M	65		
	5	M	66		
	6	F	70		
Localised ccRCC	1	M	47	I	2
	2	M	53	I	3
	3	F	58	I	2
	4	M	59	I	2
	5	F	65	I	2
	6	M	69	I	3
Metastatic ccRCC	1	M	49	IV	2
	2	F	55	IV	3
	3	M	59	IV	3
	4	F	63	IV	3
	5	M	66	IV	2
	6	M	71	IV	2

7.3.2 Sample processing for iTRAQ

The serum samples were immunodepleted, diafiltrated, reduced, alkylated and trypsin digested according to the iTRAQ protocol (AB Sciex, USA). Fourteen abundant proteins were removed from the samples using a Multiple Affinity Removal System (MARS) Human 14 column (Agilent Technologies, USA). Table 7.2 lists the abundant proteins removed by the MARS 14 column. The samples (100ug protein/group) were then labelled with the iTRAQ reagent as follows : 114 – Controls, 115 – Localised ccRCC, 116 – Metastatic ccRCC. All labelled samples were then pooled before analysis.

Table 7.2. List of abundant proteins depleted by MARS 14 column

No	Proteins depleted by MARS 14
1	Serum Albumin
2	Alpha-1-antitrypsin
3	Alpha-2-Macroglobulin
4	Alpha-1-Acid Glycoprotein
5	Apolipoprotein A-I
6	Apolipoprotein A-II
7	Complement C3
8	Fibrinogen
9	Haptoglobin
10	Ig alpha
11	Ig gamma
12	Ig mu
13	Serotransferrin
14	Transthyretin

7.3.3 High performance liquid chromatography (HPLC) and mass spectrometry (MS/MS)

Peptides were desalted on a Strata-X 33 μm polymeric reversed phase column (Phenomenex) and dissolved in a buffer containing 10mM KH_2PO_4 pH3.0 in 10% acetonitrile before separation by strong cation exchange liquid chromatography (SCX) on an Agilent 1100 HPLC system using a PolySulfoethyl column (4.6 x 100 mm, 5 μm, 300 A). Peptides were eluted with a linear gradient of 0-400mM KCl. Eight fractions containing the peptides were collected and desalted on Strata-X columns. The fractions were then analysed by electrospray ionisation mass spectrometry using the Agilent 1260 Infinity HPLC system (Agilent Technologies, USA) coupled to an Agilent 1260 Chipcube Nanospray interface (Agilent Technologies, USA) on an Agilent 6540 mass spectrometer (Agilent Technologies). Peptides were loaded onto a ProtID-Chip-150 C18 column (Agilent Technologies) and separated with a linear gradient of water/acetonitrile/0.1% formic acid (v/v).

7.3.4 Peptide identification

Spectral data were analysed against the SwissProt database for Homo sapiens (Human) using ProteinPilot™ 4.5 Software (AB Sciex). The database contained 548,872 sequences. Results were presented as fold difference of localised and metastatic ccRCC groups compared to the controls. Proteins were considered differentially expressed when they were >1.5 fold higher or <0.65 fold lower than the control group. Proteins which were >1.5 fold higher or <0.65 fold lower in both localised and metastatic ccRCC groups compared to the control group were selected for validation.

7.3.5 Protein validation

Proteins were validated in serum samples of 39 ccRCC patients and 39 healthy controls via the ELISA technique. ELISA kits used were Factor B (Abcam, USA), Factor H (Abcam), ceruloplasmin (Abcam) and soluble CD14 (R&D Systems, USA). The tests were performed according to the manufacturer's instructions. The examples of standard curves for ELISA analysis are displayed in Appendix E. ANOVA and T-test were used to compare serum protein levels between controls and ccRCC patients as well as concentration in different stages/grades of ccRCC. ROC curve analysis was performed to determine the reliability of CD14 as a biomarker. All statistical tests were done using SPSS software (IBM, USA).

7.4 Results

7.4.1 iTRAQ Analysis

A total of 84 proteins were identified from the iTRAQ run (Appendix J). Five proteins levels were higher (>1.5 fold) in the localised and metastatic ccRCC groups compared to the control group. One protein level was lower (<0.65 fold) in the localised and metastatic ccRCC groups compared to the control group (Table 7.3).

Table 7.3. List of proteins with >1.5 or <0.65 fold difference for localised and metastatic RCC groups compared to the control group

Protein	Fold change compared to control group	
	Localised ccRCC	Metastatic ccRCC
Ceruloplasmin	1.92	2.25
Complement Factor B	1.85	3.70
Complement Factor H	2.05	2.60
Haemoglobin subunit beta	1.80	2.86
Monocyte differentiation antigen CD14	1.89	2.65
Lumican	0.63	0.51

7.4.2 Validation of proteins

Validation was performed for up-regulated proteins ceruloplasmin, complement factor B, complement factor H and soluble CD14 (sCD14). The soluble from of CD14 is sCD14 which can be found in the serum. A literature search was performed on these proteins and they were shown to be potential biomarkers in other studies.

The controls and ccRCC patients were matched evenly according to age, gender and race (Table 7.4). Validation was performed using ELISA in the serum samples of these subjects and ceruloplasmin, factor B and sCD14 were found to be significantly elevated in the ccRCC patients group (Table 7.5).

Table 7.4. Subject demographics and clinical characteristics of controls and ccRCC patients for validation

Characteristics	**Controls (N=39)**	**ccRCC patients (N=39)**	**p Value**
Age (mean ± SD)	55.8 ± 9.7	57.2 ± 10.4	0.537
Men/women (N)	24/15	24/15	1.000
Race (%) Malay Chinese Indian	 17 (43.6) 14 (35.9) 8 (20.5)	 17 (43.6) 14 (35.9) 8 (20.5)	 1.000
Stage (TNM) (%) I II III IV	 -	 12 (30.8) 8 (20.5) 10 (25.6) 9 (23.1)	 -
Grade (%) 1 2 3 4	 -	 4 (10.3) 19 (48.7) 12 (30.8) 4 (10.3)	 -

p Value obtained using t-test and Chi-square test

Table 7.5. Protein concentrations of selected proteins validated by ELISA

Protein	**Controls (N=39)**	**ccRCC patients (N=39)**	**p Value**
	Protein concentration in serum (Mean ± SE)		
Ceruloplasmin (µg/ml)	389.38 ± 7.14	411.36 ± 6.58	**0.026**
Factor B (µg/ml)	359.29 ± 7.71	400.71 ± 9.05	**0.001**
Factor H (µg/ml)	700.69 ± 28.05	725.13 ± 39.66	0.620
sCD14 (ng/ml)	1853.92 ± 83.53	2351.21 ± 119.73	**0.001**

pValue obtained using t-test

Only sCD14 was significantly associated with ccRCC stage. Patients with stage IV ccRCC had significantly higher serum sCD14 than stage I-II patients (p=0.006). There was also an increasing trend in sCD14 levels with increasing tumour grade (Figure 7.1).

However, the difference did not reach statistical significance. ROC curve and AUC analysis revealed that sCD14 serum concentration is a moderate indicator of ccRCC compared to healthy controls (AUC value 0.719). sCD14 serum levels is a better indicator of ccRCC metastasis (AUC 0.878) when compared with controls and localised ccRCC (Figure 7.2).

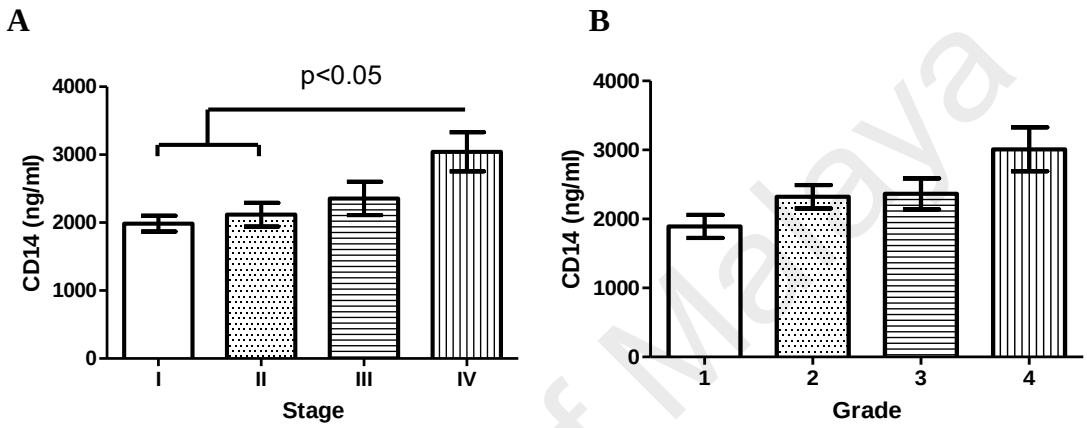


Figure 7.1. Concentration of sCD14 in RCC patients
(A) Soluble CD14 concentration in serum of ccRCC patients according to stage. (B) CD14 concentration in serum of ccRCC patients according to grade (p=0.237).

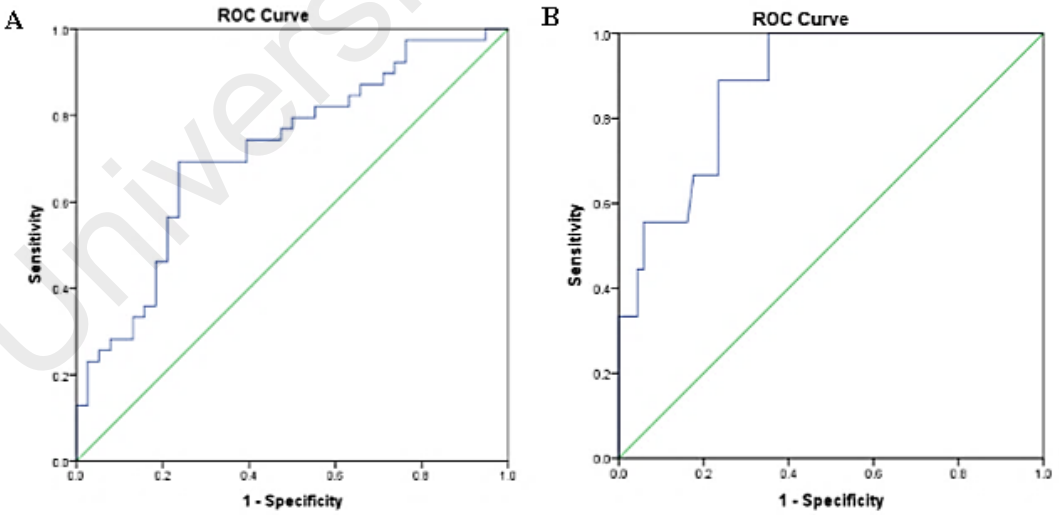


Figure 7.2. ROC curves for sCD14 serum concentration
(A) ROC curve of CD14 serum concentration in controls vs ccRCC patients. The area under the curve (AUC) is 0.719 (95% CI 0.605-0.834). (B) ROC curve of CD14 serum concentration in controls and localised ccRCC vs metastatic ccRCC patients. The AUC value is 0.878 (95% CI 0.783-0.973).

7.5 Discussion

There were 84 proteins identified during the iTRAQ run and five proteins (ceruloplasmin, complement factor B, complement factor H, CD14, haemoglobin subunit beta) were elevated (>1.5) while lumican was decreased (<0.65) in both localised and metastatic ccRCC groups compared to the controls. CRP, which was detected in the iTRAQ run, was elevated in the metastatic ccRCC group (14.06 fold)(Appendix J) compared to the controls but was not further validated here as it is a known and well-studied serum marker in RCC. Out of the proteins which were elevated, ceruloplasmin, factor B, factor H and sCD14 were validated using ELISA method. Haemoglobin subunit beta was not validated as it could be a protein found abundantly in red blood cells. Lumican was also not validated here, but it is an extracellular matrix protein and member of the proteoglycan family which can be found in the corneal, dermis or tendon tissue (Yamamoto, Matsuda, Kawahara, Ishiwata, & Naito, 2012). The expression of lumican is contradictory in cancers. Increase in lumican expression was associated with aggressive tumour traits in breast (cell proliferation, migration and invasion) and pancreatic cancers (higher TNM staging) (Karamanou et al., 2017; Yang, Lu, Yang, Dou, & Tao, 2013). However, a study in metastatic prostate cancer cells indicated that lumican inhibited cell migration and invasion while increased expression of lumican inhibited attachment and growth of human embryonic kidney cells (HEK 293) (Coulson-Thomas et al., 2013; Ishiwata et al., 2010).

In the ELISA analysis of this study, ceruloplasmin, factor B and sCD14 were found to be significantly elevated in ccRCC patients serum compared to controls. Ceruloplasmin is a copper carrying protein in the blood which is also responsible for oxidase or antioxidant activity and could be elevated in conditions of increased oxidative stress such as cancer (Esme et al., 2008). Ceruloplasmin gene expression was

found to be increased in ccRCC tissue (Osunkoya et al., 2009). Factor B is involved in the alternative complement pathway and was identified as a potential biomarker in pancreatic ductal adenocarcinoma plasma using the 2-D Difference Gel Electrophoresis and Image Analysis (2-D DIGE) method but was never studied in RCC (M. J. Lee et al., 2014).

Among the three elevated proteins, sCD14 was a potential prognostic biomarker as there was significant association with stage and a positive trend with grade. However, ceruloplasmin and factor B levels had no association with clinical parameters (stage, metastasis or grade) in the serum. ROC analysis showed that sCD14 serum concentration is a moderate indicator of ccRCC compared to controls without ccRCC (AUC value 0.719) and good indicator of ccRCC metastasis (AUC 0.878) when compared with controls and localised ccRCC. AUC is used to assess the diagnostic accuracy of a test and an AUC range of 0.7 to 0.8 is considered acceptable, 0.8 to 0.9 is excellent and more than 0.9 is considered outstanding (Mandrekar, 2010). sCD14 may be useful in a combination with other serum or plasma biomarkers mentioned in the literature review (Section 7.2), including TRAF1 which was analysed in Chapter 4 to improve accuracy of detection or prognostication.

CD14 was first discovered on the surface of monocytes and macrophages and takes part in innate immunity regulation (Jersmann, 2005). On monocytes and macrophages, CD14 acts as a co-receptor with Toll-like receptor 4 (TLR4) for lipopolysaccharide (LPS), found on gram negative bacteria. In the conventional CD14/TLR inflammatory pathway, binding of LPS can lead to NF-κB activation and transcription of cytokines such as IL-6 and TNF-α (Nijland, Hofland, & van Strijp, 2014). CD14 occurs in two forms, the membrane bound (mCD14) or the soluble form (sCD14), which results from

cleavage of mCD14 from the cell surface or is released from intracellular pools (Shive, Jiang, Anthony, & Lederman, 2015). Hence, monocyte activation can increase sCD14 in the serum (Shive et al., 2015). CD14 has since been reported in other cell types, such as cornea, skin, intestine, uro-epithelial, smooth muscle and fibroblast cells, among others, albeit at lower levels and has recently been linked to cancer progression (Jersmann, 2005).

In bladder cancer cells, CD14 was found to promote inflammation in tumour microenvironment which enhanced proliferation to encourage tumour growth (Cheah et al., 2015). Bladder cancer cells with high CD14 expression produced higher levels of cytokines and growth factors such as IL-6, IL-8, M-CSF (monocyte colony stimulating factor), VEGF-A and FGF-2 (Cheah et al., 2015). Nude mice injected with high CD14 bladder cancer cells produced more vascularised tumours with high inflammatory cell infiltration (Cheah et al., 2015). The authors reported that since LPS stimulation did not increase cytokine secretion by the bladder cancer cells, other ligands for CD14 and TLR such as heat shock protein 60 (HSP60), peroxiredoxin 1 (Prdx1) or high mobility group box 1 (HMGB1) proteins may be responsible for inducing CD14/TLR inflammatory response (Cheah et al., 2015). Another study demonstrated the presence of TLR4 on lung cancer cell lines (H1299 and A549) (He et al., 2007). The binding of ligands induced VEGF, transforming growth factor beta (TGF-β) and IL-8 secretion via p38 mitogen-activated protein kinases (MAPK) and NF-κB pathways activation which promoted apoptosis resistance and tumour immune escape (He et al., 2007).

CD14 was also reported to be involved in the regulation of epithelial-mesenchymal transition (EMT) and invasiveness of gastric cancer cells (K. Li et al., 2013). Gastric cancer cell line (MGC-803) with CD14 knockdown resulted in enhanced E-cadherin

expression but reduced N-cadherin and vimentin expressions and decreased invasive activity compared to the negative control (K. Li et al., 2013). Reduction in E-cadherin promotes EMT while N-cadherin and vimentin may increase EMT and tumour invasiveness (K. Li et al., 2013). A study in RCC found that the presence of tumour associated CD14 positive monocytes was an independent survival prognostic factor, favouring an adverse outcome (Gustafson et al., 2015). In addition, the level of peripheral blood CD14 positive monocytes correlated with the intensity of CD14 staining in RCC tumours (Gustafson et al., 2015). Besides this report from Gustafson et al., there has been no other publications directly linking CD14 expression and RCC. There were also no publications specifically on sCD14 and RCC to date.

The role of CD14 in RCC is still unclear and further immunohistochemistry and cell culture studies are needed to elucidate the mechanistic pathways of CD14 in RCC pathogenesis. Additionally, an increase in sample size for the ELISA assay would strengthen the confirmation of detection/prognostic value of CD14 levels in RCC serum. Future studies could also include ELISA validation of lumican, which was found to be lower in ccRCC serum compared to the controls.

7.6 Conclusion

In summary, iTRAQ analysis in ccRCC serum samples identified CD14 as a potential detection or prognostic marker. However, immunohistochemistry and functional studies using cell lines are required to fully understand the role of CD14 in RCC progression.

CHAPTER 8 : CONCLUSIONS AND RECOMMENDATIONS

This project explored the possible clinical predictors and protein biomarkers for the detection or prognostication of RCC using a cohort of patients treated at UMMC. Demographics and clinical data was first gathered from these RCC patients and the various presenting symptoms or clinical features (stage, grade, blood test results) were analysed for survival prognosis. Clinical and survival information obtained was also used for subsequent prognosis analysis for protein biomarkers. TRAF1 and NF-κB subunit proteins were evaluated as potential prognostic indicators in RCC tissue or serum. Knock down of TRAF1 and NF-κB p65 in cell line experiments were conducted to determine the effects of reduced TRAF1 and p65 protein levels on apoptosis and proliferation of RCC cells. Further protein biomarkers in RCC serum samples were discovered using the iTRAQ analysis. The results conclusions and future recommendations of the project are:

1. Analysis of RCC UMMC patients' clinical data revealed that 76.8% of patients treated from 2003-2012 were symptomatic at diagnosis, and symptomatic patients had an unfavourable survival outcome compared to asymptomatic patients. The five year survival rate of RCC patients with localised RCC (TNM stages I and II) was ≥90% but was only 13% for patients with stage IV disease. TNM staging and palpable abdominal mass were independent predictors for survival. Information gathered from this study is useful as a starting point for the centre's RCC registry which can help in future research involving RCC patients/samples and aid in improving patient monitoring and management. Further investigations should include a multicentre cohort to increase sample size and improve representation from other urban/suburban/rural areas or states

in the country. Furthermore, future studies could include a longer follow up and a ten year survival analysis.

2. TRAF1 concentration in ccRCC serum was significantly higher than controls without RCC. In addition, TRAF1 was higher in ccRCC patients with metastasis compared to localised ccRCC and controls. The AUC for mRCC was 0.809, indicating that it was a good predictor for distinguishing mRCC from localised ccRCC and controls. In ccRCC tissue, TRAF1 was found to be lower in expression compared to adjacent normal kidney tissue and not associated with prognostic features. Therefore, it is possible that the high serum TRAF1 might be from active secretion of the RCC cells from the metastatic sites, from the highly expressing tumour-related inflammatory cells or as a result of systemic inflammation in metastatic disease. Based on the results here and previous preliminary cell line studies, decreased TRAF1 expression in ccRCC compared with normal kidney may have a modulatory role on apoptosis. In future studies, TRAF1 concentration in serum samples could be explored in larger sample sizes which can include a cohort of Australian RCC patients and controls from the collaborators of this study.
3. Immunohistochemistry analysis of NF-κB subunits in RCC tissue showed that the p65 subunit was overexpressed in RCC tissue compared to the adjacent normal kidney while p50, p52 and cRel expressions were lower in RCC compared to normal kidney tissue. The differential expressions of p50, p52, p65 and c-Rel in RCC tissue compared to the adjacent normal kidney indicates that there is dysregulation of these proteins in RCC which may have contributed to the formation or progression of the disease. Cox regression analysis revealed that p65 and p52 in RCC tissue was associated with patients' survival and could be possible prognostic indicators in RCC. In order to understand the effects of

differential expressions of p50, p52, p65 and cRel on RCC, knock down or knock up experiments using cell lines can be performed to determine the effects of cell proliferation, apoptosis or invasiveness.

4. A total of eleven spontaneously immortalised RCC cell lines were established from UMMC patients. The first two immortalised cell lines along with commercially available ATCC cell lines (ACHN and caki-1) were used for TRAF1 and p65 silencing studies. TRAF1 expression knock down by siRNA in RCC cell line (ACHN) increased pro-survival cIAP-1 and cIAP-2 proteins whereas p65 knock down in RCC cell lines decreased levels of proliferation proteins, cyclin D1 and IL-6. Low TRAF1 in addition to high NF-κB p65 concentration in RCC tissue might cause increased cell proliferation and reduced apoptosis, inducing tumour growth. Future studies could include evaluating the effects of TRAF1 or p65 knock down on sensitivity to RCC targeted therapy drugs such as sunitinib, pazopanib or temsirolimus.
5. Proteomics iTRAQ analysis identified ceruloplasmin, complement factor B, factor H, haemoglobin subunit beta and CD14 as up-regulated (>1.5) proteins while lumican was down-regulated (<0.65) in both localised and metastatic ccRCC groups compared to controls. Ceruloplasmin, sCD14, factor B and factor H concentrations were determined in ccRCC (N=39) and control (N=39) serum samples using ELISA. Serum sCD14 was identified as a potential biomarker as it was significantly higher in ccRCC patients compared to controls and associated with stage. Serum sCD14 was a potential indicator of metastatic ccRCC compared to localised ccRCC and controls with an AUC of 0.878. Hence, sCD14 may potentially be useful in a combination with other serum or plasma biomarkers, including TRAF1, to improve the accuracy of detection or prognostication of ccRCC. In order to fully understand the role of CD14 in RCC

progression, IHC analysis to determine expression pattern and intensity in RCC tissue compared to normal kidney as well as functional studies using cell lines can be performed.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Conferences

1. **Yap, N. Y**., Rajandram. R., Pailoor, J., Razack,,A. H. A., Ong, T. A., Ng, K. L., Morais, C., & Glenda Gobe. (2013, September). *Evaluating the characteristics of newly diagnosed Renal Cell Carcinoma to improve patient outcome*. Paper presented at 49th Annual Scientific Meeting of the Australian and New Zealand Society of Nephrology conference, Brisbane. Oral presentation by Yap Ning Yi
2. **Yap, N. Y.**, Ng, K. L., Small, S., Pailoor, J., Ong, T. A., Razack, A. H. A., Morais, C., Rajandram, R., & Gobe, G. 2014. (2015, April). *Immunopositivity of NF-κB p65 as a prognostic marker in renal cell carcinoma*. Paper presented at 2nd International Conference on Advances in Medical Science (ICAMS), Kuala Lumpur. Poster presentation by Yap Ning Yi
3. **Yap, N. Y.**, Ng, K. L., Small, S., Pailoor, J., Ong, T. A., Razack, A. H. A., Morais, C., Rajandram, R., & Gobe, G. (2015, November). *NF-κB p65 Immunopositivity as an indicator of Renal Cell Carcinoma progression*. Presented at 24th Malaysian Urological Congress (MUC), Johor Bahru. Poster presentation by Yap Ning Yi
4. **Yap, N.Y.**, Ong, T.A., Razack, A. H. A., Gobe. G., & Rajandram, R. (2016, November). *CD14 as a Novel Prognostic Marker in Renal Cell Carcinoma Serum.* Presented at 25th Malaysian Urological Congress (MUC), Kuala Lumpur. Poster presentation by Yap Ning Yi

Publications from PhD project

1. **Yap, N. Y.**, Ng, K. L., Ong, T. A., Pailoor, J., Gobe, G. C., Ooi, C. C., …Rajandram R. (2013). Evaluation of clinical prognostic factors and survival outcome of Renal Cell Carcinoma patients A Malaysian single centre perspective. *Asia Pacific Journal of Cancer Prevention*, *14*(12), 7497-7500.
2. Rajandram, R., **Yap, N. Y.**, Pailoor, P., Razack, A. H. A., Ng, K. L., Ong, T. A.,… Gobe, G. C. (2014). Tumor necrosis factor receptor-associated factor-1 (TRAF-1) expression is increased in renal cell carcinoma patient serum but decreased in cancer tissue compared with normal: potential biomarker-significance. *Pathology*, *46*(6), 518-522.

Publications on topics related to PhD project

1. Pailoor, J., Rajandram, R., **Yap, N. Y.**, Ng, K. L., Wang, Z., Iyengar, K. R. (2013). Chromosome 7 aneuploidy in clear cell and papillary renal cell carcinoma: detection using silver-in-situ hybridisation technique. *Indian Journal of Pathology and Microbiology*, *56*(2), 98-102.
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4. Abd Ghafar, N. K., Alip, A., Ong, T. A., **Yap, N. Y.**, & Saad, M. (2016). Efficacy, safety, and prognostic indicators of first-line sunitinib in patients with metastatic renal cell carcinoma: A single center experience. *Journal of Cancer Research and Therapeutics*, Article online ahead of print.