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**THE ROLE OF SRC KINASE IN RENAL CELL
CARCINOMA**

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Doctor of Philosophy

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Lastly I would like to thank my family especially my mother for their support and prayers.

Abstract

Renal cancer is a malignancy which is not only increasing in incidence but there has also been an increase in mortality rates. There are various prognostic factors in renal cell cancer. We have demonstrated that some of these such as nuclear grading, tumour necrosis and systemic inflammatory response can be further refined to aid in prognosis but cannot be utilised at present to assess which would benefit from therapeutic agents when recurrence occurs.

We investigated if SFK members are expressed in renal cancer. Eight SFK members were found to be expressed in renal cancer and were present to varying degrees. Furthermore, expression differed in organ confined disease and metastatic disease.

Immunohistochemistry was employed to assess protein expression and activation of c-Src and SFK activity as well as the downstream marker FAK Y⁸⁶¹. Analysis demonstrated that c-Src expression was associated with improved survival and expression of the downstream marker FAK Y⁸⁶¹ was associated with poor survival and demonstrated a positive relationship with known prognostic factors. This would suggest that another SFK member was associated with poor survival. Dasatinib, a SFK inhibitor was utilised on renal cell lines, demonstrating a dose dependant reduction on cellular metabolic activity as well an increase in apoptotic rates. This would support that Dasatinib may be a useful therapeutic drug for RCC. Treatment with Dasatinib also demonstrated that expression of c-Src, SFK activity and FAK Y⁸⁶¹ reduced in a dose dependant manner. It was necessary to further assess that another SFK member was responsible for poor prognosis and this was undertaken by silencing c-Src. Cellular metabolic activity rates increased following silencing c-Src and assessment of SFK activity (Src Y⁴¹⁶) and FAK Y⁸⁶¹ on cell pellets demonstrated no change suggesting that another SFK member is responsible for the phosphorylation of FAK Y⁸⁶¹ and therefore responsible for poor survival.

This would suggest that another SFK inhibitor and not c-Src inhibitors may play a role in the treatment of renal cell cancer and further work is required to ascertain which SFK member is responsible so that this can be targeted for treatment.

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List of Abbreviations

4E-BP1	Eukaryotic translation initiation factor 4E-binding protein 1
AI	Apoptotic Index
Bcl-xL	B-cell lymphoma-extra large
BMI	Body Mass Index
BSA	Bovine Serum Albumin
c-KIT	Mast/stem cell growth factor receptor
CA IX	Carbonic Anhydrase IX
CI	Confidence Interval
CO ₂	Carbon Dioxide
CRP	C-Reactive Protein
CT	Computed Tomography
c-Src	Src
Cyto	Cytoplasm
DAB	Diaminobenzidine chromagen
dH ₂ O	Distilled water
DMSO	Dimethyl Sulphoxide
DPX	Dibutyl Phtalate containing Xylene 19
ECOG	Eastern Co-operative Oncology Group
EDTA	Ethylenediaminetetraacetic acid
eIF-4E	Eukaryotic initiation factor 4E
ERK1/2	Extracellular signal-regulated kinase 1/2
FAK	Focal Adhesion Kinase
FKBP	Family of Immunophilins Binding Proteins
FLT-3	Fms-like tyrosine kinase 3
FNA	Fine Needle Aspiration
GAPDH	Glyceraldehydes-3-Phosphate Dehydrogenase
GβL	G Protein β subunit-like
HBBS	Hanks Balanced Salt Solution
HIF α	Hypoxia Inducible Factor α
H ₂ O ₂	Hydrogen peroxide
HPRT	Hypoxanthine-guanine Phosphoribosyl-Transferase
HR	Hazard Ratio
HRP	Horseradish Peroxidase
HU	Hounsfield Unit
IARC	International Agency for Research on Cancer
ICCC	Interclass Correlation Coefficient
IHC	Immunohistochemistry
K/M	Klintrup-Makinen
Mem	Membrane
mGPS	modified Glasgow Prognostic Score
mLST8	Mammalian Lethal with SEC13 protein 8
mins	Minutes
mRNA	Messenger Ribonucleinacid
MRI	Magnetic Resonance Imaging
mTOR	Mammalian Target of Rapamycin
NSS	Nephron Sparing Surgery
Nuc	Nuclear
P13K-Akt-mTOR	Phosphoinositide 3-kinase/Akt Pathway
O.D. 595	Optical Density at 595 nm

PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
PDGFR	Platelet Derived Growth Factor Receptor
PI	Proliferation Index
PI3-K	Phosphatidylinositol 3-kinase
PM	Peter McArdle
PTEN	Phosphatase and Tensin homolog (gene)
PVDF	Polyvinylidene Difluoride
RCC	Renal Cell Carcinoma
RIPA	Radio-Immunoprecipitation Assay
RPM	Rounds Per Minute
RPMI	Roswell Park Memorial Institute
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulphate
SFK	Src Family Kinase
SH	Conserved Protein Domains
SSIGN	Stage Size Grade Necrosis
S.T.W.S.	Scots Tap Water Substitute
TBS	Wash buffer for IHC and Western Blotting
TBST	Wash buffer and Tween for Western Blotting
TCE	Trichloroethylene
TdT	Terminal Deoxynucleotidyl Transferase
TKI	Tyrosine Kinase Inhibitor
TNM	Tumour Node Metastases
TQ	Tahir Qayyum
TUNEL	Terminal deoxynucleotidyl transferase dUTP Nick end Labelling
TKI	Tyrosine Kinase Inhibitor
TNM	Tumour Node Metastases
Tris	Tris(hydroxymethyl)aminomethane
Tyr (Y)	Tyrosine
UICC	Union Internationale Contre le Cancer
UISS	UCLA Integrated Staging System
V	Volt
VEGF	Vascular Endothelial Growth Factor
VHL	von Hippel Lindau
WST	Water Soluble Tetrazolium

List of Publications

Qayyum T, McArdle PA, Going JJ, Orange C, Seywright M, Aitchison MA, Edwards J
Is the presence or absence of tumour necrosis a significant predictor of survival in Renal Cell Cancer?

Urol Int. 2012;88(1):79-83

Qayyum T, McArdle PA, Lamb GW, Going JJ, Orange C, Seywright M, Horgan PG, Oades G, Aitchison MA, Edwards J

A prospective study of the role of inflammation in renal cancer

Urol Int. 2012 2012;88(3):277-81

Qayyum T, McArdle PA, Lamb GW, Jordan F, Orange C, Seywright M, Horgan PG, Jones RJ, Oades G, Aitchison MA, Edwards J

Expression and Prognostic significance of Src Family Members in Renal Clear Cell Carcinoma

Br J Cancer. 2012 Jul 19. doi: 10.1038/bjc.2012.314

Qayyum T, Oades G, Horgan PG, Aitchison MA, Edwards J

The epidemiology and risk factors for Renal Cancer

Current Urology 2012;6:169-174

Qayyum T, McArdle PA, Orange C, Seywright M, Horgan PG, Oades G, Aitchison M, Edwards J

Re-classification of the Fuhrman grading system-Does it make a difference?

Springerplus. 2013 Aug 10;2:378

Chapter 1-Renal Cell Cancer

1.1 Incidence and survival rates in renal cell carcinoma

Worldwide, renal cancer is the 13th most common malignancy with over 270,000 new cases diagnosed in 2008 [1]. In the UK alone, approximately 9000 new cases of renal cancer are diagnosed each year [2]. Incidence rates have increased in the UK (Figure 1.1) with age standardised incidence rates more than doubling between 1975-1977 and 2007-2009 [2]. Age standardised ratios for both incidence and mortality is observed to be 50% lower in women compared to men [2]. Renal cell carcinoma (RCC) accounts for nearly 90% of all renal malignancies. There has been much debate that the increased incidence rates is due to the vast improvement and use of imaging modalities such as magnetic resonance imaging (MRI) and computed tomography (CT). It has been reported that there has been an increased rate of detection of incidental tumours which are asymptomatic and localised [3-6] but there has also been an increase detection of more advanced tumours and that the increase in incidence is real and cannot be solely accounted by incidentally detected tumours [3, 7].

Figure 1.1: Renal cancer incidence rates between 1993-2009 for the UK.

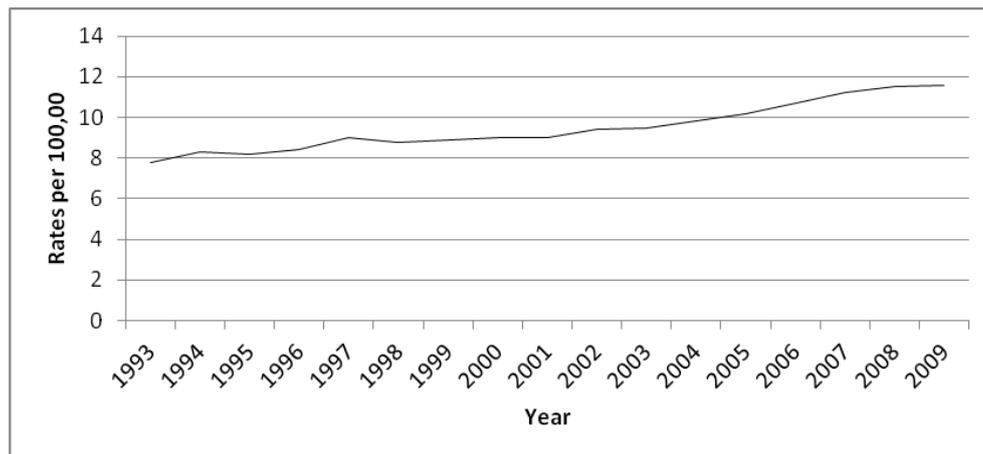


Figure 1.1 demonstrates that the incidence rates of renal cancer have increased over the 16 years [2].

Globally, renal cancer was responsible for over 110,000 deaths in 2008 [1]. Nearly 4000 patients died from renal cancer in 2008 accounting for 2% of all cancer deaths in the UK [2] with mortality rates increasing (Figure 1.2). 20-30% patients present with metastatic disease [8] with another 20% of patients undergoing nephrectomy developing metastases during subsequent follow up [9]. Various factors contribute to survival such as tumour involvement and overall health, but there is still only a 50% chance of survival at five years following diagnosis [2].

Figure 1.2: Renal cancer mortality rates between 1993-2008 for the UK.

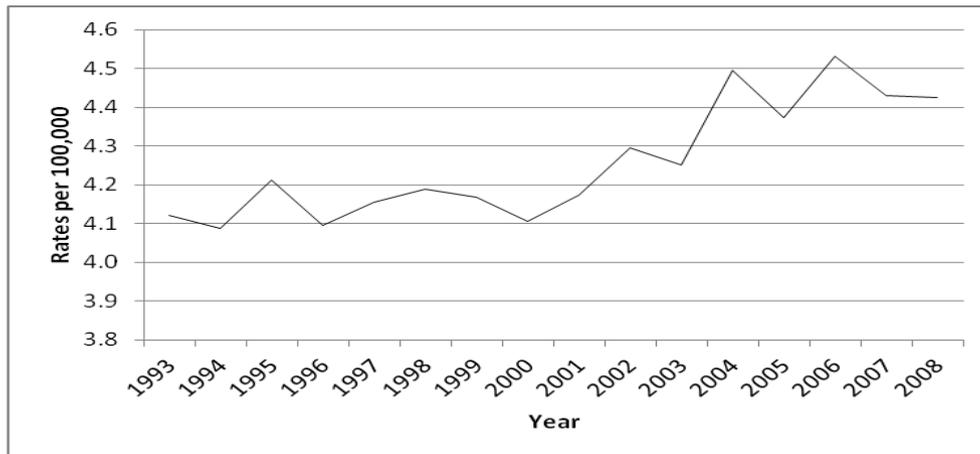


Figure 1.2 demonstrates that mortality rates are increasing [2].

1.2 Risk Factors

Approximately 75% of those diagnosed are over 60 years of age with the disease being rare in those under 50 years of age [2] and the disease reaching a plateau around 70-75 years of age [10]. Incidence rates have increased in all age groups but this increase is predominantly in those over 75 years of age (Figure 1.3), in addition mortality rates have predominantly increased in those over 75 (Figure 1.4) confirming that renal cancer is predominantly a disease of the elderly.

Figure 1.3: Kidney cancer incidence rates as shown by various age groups.

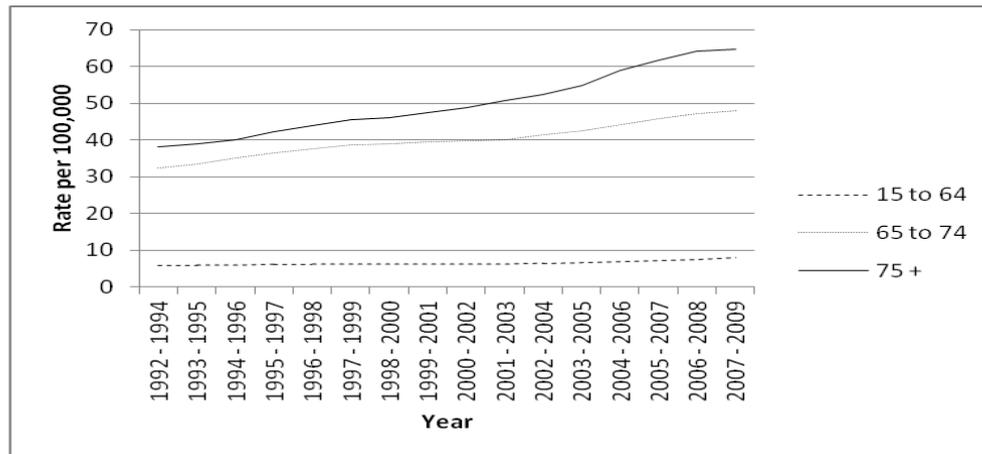


Figure 1.3 demonstrates that the incidence rates of renal cancer increased in all age groups but the largest increase in incidence was in those over 75 years of age [2].

Figure 1.4: Kidney cancer mortality rates as shown by various age groups.

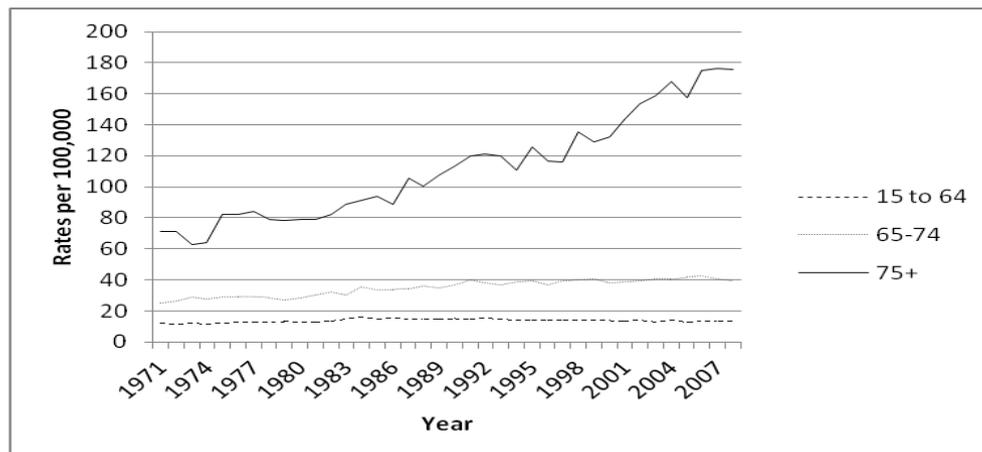


Figure 1.4 demonstrates that the mortality rates of renal cancer increased in all age groups but the largest increase in mortality was in those over 75 years of age [2].

Age standardised incidences suggest that men are at an increased risk of RCC [1] with it being the 6th most common cancer in men and the 9th most common in women in the UK [2]. Whilst there has been an increase in the overall incidence in RCC (Figure 1.1), there has been a higher incidence in males than females (Figure 1.5) accounting for the overall

increase in incidence suggesting that there is a higher predisposition of renal cancer in males than females. It has been reported that between 2007-2030 there will be a 27% and 18% increase in the incidence of renal cancer in males and females respectively [11]. In 2009 the age standardised incidence risk per 100,000 was 15.5% in men compared to 8.2% in women [2].

Figure 1.5: Renal cancer incidence rates between 1993-2009 according to sex in the UK.

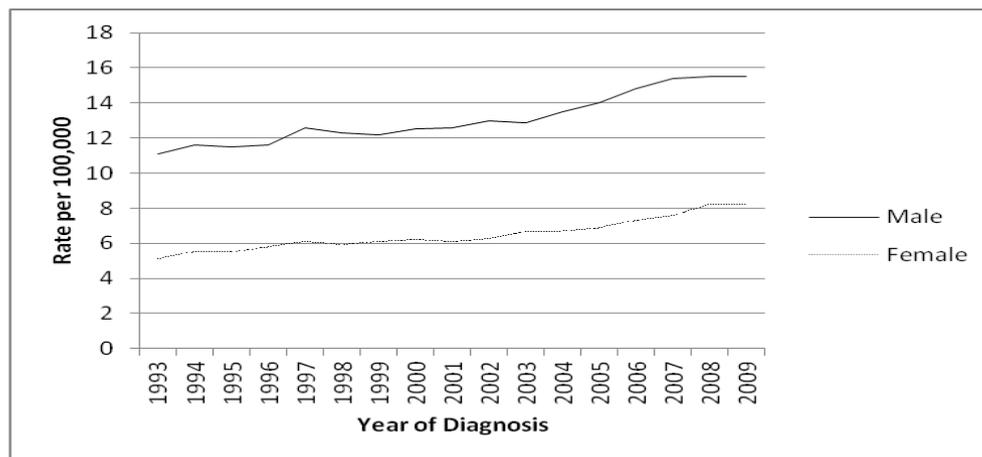


Figure 1.5 demonstrates a marked difference in the incidence rates in renal cancer between males and females [2].

Mortality rates are also higher in males than in females (Figure 1.6), this may be expected given the obvious difference in incidence rates in both sexes. In 2008 the age standardised mortality rate per 100,000 was 6% in men compared to 3.1% in women [2]. It has long been thought that incidence and mortality rates have been higher in males due to lifestyle factors such as cigarette smoking which has been historically higher in males and also exposure to industrial carcinogens due to differing occupational bias between the sexes.

Figure 1.6: Kidney cancer mortality rates between 1993-2009 according to sex.

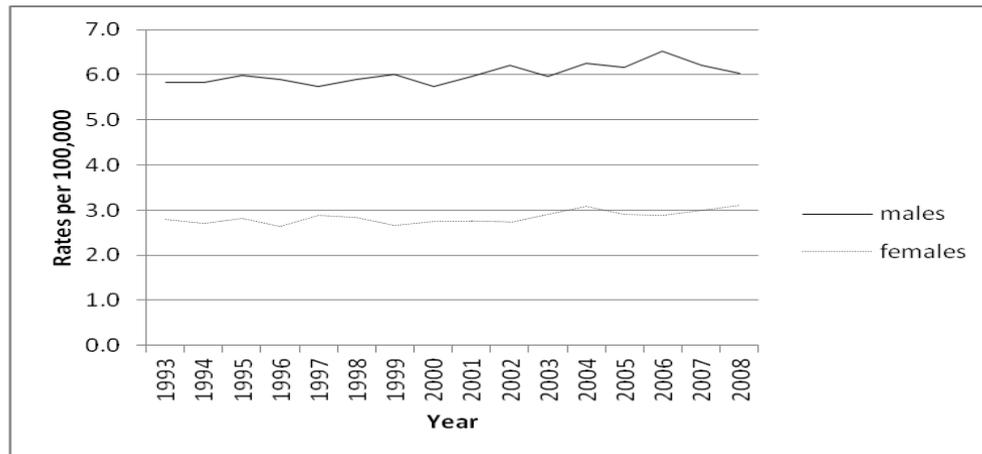


Figure 1.6 demonstrates that the mortality rates have increased in both sexes over the last 15 year [2].

Smoking is a well-established risk factor for RCC with a meta-analysis reporting not only a difference in a smoker and a non-smoker but also a dose dependant risk with the number of cigarettes smoked [12]. Compared to those whom never smoked, there was a 50% increase in the risk for males and a 20% increase risk for females [12]. This risk can be reduced after smoking cessation for more than 10 years [10, 12, 13]. It is thought that cigarette smoking increases the risk of RCC through chronic tissue hypoxia due to carbon monoxide exposure [14] as well as evidence suggesting higher levels of DNA damage in peripheral blood lymphocytes in those with RCC compared to controls [15].

It has been suggested that the different incidence rates observed between males and females may be due to exposure to potential occupational carcinogens. The most extensively studied is the solvent Trichloroethylene (TCE) which is widely used as a metal degreaser and has been considered a human carcinogen by the International Agency for Research on Cancer (IARC) as well as a common environmental contaminant [16]. A case controlled series in Europe reported an increased risk following exposure to TCE [17] with one review reporting increased risk of various malignancies including renal following

exposure [18] and a meta-analysis suggesting a weak association with exposure to TCE [19] whilst others have reported that given the complexities of TCE pharmacokinetics and limitation of studies this prevents a definitive relationship [16, 18, 20]. Various other compounds have been investigated with one study reporting an association with lead which requires further investigation whilst associations have been reported for glass and wool fibres as well as brick dust [21, 22]. Exposure to industrial agents such as cadmium and uranium [10, 23, 24] have shown no relationship to RCC risk and neither have arsenic, nitrate and radon in drinking water [10]. Interestingly an association between agricultural workers and RCC was reported [25] and an inverse relationship between exposure of ultraviolet light in men and RCC risk was observed [22].

Excess body weight has been established as a risk factor for RCC with it accounting for 30% of cases in Europe [26]. Various prospective studies conducted worldwide have reported that overweight and obese individuals were found to have an elevated subsequent risk of RCC [27-30] with a meta-analysis of this work also suggesting that an association between body mass index (BMI) and risk of RCC exists [31]. Some have suggested that body fat distribution is associated with an increased risk of RCC [28, 30] but evidence is limited suggesting that abdominal obesity is independent of BMI with the association with RCC. Two factors closely related to each other and obesity are diet and physical activity. The majority of studies have demonstrated an inverse relationship between physical activity and RCC risk [32-40] with some authors reporting a dose response with further reduction of risk with increasing levels of activity [35, 37, 38]. Assessing dietary intake has reported mixed results with association with RCC. The role of vitamins that are abundant in fruit and vegetables has produced variable results with the risk of RCC with some reporting an association with RCC [41] whilst others have reported no correlation [42-44] whilst analysis of cohort studies has reported that diets rich in fruits and vegetables are inversely related to RCC [45]. High consumption of fat and protein has not been shown to be associated with an increased risk of RCC [46-48]. The consumption of alcohol has

also been demonstrated to have a negative relationship with risk of RCC in a dose response manner [49] whilst in contrast no correlation was demonstrated with total fluid intake from any fluids in total or from individual types of fluids [50, 51] suggesting that it is not duration of contact with any potential carcinogens which prevents RCC risk with alcohol consumption.

Of the various subtypes of RCC, each has a corresponding hereditary component caused by a distinct genetic alteration [52]. The majority of clear cell renal cancer is sporadic with 2-4% of all RCC being familial [53-55] with a two-fold increase in a first degree relative [56]. The most common familial syndrome for RCC is von Hippel Lindau (VHL) syndrome [57] which is transmitted in an autosomal dominant manner on chromosome 3p which can also cause patients to develop pheochromocytomas, retinal angiomas and haemangioblastomas of the central nervous system [58] with 50% suffering from RCC with VHL. It has been demonstrated that inactivation of the VHL gene is an early step in the development of clear RCC in those suffering from VHL syndrome [58]. The VHL tumour suppressor gene is mutated in all cases of hereditary RCC with 50% of sporadic cases also manifesting with this mutation [59]. The VHL gene is responsible for the degradation of hypoxia inducible factors [60] without which leads to up-regulation of factors which promote angiogenesis and tumour growth such as vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF) [61-63]. In those with VHL syndrome, RCC is the most common cause of death.

Various medical conditions have been demonstrated to have an association with an increased risk of RCC. Types of renal tumours have been shown to cause hypertension [64]. Interestingly however, several studies have reported an association with long term hypertension and risk of RCC [32, 33, 40, 65] as well as some reporting a dose response relationship [32] with the risk of RCC increasing with further elevation of blood pressure and decreasing with a reduction in blood pressure [65]. There have been reports that usage of anti-hypertensive treatment is also associated with an elevated risk of RCC but it is

thought that it is hypertension causing this increased risk and not the actual treatment [32, 35, 36, 39, 66]. Obesity as a risk factor has already been mentioned but it has been reported that despite the relationship between obesity and hypertension, both are independent with their association with RCC and risk is higher in amongst those suffering from both conditions than those with only one [32, 36, 39, 65].

Diabetes mellitus is known to be associated with an increased risk of several cancers. Its relationship in RCC has not been demonstrated to be an independent factor but was closely associated with obesity and hypertension [35, 36, 40, 66-71].

It has been demonstrated that acquired renal cystic disease develops in those with end stage renal disease and in those on haemodialysis [72]. The incidence of RCC is higher in those with cystic disease [73, 74] but the evidence suggesting that these cysts undergo malignant change is not conclusive [74, 75]. Those who are undergoing haemodialysis are at a higher risk of RCC [75-77] as well as there being an increased risk of RCC after renal transplantation [77-79].

There has been some controversy surrounding an association between urinary tract infections and RCC, one study suggests that a history of a urinary tract infection increases the risk of RCC and this risk is further exacerbated with a history of smoking [80] whilst another report has demonstrated that no relationship between these parameters is present [81].

1.3 Symptoms

Traditionally the majority of patients presented with the classical triad of flank pain, haematuria and abdominal mass with nearly half of these having advanced or metastatic disease [82]. Prior to improvements in imaging modalities and the frequency with which they were utilised, less than 10% of tumours were incidental findings [83]. This classical

triad is now a rare presentation accounting for less than 10% of cases [84, 85] with >50% of cancers now being detected incidentally [86-88].

The majority of these asymptomatic cancers are detected following ultrasound examination of the abdomen following a variety of non-specific symptoms. The difficulty arises trying to differentiate between benign and malignant lesions. A classification system was devised to outline the features of benign lesions which do not require any further follow up and those that require further investigation [89] as renal cysts which are often benign, are the most common space occupying lesions and therefore do not require further follow up or investigation [90]. For those solid renal masses which require differentiation from malignant lesions, the most important way to distinguish between these lesions is the presence of enhancement [91]. CT imaging is generally accepted as the imaging of choice remaining the most widely available and single most effective modality for staging [92, 93] with radiation exposure being the greatest disadvantage. Enhancement is determined by comparing Hounsfield Unit (HU) prior and after contrast administration with a change of 20 HU evidence of enhancement [94] and therefore indicative of malignancy. Where CT cannot be utilised due to renal impairment, contrast enhanced ultrasound can be utilised with a relative contraindication for contrast [95-97]. MRI can also be utilised in cases of renal impairment [98, 99] or with an absolute contraindication to contrast such as pregnancy [100]. There have been reports that MRI can potentially improve imaging and assessment of a renal mass [101-104] but imaging with CT has the advantages of widespread availability, shorter examination time and lower cost in comparison to MRI.

1.4 Histological Diagnosis

A percutaneous biopsy is rarely required for large renal masses prior to undergoing nephrectomy as the positive predictive value of imaging is so high that a negative biopsy would not alter management [105]. Cytology can be obtained via fine needle aspiration

(FNA) or with core biopsy. FNA has been demonstrated to provide no additional information with large masses as imaging is diagnostic and the low sensitivity also makes FNA unreliable [106]. The role of pre-operative biopsy requires further validation before becoming a tool that is widely accepted [107]. Despite FNA being considered less invasive than percutaneous biopsy, its accuracy and diagnostic yield has not been able to match that of a core biopsy utilising an 18Gauge needle [108] whilst another study investigating the accuracy of various gauges of core biopsy size in terms of accuracy reported that the 18Gauge needle was the most accurate in determining histological diagnosis [109]. One report demonstrated that biopsies can be utilised safely to distinguish between benign and malignant lesions in small asymptomatic cases but biopsies of these small lesions was associated with a high rate of technical biopsy failure [110] as well as reports that the rates of a biopsy being inconclusive can vary between 3-21% of cases [111]. Despite core biopsies being utilised frequently for the purposes of follow up, ablative therapies and diagnosis in cases of metastatic disease prior to systemic therapy [105, 106, 110-112], it is still not considered a requirement for definitive diagnosis in terms of histology prior to nephrectomy as a negative biopsy would not alter treatment in cases where imaging is suggestive of a malignancy [105].

The histological diagnosis of RCC is confirmed either at time of biopsy or after nephrectomy. RCC's are thought to arise from various specialised cells located within the nephron with the Heidelberg classification utilised for the various sub-types with clear cell and papillary which arise from the epithelium of the proximal tubule being the most common whilst those of lesser frequency which includes chromophobe and collecting duct RCC arising from the epithelium of the distal collecting duct [59, 113, 114]. Each of the different sub-types have different cytogenetic and immunohistochemical profiles as well as differing prognoses. Clear cell which accounts for 80-90% of RCC displays large uniform cells with abundant clear cytoplasm and is typically highly vascular (Figure 1.7). Papillary RCC has small cells with scanty cytoplasm and consists of two sub-types with type I

occurring sporadically and metastasising later than type II which are more likely inherited, can be multiple and are of a higher grade (Figure 1.8). Collecting duct tumours which arise from the medullary collecting duct tend to occur in younger people and have an overall poor prognosis. Chromophobe RCC arises from collecting duct epithelium (Figure 1.9) and has been demonstrated to have a more favourable prognosis over papillary carcinoma which has a favourable prognosis over clear cell cancer [115, 116]. For the purposes of this thesis, the main focus will be on clear cell carcinoma as it can account for nearly 90% of all renal cancers and will be referred to as renal cell cancer (RCC) for the purposes of this manuscript from this point.

Figure 1.7: Histopathological slide of clear cell carcinoma (RCC).

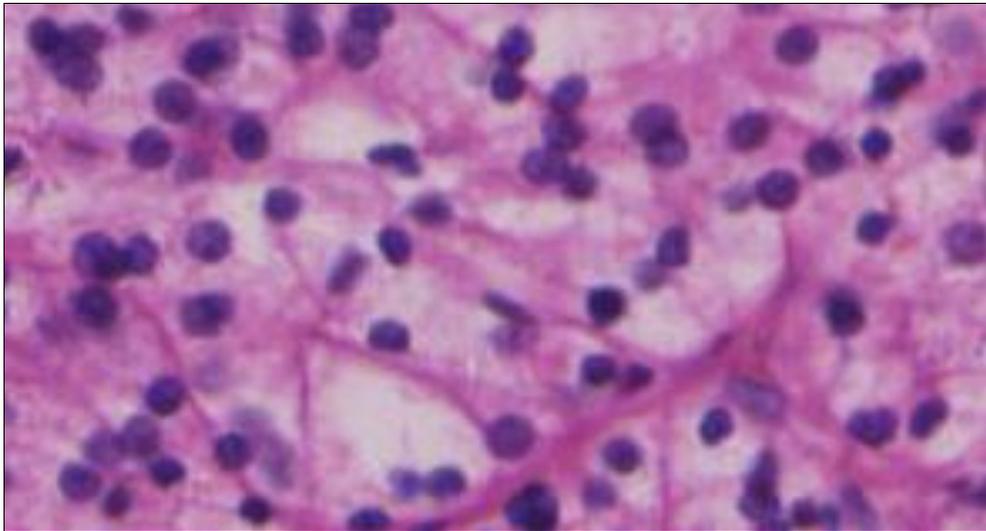


Figure 1.7 demonstrates the large uniform cells abundant with cytoplasm associated with clear cell carcinoma.

Figure 1.8: Histopathological slide of papillary renal carcinoma.

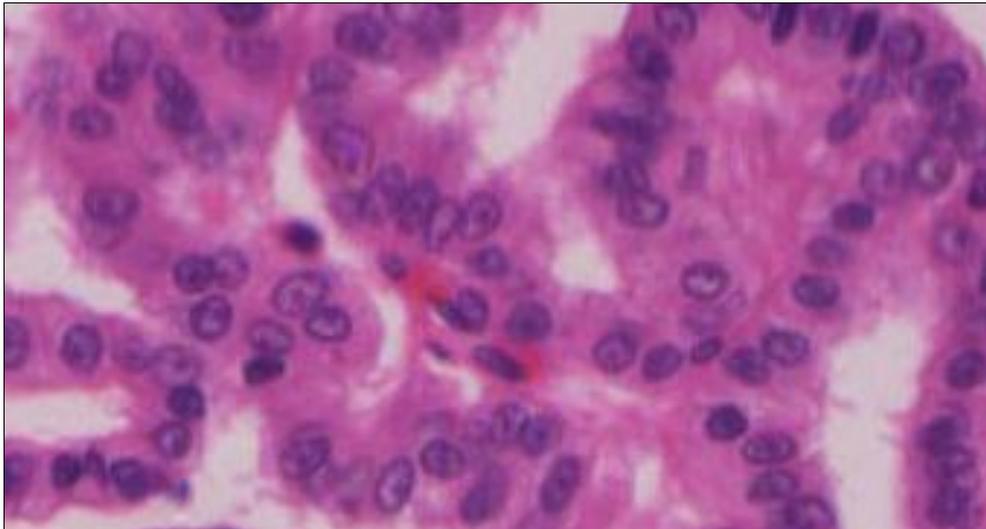


Figure 1.8 demonstrates small cells with scanty cytoplasm.

Figure 1.9: Histopathological slide of chromophobe renal carcinoma.

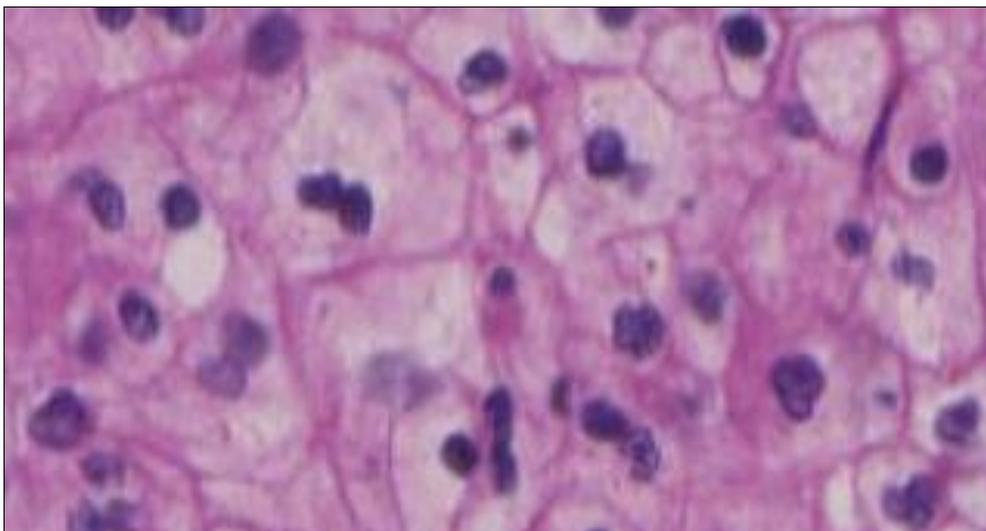


Figure 1.9 demonstrates the abundant pale cytoplasm and prominent cell membrane associated with chromophobe carcinoma.

1.5 Classification and Prognostic Factors

1.5.1 Anatomical Factors

The TNM (Tumour Node Metastases) classification is a global, well established classification system accepted for the staging of diverse solid tumours including renal cancer. It is recommended for clinical and scientific use, representing anatomical factors [117] (Table 1).

Table 1.1: The 2009 TNM staging classification system.

T - primary tumour
TX Primary tumour cannot be assessed
T0 No evidence of primary tumour
T1 Tumour < 7 cm in greatest dimension, limited to the kidney
T1a Tumour < 4 cm in greatest dimension, limited to the kidney
T1b Tumour > 4 cm but < 7 cm in greatest dimension
T2 Tumour > 7 cm in greatest dimension, limited to the kidney
T2a Tumour > 7 cm but < 10 cm in greatest dimension
T2b Tumours > 10 cm limited to the kidney
T3 Tumour extends into major veins or perinephric tissues but not into the ipsilateral adrenal gland and not beyond Gerota's fascia
T3a Tumour grossly extends into the renal vein or its segmental (muscle-containing) branches or tumour invades perirenal and/or renal sinus (peripelvic) fat but not beyond Gerota's fascia
T3b Tumour grossly extends into the vena cava below the diaphragm
T3c Tumour grossly extends into vena cava above the diaphragm or invades the wall of the vena cava
T4 Tumour invades beyond Gerota's fascia (including contiguous extension into the ipsilateral adrenal gland)
N - Regional lymph nodes
NX Regional lymph nodes cannot be assessed
N0 No regional lymph node metastasis
N1 Metastasis in a single regional lymph node
N2 Metastasis in more than 1 regional lymph node
M - Distant metastasis
M0 No distant metastasis
M1 Distant metastasis

Table 1.1 demonstrates the TNM staging classification with the various breakdown of each clinical parameter.

The first formal staging system in renal cancer was based on physical tumour characteristics and the location of tumour spread [118] which was later modified to include vascular involvement [119]. It was based on these characteristics that led to the development of the TNM system by the Union Internationale Contre le Cancer (UICC) allowing patients to be categorised [120]. The T component reflects the local extension of the primary tumour, the N component represents extent of lymphatic involvement and the M component indicates distant metastases. The primary size of the tumour is a key component of the TNM staging system and has been demonstrated to be one of the most prognostic factors for renal cancer [121, 122]. Furthermore there has been a large body of evidence demonstrating the prognostic benefit of the various T stage stratifications for T1 [123], T2 [124], T3 [125, 126] and T4 [127-129] disease. The risk of lymph node involvement varies depending on primary tumour stage, vascular involvement, metastases and the extent of lymphadenectomy performed [130, 131]. It has been reported that patients without preoperative lymph node involvement had a significantly longer survival rate than those with lymph node involvement [132]. It has also been demonstrated that in those with metastatic diseases, presence of lymph node involvement resulted in worse outcome than presenting with metastases alone [133]. The presence of metastatic disease would naturally suggest a poor prognosis. It has been demonstrated that the presence of metastases is a predictor of poor survival [134, 135].

1.5.2 Histological Factors

Various grading classifications for RCC based on morphological features have been proposed [83, 136-141] and of these the Fuhrman grading system [138] has achieved widespread usage in pathology practise. This 4-tiered grading system is essentially based on nuclear size and morphology and on the presence or absence of nucleoli. G1 tumours consist of cells with small (approximately 10 μm), uniform, round nuclei with inconspicuous or absent nucleoli; G2 tumours have irregular morphology, larger nuclei

(approximately 15 μm) with small nucleoli when examined under high power ($\times 400$ magnification); G3 tumours have irregular outlines with even larger nuclei (approximately 20 μm) and large, prominent nucleoli that are evident even at low power ($\times 100$ magnification); and G4 tumours differ from G3 lesions in that they contain bizarre, multilobed nuclei and heavy chromatin clumps [138] (Figure 1.10). Nuclear grading is an important predictor of survival in RCC with increasing grade a predictor of metastases [138]. This grading system has been demonstrated to be an independent predictor of survival [127, 142, 143] and acknowledged as optimal for predicting outcome [144]. Therefore this has been incorporated into the majority of prognostic algorithms including SSIGN [145], UISS [146] and Leibovich [147].

Figure 1.10: Classification of the Fuhrman grading system.

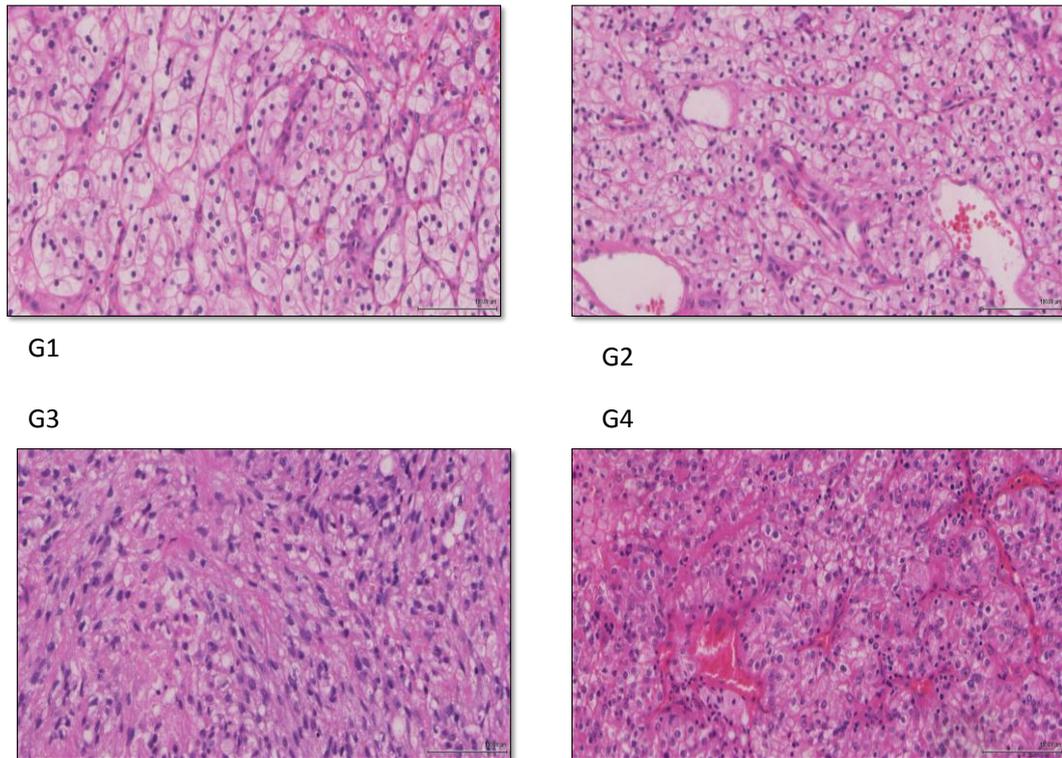


Figure 1.10 demonstrates the various grades of clear cell carcinoma as classified by the Fuhrman nuclear grading system.

There has been a suggestion that the Fuhrman grading system has low-moderate inter-observer agreement [148-151] and that a simplified system improves inter-observer agreement [148, 149] as well as demonstrating as much accuracy as the conventional grading system [152, 153]. Furthermore there are those suggesting that the ideal grading system is yet to be defined and should consist of three tiers [151] whilst a three tiered system has been shown to be an independent predictor of survival [154, 155].

Tumour necrosis occurs when tumours outgrow their blood supply therefore reflecting aggressive tumour biology and rapid proliferation and progression [156]. Tumour necrosis has been investigated in malignancies such as breast, colorectal and renal cancer. It is often assessed reviewing haematoxylin and eosin stained sections at low magnification using a

semi-quantitative method to classify necrosis as simply being absent or present and is demonstrated by an area devoid of recognised pathology (Figure 1.11).

Figure 1.11: Tumour necrosis on a haematoxylin and eosin stained section.

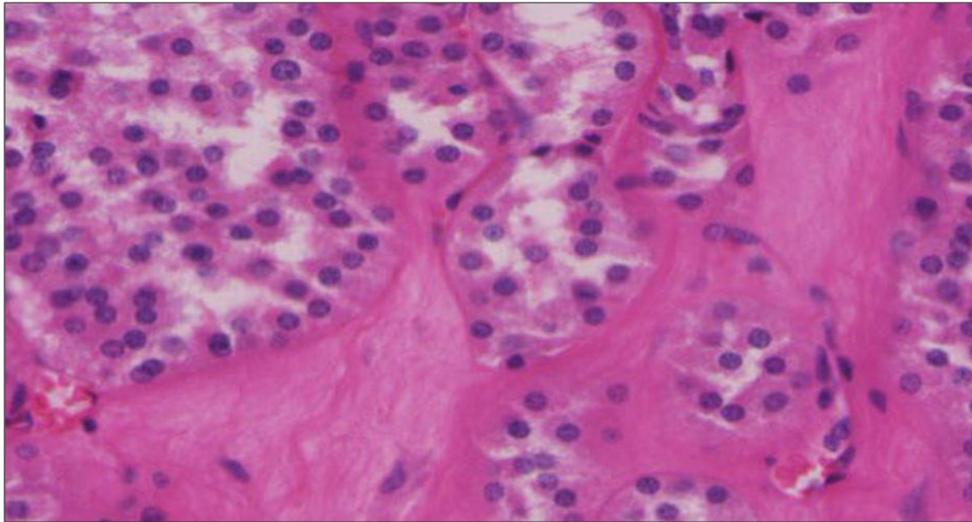


Figure 1.11 demonstrates areas completely devoid of recognised pathology which is the feature of tumour necrosis.

Some studies investigating necrosis in breast cancer have reported that necrosis is independently associated with tumour recurrence [157] and overall survival [158] whilst others have suggested that necrosis predicts survival but is not an independent factor [159, 160]. Despite using a quantitative method to classify necrosis in breast cancer with a 15 year follow up, necrosis was not associated with overall survival [161]. Evidence investigating the role of tumour necrosis in colorectal surgery is more supportive. Necrosis has been shown to be an independent predictor of disease specific survival in colorectal cancer of various stages [162, 163]. Studies investigating the role of necrosis in organ confined disease in colorectal cancer, a group that would have the greatest benefit of additional prognostic information being utilised, have conflicting results with one study observing no relationship between necrosis and survival [164] whilst another reported it as an independent predictor of survival [165]. The role of tumour necrosis has been

investigated in renal cancer with some conflicting results. The majority of studies have reported that necrosis adds prognostic information as it is associated with reduced disease specific survival, recurrence and progression to metastatic disease [145, 147, 166-168]. Others have shown either no correlation or independent relationship between necrosis and outcome [137, 169-172]. Despite conflicting results regarding the significance of necrosis in renal cancer, a semi-quantitative method of assessment of this pathological parameter is incorporated into prognostic algorithms such as SSIGN [145] and Leibovich [147] scores. Necrosis is evaluated on a simple absence/presence basis and this therefore does not take into account the extent of necrosis. It has been suggested that an extent based classification is superior to a simple absence/presence response and was retained as an independent prognostic factor in renal cancer [173].

It is now established that disease progression in cancer patients is not solely determined by the tumour characteristics but also by the host response. There is increasing evidence that both local and systemic inflammatory responses play an important role in the progression of various solid tumours [174, 175]. When inflammation does not resolve, it promotes tumour cell growth, survival and angiogenesis as well as playing a critical role in the metastases of cancers [176]. When attempts are made to reduce this inflammation by the administration of non-steroidal anti-inflammatory drugs, it has led to the reduction of tumour involvement in colon and lung cancers [174, 177-179].

Evidence suggests that intensity of local inflammatory infiltrate within the tumour bed predicts prognosis [180]: a pronounced lymphocytic infiltration in colorectal cancer is associated with improved survival [181-183]. Also quantifying the degree of infiltration by lymphocyte subsets such as CD8+ and CD4+ T cells provides prognostic information in various tumour types [184, 185] including renal cancer [186]. Identifying and quantifying the various inflammatory cells on haematoxylin and eosin stained sections is a difficult process with obvious differing features between the different cells. Neutrophils are identified as those inflammatory cells with multi-lobulated nuclei, lymphocytes are those

with a thin rim of cytoplasm and macrophages are irregular in shape with a vacuolated cytoplasm (Figure 1.12). The process of assessing inflammatory cell infiltration is time consuming and has not been adopted into routine clinical practice.

Figure 1.12: Local inflammatory response.

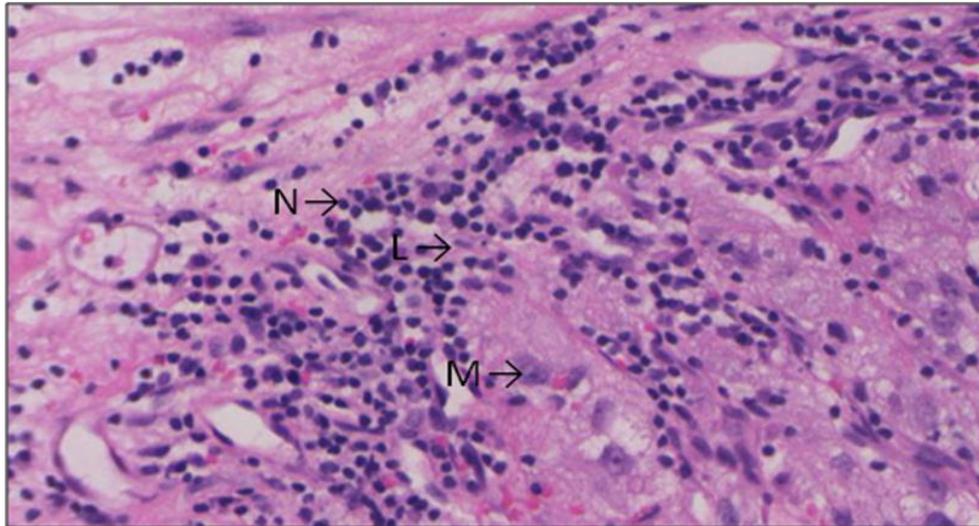


Figure 1.12 demonstrates the various inflammatory cells, N-neutrophil, L-lymphocyte, M-macrophage.

It is therefore of interest that Klintrup and colleagues have reported a simplified method of assessing the inflammatory cell infiltrate at the tumour margin [187], shown on routine haematoxylin and eosin stained sections, that tumour inflammatory infiltrate, including all white cell types, can be graded high or low grade. It has been shown that a high grade infiltrate is associated with improved survival in colorectal cancer [187, 188] and gastroesophageal cancer [189].

Whilst the systemic inflammatory response is not a histological response per se, peripheral blood markers are a systemic response to the histological process. Neutrophil/lymphocyte ratio has been demonstrated as a prognostic marker of systemic inflammation in colorectal, hepatocellular, ovarian and pancreatic cancers [190-193]. Increasing evidence supports a role of the systemic inflammatory response, indicated by elevated levels of C-reactive protein (CRP) being an independent predictor of survival in patients with a variety of

common solid tumours including gastrointestinal, lung, prostate, bladder and renal cancer [194-199]. The modified Glasgow Prognostic Score (mGPS), incorporates CRP and albumin serum levels [200]. The mGPS score has provided additional prognostic information in patients with various solid malignancies including lung, gastroesophageal and colorectal cancers [201-203].

1.5.3 Molecular Factors

It is felt that the incorporation of molecular markers into prognostic algorithms will increase their predictive accuracy. A wide variety of molecular markers have been reported to influence prognosis in renal cancer including cell cycle regulators, mediators of cellular proliferation and apoptosis.

Vascular endothelial growth factor (VEGF) is an important angiogenic factor with significant effects on tumour angiogenesis. In recent years there has been increasing evidence showing that treatment with vascular endothelial growth factor-tyrosine kinase inhibitors (TKI, e.g Sunitinib, Sorafenib) conveys additional survival benefit in metastatic or recurrent renal cancer [204-208]. Studies examining plasma levels of VEGF prior to nephrectomy have shown conflicting results. Some studies have shown that plasma levels of VEGF as an independent predictor of survival [205] whilst others have suggested that VEGF levels might be utilised as a biomarker for clinical efficacy [209]. Others have reported that VEGF plasma levels have prognostic significance but failed to be independent of other clinic-pathological parameters [210, 211], and it is also reported to have no correlation to survival [212]. One of these studies despite showing that plasma VEGF levels were an independent predictor of survival, further demonstrated that those patients with both high and low levels of VEGF benefited from treatment with a TKI [205] suggesting that plasma levels may not be used as a biomarker for clinical efficacy. Studies have examined expression of VEGF in renal cancers with varying results. Some have shown that expression of VEGF as an independent predictor of survival [213-216] whilst

another showed it to be prognostic but not an independent predictor [217] with another study showing no correlation to survival [218].

The mammalian target of rapamycin (mTOR) pathway (phosphoinositide 3-kinase/Akt pathway, (P13K-Akt-mTOR)) has a central role in the regulation of cell growth and survival, playing a critical role in tumour progression [219, 220]. Treatments with inhibitors of the mTOR pathway (e.g. temsirolimus, everolimus) have been shown to convey additional progression free survival in renal cell cancer [221, 222] and are currently utilised to treat recurrence and metastatic disease. Efforts have been made to characterise biomarkers such as members of the mTOR pathway which may predict benefit from mTOR inhibitors but with little success. Studies have reported that mTOR is more highly expressed in renal cancer [223-226] as well as demonstrating that the P13K-Akt-mTOR pathway is activated in renal cell cancers [224, 226-229]. Studies examining the activation status of the mTOR pathway have observed an association with tumour stage, grade and disease specific survival as well as being an independent predictor of disease specific survival [226, 230, 231] suggesting that this could allow targeted therapy.

It is known that inactivation of PTEN (phosphatase and tensin homologue), a tumour suppressor gene, results in activation of the P13K-Akt-mTOR pathway [232, 233].

Expression levels of PTEN could be assessed with the possibility of expression guiding treatment with mTOR inhibitors. Studies have suggested that PTEN levels are reduced in clear cell cancer [225, 230, 231, 234-236] and have therefore opened the possibility of expression levels being utilised in targeted therapy with mTOR inhibitors. A study examined this possibility investigating baseline levels of PTEN in response to therapy with temsirolimus and showed no correlation in response to therapy [237].

Carbonic anhydrase IX (CA IX) is a protein that is thought to play a key role in maintaining cellular pH allowing cell survival in hypoxic conditions [238] allowing cancer cells to grow and spread out with the organ [239] and is up regulated when the VHL gene is inactivated resulting in accumulation of hypoxia inducible factor [62]. Studies have

investigated the role of CA IX in renal cancer with reports showing that it is up regulated in renal cancer [240] and is independently associated with survival [241-243] whilst in another study it was reported to correlate to cancer staging but not survival [244] and to be associated with disease specific survival but not an independent factor [245] and in another no correlation was found [216].

Markers of cellular proliferation and apoptosis can be utilised to examine if expression provides any prognostic information. Ki 67, a marker of active cellular proliferation is a nuclear protein expressed during all active phases of the cell cycle (G1, S, G2 and M-phases) but absent in the resting phase of the cell cycle (G0).

Ki 67 has been investigated in renal cancer; some studies have shown this marker to be associated with increased nuclear grade and a predictor for disease specific survival [216, 218, 242, 243, 246-251] whilst in other studies it has not been shown to add prognostic information [252-255]. Apoptosis on the other hand is also a highly regulated cellular process but is involved in maintenance of tissue homeostasis and elimination of unwanted cells [256]. Acquired resistance to apoptosis is thought to be one of the salient features of cancer [257]. Apoptosis has been shown to be a prognostic marker for survival in renal cancer as it has been shown to correlate to tumour grade, stage and size [258-260] as well as being independently associated with survival [261]. While it has been suggested that apoptosis may be a prognostic marker in its own right, others have shown it to either have no correlation to established prognostic factors or it to not be an independent predictor of survival [236, 262-265].

Despite varying degrees of evidence in the literature showing that molecular markers can aid in prognosis and guide treatment strategies, efforts have been made to utilise molecular markers in prognostic algorithms [216, 218, 266]. Nevertheless, there are still no biomarkers in routine clinical use in RCC [267].

1.6 Treatment of Renal Cell Cancer

1.6.1 Nephron Sparing Surgery

Radical nephrectomy has been the standard treatment of renal cell carcinoma [119]. Nephron sparing surgery (NSS) has however been accepted as the ideal treatment in those with a single functioning kidney or in those at high risk of requiring renal replacement therapy [268]. NSS for localised RCC has been demonstrated to have similar oncological outcome to radical surgery [269-273]. NSS is not a feasible option in those with locally advanced tumour burden, technical unfeasibility due to unfavourable location or when it is detrimental to the patients' health. In this situation, radical nephrectomy either by open or laparoscopic surgery remains the gold standard [125, 274-277]. NSS in those with T1a disease has been demonstrated to provide similar recurrence free and survival compared to those undergoing radical surgery [269-273, 278, 279]. Even in those with T1b disease, NSS has also demonstrated that overall and cancer specific survival is not compromised in comparison to radical surgery [280-283]. Furthermore studies have demonstrated that renal function is preserved with NSS and this would demonstrate improved overall survival [284-287]. The complication rates for NSS are higher than those undergoing radical surgery [288] especially in those undergoing NSS for solitary kidney [289, 290].

1.6.2 Laparoscopic Surgery

Since its initial introduction, laparoscopic radical surgery has now become standard practise for those patients with T2 disease and those with smaller disease not amenable for NSS [291-295]. Laparoscopic radical surgery has not only been demonstrated to have similar cancer free survival rates to open surgery [274-276, 293, 294] but in a very small cohort of patients has been demonstrated to have lower morbidity in comparison to open surgery [296]. In certain cases and in experienced hands, laparoscopic NSS is an alternative option to open NSS in those with small peripheral tumours [272]. In those

undergoing NSS, long term renal function correlates inversely to ischaemia time [297]. In those undergoing NSS, intraoperative ischaemia time is longer with laparoscopic surgery than with open surgery [272, 298, 299]. Limited evidence is available suggesting similar oncological outcomes in those undergoing NSS either with laparoscopic or open surgery [272, 300] whilst it has been demonstrated that there is a higher complication rate in those undergoing laparoscopic than open NSS [301] especially in those with a solitary kidney [272, 297, 302].

1.6.3 Metastatic disease

Performing a nephrectomy is only curative when there is no evidence of metastatic disease and therefore is palliative when performed in these patients. Nephrectomy in this group of patients is only indicated when patients are suitable and fit for surgery [303]. It has been demonstrated that in those patients receiving immunotherapy, there was a modest survival in those that had a cytoreductive nephrectomy performed [304].

RCC develop from the proximal tubules. These proximal tubules have high levels of P-glycoprotein which is a multi-drug resistant protein making treatment with chemotherapeutic agents not possible and therefore chemotherapy as a single agent is not utilised. It has been demonstrated that treatment with the chemotherapeutic agent 5-fluorouracil when combined with treatment with immunotherapy is moderately effective [305].

Following disappointing results with hormonal therapy and conventional chemotherapy and the notion that spontaneous remission may be immune related, trials investigating treatment with immunotherapy were commenced with Interferon- α and Interleukin-2 [306]. Immunotherapy treatment has demonstrated a decrease in the risk of tumour progression as well as increased survival [306-308] with toxicity of Interleukin-2 being higher than Interferon- α and treatment with Interferon- α as monotherapy demonstrating similar efficacy than with combination of the two in addition with chemotherapy [309].

In recent years there have been recent advancements in the molecular aspects of renal cancer leading to the development of novel agents in the treatment of metastatic disease (Figure 1.13).

Figure 1.13: Biological pathways allowing targeted therapy in RCC.

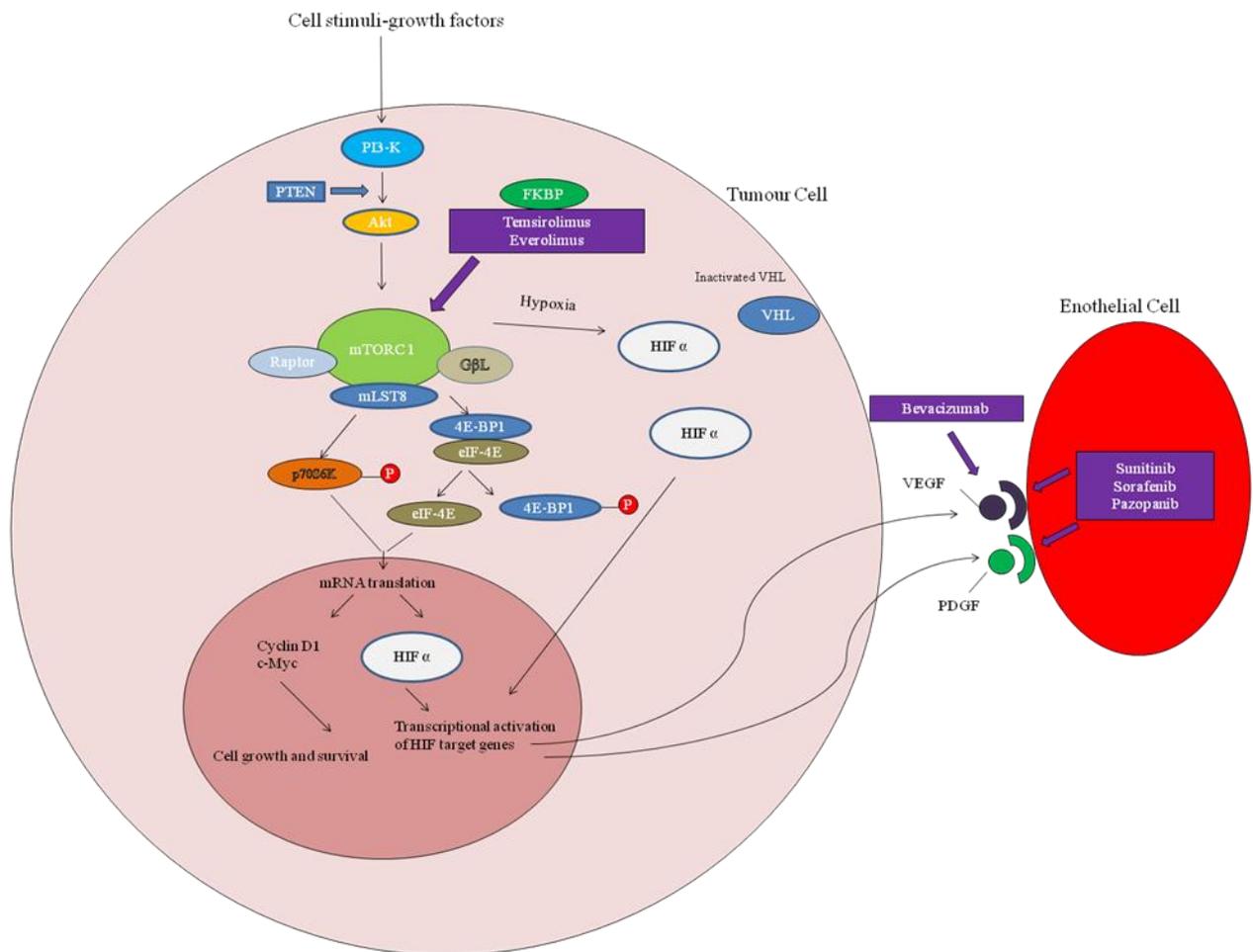


Figure 1.13 demonstrates the various pathways in the biology allowing targeted therapy. Loss of VHL gene results in the accumulation of angiogenic substances such as VEGF and PDGF. The mTOR pathway can promote cell survival and growth as well the accumulation of HIF.

The VHL gene is responsible for the degradation of hypoxia inducible factors [60] and if not broken down causes accumulation of substances which promote angiogenesis [61-63]. TKI inhibitors such as Sunitinib and Sorafenib have been demonstrated to convey additional survival in those with metastatic or recurrent renal cancer. Sunitinib, an oxindol

TKI which inhibits PDGF-receptor, VEGF-receptor, c-KIT and FLT-3 and has anti-tumour and anti-angiogenic activity has been demonstrated to result in longer progression free times and higher response rates [206-208]. Sorafenib, an oral multikinase inhibitor with activity against Raf-1 serine/threonine kinase, B-Raf, VEGF-receptor-2, PDGF-receptor, FLT-3 and c-KIT has been demonstrated to prolong progression free survival [204, 205]. Pazopanib and Bevacizumab are another two angiogenesis inhibitors. Pazopanib targets VEGF-receptor, PDGF-receptor and c-KIT and has been demonstrated to improve survival in those with metastatic cancer [310] whilst Bevacizumab which is a humanised monoclonal antibody to VEGF and has been demonstrated an increase in overall response in comparison to a placebo [63] and in those when combined with Interferon- α [311]. Temsirolimus and Everolimus are mTOR inhibitors. Temsirolimus has been demonstrated to improve survival by three months in those that are deemed high risk patients in comparison to immunotherapy [221] whilst Everolimus has shown progression free survival in comparison to placebo in those patients who have progressed despite commencing Sunitinib/ Sorafenib [222].

1.7 Src kinase family members

One potential molecular target in those to prevent progression to metastatic disease is the non-receptor tyrosine kinase Src, the first identified human proto-oncogene. The origins of the discovery of Src began in the 1900's when Rous proposed that viruses could cause cancer [312]. Material from centrifuged chicken sarcomas was injected into chicks that went on to developing sarcomas. The gene implicated, viral Src (v-Src), was identified [313] and it later transpired that this virus actually acquired the cancer causing gene from a normal cellular gene, c-Src (referred to as Src) [314-316]. Src has a role in signal transduction of multiple oncogenic cellular processes including migration, adhesion, invasion, angiogenesis, proliferation and differentiation and has significant interactions

with other cellular proteins such as growth factor receptors [317]. Src is the prototypical member of the Src kinase family (SFK), There have been 12 SFKs identified: Src, Fyn, Yes, Yrk, Lyn, Hck, Fgr, Blk, Lck, Brk, Srm, and Frk, 11 of which are found in humans [318-323]. Src, Fyn, and Yes are expressed ubiquitously, with concentration levels of Src being five to 200 times higher levels in platelets, neurons, and osteoclasts [324] whereas the others are relatively concentrated in hematopoietic cell lineages [325].

The role of SFK in various malignancies is well reported. Lyn is the predominant SFK expressed in normal B lymphocytes [323, 326] which are required for the production of antibodies to allow for the eradication of extracellular pathogens. Lyn has been implicated in haematological malignancies where levels of other SFK's were no different in these malignant cells in comparison to non-malignant cells [327, 328]. Lyn has also been implicated in various other malignancies such as colon, prostate and breast cancer [329-332] whilst inhibition of Lyn has a negative impact on proliferation on prostate cancer cells [333] as well as inhibiting tumour growth and metastases in Ewing's sarcoma [334].

It has been demonstrated that increased levels of Src are expressed in breast [335-341], pancreatic [342-347] prostate [348-350], colorectal [351-356], bladder cancers [357-362] as well as other malignancies such as skin, lung, liver and ovarian [363-369]. Furthermore activation of Src is associated with poor prognosis/ metastatic disease in various cancers [337, 340, 353, 368, 370-374] whereas in bladder cancer, Src expression and activity decreases with tumour stage [357-362]. This would suggest that Src expression/activity does not necessarily constitute poor prognosis in all cancers and the utilisation of Src inhibitors may not necessarily result in improved survival in patients. Furthermore it has been demonstrated that inhibition of Src reduces proliferation, invasion and migration of cancer cells [375-378].

1.7.1 Src Structure and Activation

SFK's share a conserved domain structure. Src kinase is composed of a C-terminal tail, kinase domain, two protein-protein interaction domains (SH2, SH3) and a unique amino-terminal domain that varies between SFK members (Figure 1.14).

Figure 1.14: Structure of Src.

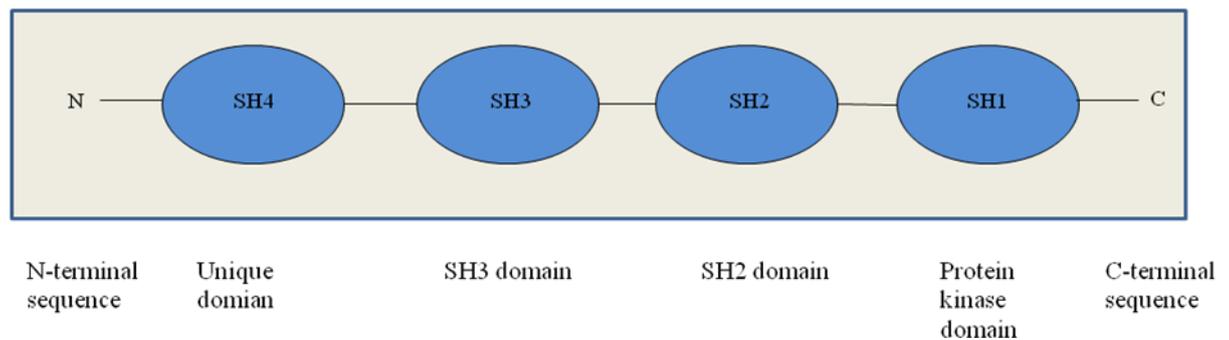


Figure 1.14 demonstrates a basic overview of the structure of Src.

The four Src homology domains (SH1-4) are involved in auto regulating SFK activity and form intracellular signalling complexes following interaction with substrates [379]. The N-terminal domain contains signals for lipid modification: myristylation (in all SFKs) and palmitylation (in all but Src and Blk) signals, both of which are required for membrane association of SFK with the N-terminal myristylation of Src, essential for the transformation of oncogenic Src mutants [380-382].

Activation of Src is highly dependent upon the phosphorylation state of various tyrosine residues on the SH domains. Classical activation of Src kinase occurs by an initial dephosphorylation of a conserved tyrosine residue in the C-terminal domain known as the negative regulatory region (Tyrosine (Tyr) 530) and followed by a subsequent autophosphorylation of the Tyr 419 site in the kinase domain [383, 384]. Both these events are required to occur before the kinase can be considered fully activated. In normal cells, the kinase activity of SFKs is negatively regulated by the phosphorylation of its C-terminal

regulatory Tyr residue by C-terminal Src kinase (Csk) [385, 386] and that Src is activated by dephosphorylation [387]. It has been identified that Tyr 527 in chickens (Tyr 530 in humans) in the C-terminal is the site of inhibitory phosphorylation [388] and that dephosphorylation at this site increases the activity of Src [389-392]. This would suggest that Src is normally present in an inactive form in which Tyr 530 site is phosphorylated. When Tyr 530 is phosphorylated, Src adopts an inactive configuration which is stabilised by binding of the Tyr 530 to its own SH2 domain and binding of SH2 kinase linker to the SH3 domain [379, 393-395] adopting a closed confirmation [396] (Figure 1.15). Upon dephosphorylation of Tyr 530, Src undergoes a transformational change exposing the Tyr 419 which is required to undergo autophosphorylation prior to Src being fully active [383, 384] (Figure 1.15).

Figure 1.15: Configuration of Src when inactive and active.

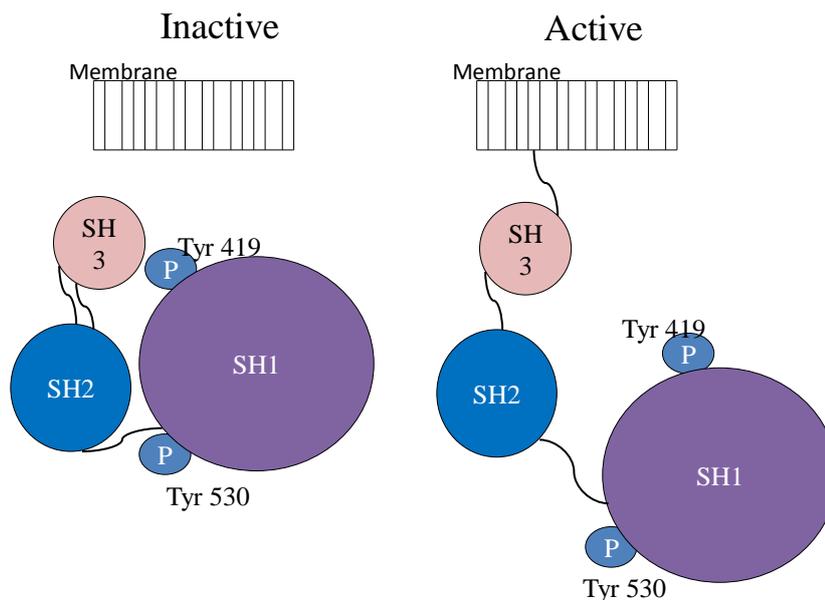


Figure 1.15 demonstrates changes in configuration when Src becomes activated following dephosphorylation of Tyr 530 and autophosphorylation of Tyr 416.

SFK's localisation has a preponderance to the cell cytoplasm with location suggesting Src inactivity [397]. Src localisation in the avian sarcoma virus has shown a preponderance to the membrane [398]. It has been demonstrated that the SH3 domain of inactive Src is localised to the perinuclear region and upon activation this domain is transported to the plasma membrane [399]. Furthermore activated Src kinase has been demonstrated to translocate to the cell membrane [318, 400-403] and therefore cellular location may also be employed as a surrogate marker of activation [404]. It has been demonstrated that membrane localisation of Src is associated with poor survival in pancreatic, breast and prostate cancers [335-337, 348, 405].

1.7.2 Src and tumourgenesis

Src has a role in multiple cellular processes in normal cells that do not result in tumourgenesis. It is through these same processes that results in cancer progression may occur through aberrant Src activation [318]. Src kinase has a role in signal transduction of multiple oncogenic cellular processes including migration, adhesion, invasion, angiogenesis, proliferation and differentiation and has significant interactions with other cellular proteins such as growth factor receptors [317]. Some of these cellular processes are discussed below.

1.7.2.1 Migration

Src is a key component in regulating cell motility as it is involved in the disruption of cell-cell contacts and increasing focal adhesion turnover [406]. Cortactin, a regulator of cell migration is an oncogene that is frequently amplified in a subset of tumours and tumour cell lines [407, 408]. Cortactin is a substrate of Src which has not only been demonstrated to promote the formation and stabilisation of the actin network that drives protrusion at the leading edge of migrating cells [409] but Src itself can directly regulate the activity of Cortactin [410]. Inhibition of Src has been demonstrated to decrease the formation of these

protrusions [411, 412] and as well as inhibiting the protrusions themselves [348].

Furthermore it has been demonstrated that during metastases, the cell-cell, cell-matrix contacts undergo changes which allow migration which is a Src dependant process [413]. It has been demonstrated that the invasive nature of cancer cells is regulated by Src as it inhibits cadherins which stabilise these cell-cell contacts as well as increasing the degradation of these proteins [414]. It has been demonstrated that over expressed and active Src increases the invasive capability of colon cancer cells and that Src inhibition decreased invasion [415]. Active Src results in the phosphorylation of downstream markers such as focal adhesion kinase (FAK) and paxillin which play a key role in the regulation of proliferation and migration of normal and tumour cells [416-420].

1.7.2.2 Angiogenesis

Vascular endothelial growth factor (VEGF) is an important angiogenic factor with significant effects on tumour angiogenesis which is essential for tumour development. The role of VEGF inhibitors in renal cancer is well established demonstrating survival benefit [204, 206, 310, 311, 421]. Src has been demonstrated to be involved in the regulation of VEGF expression [422] and that VEGF is a downstream target of Src suggesting a role of Src in promoting angiogenesis [423]. A report has demonstrated that VHL tumour suppressor gene is de-stabilised by Src [424]. VEGF has been demonstrated to mediate migration of colorectal cancer cells by activation of Src [425]. Src activation has been demonstrated to play a role in VEGF production and vascularisation in colon, breast and ovarian cancer cells [424, 426-428] and Src inhibition resulting in reduced VEGF expression [429]. Furthermore, VEGF mediated Src results in increased vascular permeability [430] as well as tumour cell extravasation and metastasis [431].

1.7.2.3 Proliferation/Apoptosis

It has been suggested that Src may regulate different stages of tumour growth in different tissues [319]. SFK inhibitors in breast cell lines has demonstrated that blocking Src activity results in the inhibition of proliferation [412] whilst in the case of prostate cancer, only those castrate resistant cell lines demonstrated the inhibition of proliferation following Src inhibition [348]. Cell death, termed anoikis, normally occurs following loss of cell-cell contact and detachment of these adherent cells from the extracellular matrix [416]. It has been demonstrated that Src activated cells do not undergo anoikis, as a result of the activation of PI3K and extracellular signal-regulated kinase 1 or 2 (ERK1/2) [432].

1.7.3 Src in Renal Cancer

In renal cancer, Src has been shown to contribute to the appearance of malignant phenotypes, particularly due to the resistance against apoptosis by Bcl-xL and angiogenesis stimulated by Src-STAT3-VEGF signalling [433]. The pleiotropic effects of Src activity are due to the multiple signal pathways engaged by Src and its accompanying kinases. Studies investigating the role and expression of Src and the downstream marker FAK are very limited. A case report has demonstrated that Dasatinib, a Src inhibitor, reduced the viability of a patients cultured renal cancer cells and demonstrated strong staining for Src [434] and that Src expression when utilising a random cut off value of 5% was associated with poor prognosis in renal cancer and renal cell lines demonstrated sensitivity to Dasatinib [435]. Despite these limited studies with obvious limitations, Src inhibitors are being utilised in clinical trials in those with renal cancer. We believe that further work is required not only to demonstrate if SFK's are expressed in renal cancer, whether assessment of the downstream marker FAK and those SFK's that are highly expressed correlates to prognosis and to assess if treatment of renal cancer with Src inhibitors causes any changes in the expression with those SFK that are highly expressed and the impact on the downstream marker FAK.

1.7.4 Downstream markers of SFK

When SFK's such as Src, Lyn and Fyn are phosphorylated; several downstream markers such as FAK are phosphorylated [436-438]. FAK is a non-receptor cytoplasmic tyrosine kinase that plays a key role in the regulation of proliferation and migration of normal and tumour cells. FAK was first identified in search of proteins that were tyrosine phosphorylated where it was demonstrated that they were present in Src transformed fibroblasts as a key substrate for Src oncoprotein [439]. It has been demonstrated that FAK is up-regulated/ activated in various malignancies by playing a role in cellular processes responsible for tumourgenesis [417, 440, 441], whilst several studies have demonstrated a further role for FAK in cancer progression and invasion. Elevated FAK phosphorylation has been observed in several cancers, including breast, endometrial, colon, thyroid, prostate, liver and ovarian [369, 442-444]. Deletion of FAK from cancer cells has resulted in decreased tumour progression [445, 446] whilst quantitative real time PCR has demonstrated elevated levels of FAK expression in gastrointestinal stromal tumours [447]. Increased FAK expression was not found in oesophageal cancer but correlated with tumour invasiveness and lymph node metastases [448], whilst in metastatic prostate cancer, it has been demonstrated that elevated levels of mRNA of FAK are expressed [449]. In vitro evidence has demonstrated that Src-FAK signalling is associated with elevated tumour cell metastases and cell invasion [448, 450]. A positive feedback loop exists between Src and FAK in which both increase the activation phosphorylation of the other [451] thereby demonstrating a relationship between the two.

Several sites of phosphorylation have been identified in FAK which modulate FAK activity including the major autophosphorylation site Y³⁹⁷ [452]. Autophosphorylation of FAK at the Y³⁹⁷ site occurs as a result of many stimuli thereby creating a high affinity binding site for the Src homology 2 domain of several proteins including Src kinase [453, 454]. Src phosphorylates FAK at several other sites as well including Y⁴⁰⁷, Y⁵⁷⁶, Y⁵⁷⁷, Y⁸⁶¹ and Y⁹²⁵ [453-456] but it has been reported that the Y⁸⁶¹ is the major site on the FAK

domain associated with activation [455, 457, 458], and therefore maybe used as a surrogate marker not only for SFK activation but also for SFK function [459]. Furthermore, Src inhibition has resulted in significant reduction of FAK at Y⁸⁶¹ [458] leading to some suggest that downstream markers of SFK activation such as FAK could therefore act as biomarkers for SFK activation [459].

1.7.5 SFK Inhibitors

Given the role of SFK in tumourgenesis, this has made them important targets for therapeutic intervention. Various inhibitors are currently utilised either in trials in those with cancer or have demonstrated efficacy in progression free survival. Below is an overview of the various inhibitors available.

Dasatinib is a tyrosine kinase inhibitor of Abl, Src and SFKs [378]. It is approved in those with chronic myeloid leukaemia [460] and is utilised as first line therapy. Whilst inhibition of lung cancer cell lines has been demonstrated with Dasatinib [461], results of phase II trials in lung cancer have demonstrated variable results with some showing a modest result at best with clinical activity less than chemotherapy [462, 463] and another phase II trial being terminated due to lack of efficacy [464]. Dasatinib has been demonstrated to reduce prostatic cancer growth and lymph node metastases in a mouse model [465, 466].

Treatment with Dasatinib has demonstrated inhibition of the downstream marker FAK in prostate cancer cell lines [459] as well as inhibiting phosphorylation of Src and FAK in hormone sensitive and naive cell lines [348]. Dasatinib treatment of those with prostate cancer in a phase I/II trial has demonstrated a high objective response [467]. Treatment in colorectal cancer cell lines has demonstrated reduced cell growth [468, 469] whilst in a phase II trial, patients treated with Dasatinib showed it to be ineffective as a single agent [470]. In melanoma cell lines, treatment has demonstrated anti-proliferative and anti-invasive effects [471, 472] with results from a phase I trial suggesting favourable survival to historical data [473]. Treatment of breast cell lines demonstrated inhibition of growth

[338, 474, 475] whilst inhibition with Dasatinib in phase II trials demonstrated limited activity [476, 477]. Treatment of those with ovarian cancer or intraperitoneal cancer in a phase II trial with Dasatinib has demonstrated minimal activity [478] and demonstrated no activity in those with mesothelioma in a phase II trial [479]. Results of Dasatinib treatment of hepatocellular carcinoma cell lines have demonstrated the induction of cell cycle arrest and apoptosis [480, 481] whilst in pancreatic cancer cell lines, treatment resulted in inhibition of cellular proliferation, migration and invasion [405]. In regards to RCC, there is one case report demonstrating that Dasatinib treatment reduced the viability of the patients cancer cells [434].

Saracatinib is a dual Src/Abl kinase inhibitor [482]. Treatment on melanoma cancer lines has demonstrated an inhibitory effect [483] whilst a phase II clinical trial has demonstrated it to have minimal activity on patients [484]. Treatment with Saracatinib on gastric cancer cell lines has demonstrated a reduction in anti-tumour activity [485, 486] whilst a phase II trial showed it to have insufficient activity [487]. Phase II trials investigating treatment in breast and head/neck cancers demonstrated such little efficacy that it was felt that there was no justification for continuation [488, 489] whilst a phase I/II trial in pancreatic cancer showed no improved efficacy in comparison to current treatment available [490] and a phase II trial with prostate cancer demonstrated little clinical efficacy [491]. Saracatinib has showed efficacy when utilised on various other cancer cell lines such as biliary tract [492], ovarian [366], lung [493] and colorectal cancers [494] whilst evidence has demonstrated that it may be of therapeutic benefit in those with metastatic disease [495]. Regarding RCC, there is no evidence demonstrating its efficacy, yet a phase II trial (COASK) has been undertaken the results of which are not yet available.

Bosutinib originally identified as a Src/Abl inhibitor is a multikinase inhibitor [496]. Bosutinib has been demonstrated to inhibit invasion, growth and metastases in breast cancer cells as well as inhibiting the downstream marker FAK [497, 498] whilst a phase II trial in those with locally advanced or metastatic breast cancer has demonstrated promising

efficacy [499]. Treatment in a mouse model of thyroid cancer demonstrated treatment resulted in reduced tumour growth, invasion and metastases [500] whilst treatment of prostate cancer cell lines has shown a reduction in cellular migration, proliferation and invasion [501] and reduced wound healing in colorectal cancer cell lines [502].

KX2-391 is a highly selective Src substrate binding site inhibitor [503]. Studies demonstrating its efficacy are limited with evidence demonstrating inhibition of breast cancer cell lines [503] as well as a reduction of breast metastases in xenograft models [504]. A phase II trial investigating its role in prostate cancer failed to show any anti-tumour activity [505].

AP 23846 is a specific Src inhibitor which has been demonstrated to affect the properties of tumour progression in pancreatic and ovarian cancer cell lines [429, 506].

1.8 Hypothesis and aims

The role of SFK's in the development and progression of cancer is well known and documented in literature. Despite the evidence of SFK's in malignancies, there is paucity in translational evidence in the role in RCC and phase II trials have commenced without identification of biomarkers to guide treatment or of the effect of the various SFK's in RCC.

We hypothesise that SFKs are involved in promoting metastatic spread of RCC and that by furthering the understanding of their role in RCC we will demonstrate that they are a RCC therapeutic target.

Project Aims

1. To assess whether utilisation of known prognostic markers in solid malignancies can be applied in RCC.

2. To identify which Src family kinase is expressed in RCC and assesses their clinical significance.
3. To select and optimise antibodies for the detection of the Src family kinase that is expressed in RCC and the downstream marker FAK utilising full tissue sections and assessing if staining intensity and cellular localisation correlate to clinical parameters.
4. To determine if SFK are associated with promoting proliferation, apoptosis or migration in RCC cell lines and assess the effect of inhibition with Dasatinib, a non-selective SFK inhibitor on Src family kinase expression, expression of the downstream marker FAK and the effect on proliferation and apoptosis in RCC cell lines.
5. To assess if silencing Src kinase in RCC cell lines alters the cells response to Dasatinib via expression of downstream marker FAK and the effect on proliferation and apoptosis on RCC cell lines.

By addressing these aims it is hoped that this project will identify novel prognostic markers of use in RCC, validation of SFKs as therapeutic targets for RCC and also identify predictive markers for Src inhibitors, allowing these drugs to provide maximum patient benefit.

Chapter 2-Role of prognostic markers used in other solid tumours, can they be applied to renal cancer?

Currently, the TNM stage and tumour grade are the most widely used tools to predict survival. Various grading classifications for RCC based on morphological features have been proposed [83, 136-141] and of these the Fuhrman grading system [138] has achieved widespread usage in pathology practise. The Fuhrman grading system has been demonstrated to be an independent predictor of survival [127] having been acknowledged as optimal for predicting outcome [144] and therefore has been incorporated into the majority of prognostic algorithms including SSIGN [145], UISS [146] and Leibovich [147].

The Fuhrman grading system is based on assessment of the uniformity of nuclear size, nuclear shape and nucleolar prominence [138]. The Fuhrman grading system has been demonstrated to correlate to metastasis with grade 1 tumours having a statistically significant lower metastases rate compared to those with grade 2 to 4 and survival rates being distinguished into 3 categories, those with grade 1, those with grade 4 and those with grades 2 and 3 [138]. Despite the popularity of this grading system, problems have been demonstrated regarding its application [137, 151, 507].

There have been suggestions that the Fuhrman grading system has low-moderate inter-observer agreement [148-151] and that a simplified system improves inter-observer agreement [148, 149] as well as demonstrating as much accuracy as the conventional grading system [152, 153]. Furthermore there are those suggesting that the ideal grading system is yet to be defined and should consist of three tiers [151] whilst a three tiered system has been shown to be an independent predictor of survival [154, 155].

There has been a long standing interest in identifying those patients most at risk of disease progression and ultimately dying from their disease. Ideally, a factor or combination of factors that could clearly stratify patients into those who do not progress and those that progress and are at a higher risk of dying from their cancer would be highly beneficial. Currently, the TNM stage which incorporates the size or invasion of the tumour as well as evidence of nodal involvement and distant spread of the cancer is amongst the widely used tool to predict survival in many cancers including renal cancer. This is utilised with surrogate markers and incorporated in prognostic algorithms for renal cancer including SSIGN [145], UISS [508] and Leibovich [147].

It is now established that disease progression in cancer patients is not solely determined by the tumour characteristics but also by the host response. There is increasing evidence that both local and systemic inflammatory responses play an important role in the progression of various solid tumours [174, 175]. When inflammation does not resolve, it promotes tumour cell growth, survival and angiogenesis as well as playing a critical role in the metastases of cancers [176]. When attempts are made to reduce this inflammation by the administration of non-steroidal anti-inflammatory drugs, it has led to the reduction in colon and lung cancers [174, 177-179].

Evidence suggests that intensity of local inflammatory infiltrate within the tumour bed predicts prognosis [180]: a pronounced lymphocytic infiltration in colorectal cancer is associated with improved survival [181-183]. Also quantifying the degree of infiltration by lymphocyte subsets such as CD8+ and CD4+ T cells provides prognostic information in various tumour types [184, 185] including renal cancer [186]. The process of assessing lymphocyte infiltration is time consuming and has not been adopted into routine clinical practice. It is therefore of interest that Klintrup and colleagues have reported a simplified method of assessing the inflammatory cell infiltrate at the tumour margin [187], showing on routine haematoxylin and eosin stained sections, that tumour inflammatory infiltrate, including all white cell types, can be graded high or low grade. It has been shown that a

high grade infiltrate is associated with improved survival in colorectal cancer [187, 188] and gastroesophageal cancer [189].

In addition, increasing evidence supports a role of a pre-treatment systemic inflammatory response, indicated by elevated levels of CRP being an independent predictor of survival in patients with a variety of common solid tumours including gastrointestinal, lung, prostate, bladder cancers [194, 196-199]. There is now increasing evidence of the prognostic role that preoperative CRP has in renal cancer [509-513]. It has been shown that when CRP is combined with tumour stage, ECOG status (Eastern Co-operative Oncology Group) or albumin concentrations, the combination of CRP with albumin is more prognostic than the combination of CRP with the other two clinical parameters in those with lung cancer [202]. This has led to the development to the development of the modified Glasgow Prognostic Score (mGPS), which incorporates preoperative CRP and albumin serum levels [200]. This score, briefly, comprises a score of 2 in those patients with both an elevated CRP (CRP >10mg/l) and hypoalbuminaemia (<35g/l), patients with only an elevated CRP (CRP >10mg/l) a score of 1 and in those with a normal CRP (<10mg/l) a score of 0. The mGPS score has provided additional prognostic information in patients with various solid malignancies including lung, gastroesophageal and colorectal cancers [201-203].

Tumour necrosis occurs when tumours outgrow their blood supply therefore reflecting aggressive tumour biology and rapid proliferation and progression [156]. Tumour necrosis has been investigated in malignancies such as breast, colorectal and renal cancer. It is often assessed reviewing haematoxylin and eosin stained sections at low magnification using a semi-quantitative method to classify necrosis as simply being absent or present. Some studies investigating necrosis in breast cancer have reported that necrosis is independently associated with tumour recurrence [157] and overall survival [158] whilst others have suggested that necrosis predicts survival but is not an independent factor [159, 160].

Despite using a quantitative method to classify necrosis in breast cancer with a 15 year

follow up, necrosis was not associated with overall survival [161]. Evidence investigating the role of tumour necrosis in colorectal surgery is more supportive. Necrosis has been shown to be an independent predictor of disease specific survival in colorectal cancer of various stages [162, 163] as well as lung cancer [514]. Studies investigating the role of necrosis in organ confined disease in colorectal cancer, a group that would have the greatest benefit of additional prognostic information being utilised, have shown conflicting results with one study showing no relationship between necrosis and survival [164] whilst in another it was shown to be an independent predictor of survival [165].

The role of tumour necrosis has been investigated in renal cancer with some conflicting results. The majority of studies have shown that necrosis adds prognostic information as it is associated with reduced disease specific survival, recurrence and progression to metastatic disease [145, 147, 166-168] whilst others have shown either no correlation or no independent relationship between necrosis and outcome [137, 169-172]. Despite conflicting results regarding the significance of necrosis in renal cancer, a semi-quantitative method of assessment of this pathological parameter is incorporated into prognostic algorithms such as SSIGN [145] and Leibovich [147] scores.

Necrosis is evaluated on a simple absence/presence basis and this therefore does not take into account the extent of necrosis. It has been suggested that an extent based classification is superior to a simple absence/presence response and was retained as an independent prognostic factor in renal cancer [173].

It is felt that the incorporation of molecular markers into prognostic algorithms will increase their predictive accuracy. A wide variety of molecular markers have been shown to influence prognosis in renal cancer including cell cycle regulators, mediators of cellular proliferation and apoptosis.

Ki 67 has been investigated in renal cancer; some studies have shown this marker to be associated with increased nuclear grade and a predictor for disease specific survival [216,

218, 242, 243, 246-251] whilst in other studies it has not been shown to add prognostic information [252-255]. Apoptosis on the other hand is also a highly regulated cellular process but is involved in maintenance of tissue homeostasis and elimination of unwanted cells [256]. Acquired resistance to apoptosis is thought to be one of the salient features of cancer [257]. Apoptosis has been shown to be a prognostic marker for survival in renal cancer as it has been shown to correlate to tumour grade, stage and size [258-260] as well as being independently associated with survival [261]. While it has been suggested that apoptosis may be a prognostic marker in its own right, others have shown it to either have no correlation to established prognostic factors or it to be an independent predictor of survival [236, 262-265]. The TUNEL method is a well-established method of assessing apoptosis in renal cell cancer [259, 260, 263-265].

Despite varying degrees of evidence in the literature demonstrating that molecular markers can aid in prognosis and guide treatment strategies, efforts have been made to utilise molecular markers in prognostic algorithms [216, 218, 266] but these are not currently utilised in clinical practise.

Given the evidence suggesting that a simplified system improves the prognostic ability of the Fuhrman grading, that the inflammatory response could provide prognostic information, quantifying the extent of tumour necrosis is superior to a simple presence/absence response and that molecular markers of proliferation/apoptosis can aid prognosis, we aim to assess these various aspects in renal cancer and to examine for any correlations to disease specific survival.

2.1 Materials and Methods

Patients with RCC were included for this study. These patients had undergone resection based on the surgical findings and the results of CT scans for staging purposes between

January 1997 and Dec 2007 in the North Glasgow NHS Trust. The Research Ethics Committee of West of Scotland has approved the study.

Various cohorts were identified to investigate the various prognostic factors. Two hundred and thirty seven patients with clear cell renal cancer were identified retrospectively that underwent nephrectomy for which nuclear grading was to be investigated. Archival slides were not available for all these patients from which the inflammatory response and tumour necrosis could be investigated. Seventy nine patients were identified for whom archival slides were available for examination as well preoperative CRP and albumin levels allowing the inflammatory response to be investigated. Due to time constraints, tumour necrosis was only evaluated in forty seven patients. The study cohorts constituted a representative sample of all surgically treated patients within this period.

Clinicopathological data including T stage, nuclear grade assessment [138] and survival for each patient was collected. Survival was determined from the time of diagnosis to the time of last follow up or death. The cause of death was determined by linkage through the Scottish Cancer Registry. In those who were deceased, if the primary cause of death was of renal cancer, these were classed as cancer specific and all other causes were non-cancer specific deaths.

The original Fuhrman grading system was investigated as well as various simplified systems utilising the Fuhrman grade. Table 2.1 shows the various simplified models that were investigated.

Table 2.1: Demonstrating the various simplified grading systems investigated.

<i>Fuhrman Grading System</i>	<i>1</i>	<i>2</i>	<i>3</i>	<i>4</i>
Model 1	1	1	2	2
Model 2	1	2	2	2
Model 3	1	1	1	2
Model 4	1	2	3	3
Model 5	1	1	2	3

Table 2.1 demonstrates how the different simplified grading systems were configured from the original Fuhrman grading system.

For investigating the role of inflammation, diagnostic haematoxylin and eosin sections from pathology archives were reviewed. A minimum of three slides from the deepest area of tumour invasion were reviewed and were scored according to the Klintrup-Makinen criteria (K/M) [187]. This method is based on scoring inflammation at the deepest point of invasion identified from the three slides. A four point scale was used. A score of 0 was given for no increase of the inflammatory cells at the invasive margin; a score of 1 denoted a mild and patchy increase of inflammatory cells. Score 2 was assigned when inflammatory cells formed a band-like infiltrate at the invasive margin. A score of 3 was given when a prominent inflammatory reaction formed a cup-like zone at the margin. Scores of 0 and 1 were combined (low grade inflammation) and scores of 2 and 3 combined (high grade inflammation).

Preoperative systemic inflammatory response was assessed using the modified Glasgow Prognostic Score (mGPS) [200]. Patients with both elevated C-reactive protein (>10mg/l) and hypoalbuminaemia (<35g/l) scored 2. Patients in whom both were normal scored 0. Patients with elevated C-reactive protein alone were scored as 1 while those with hypoalbuminaemia alone were scored as 0.

Investigating the role of tumour necrosis, routine haematoxylin and eosin stained sections were reviewed from pathology archives. Tumour necrosis was evaluated on histological sections and was graded accordingly. This was performed according to established histological criteria [156, 515]. Necrosis related to haemorrhage and foci of hyalinization was not considered. The extent of necrosis was graded with 0 when no necrosis was present; a score of 1 given when there was <25% necrosis, a score of 2 when necrosis was 25-50% and 3 for necrosis >50%.

Investigating the role of molecular markers, fifty seven patients with RCC were identified retrospectively that underwent nephrectomy and for whom pathological blocks were available for examination.

Immunohistochemistry (IHC) was utilised to assess the proliferation and apoptosis indices of these renal cell carcinomas. All IHC was performed on 5µm archival formalin fixed paraffin embedded full RCC tissue sections.

2.1.1 Proliferation Assessment

2.1.1.1 Tissue Preparation

Sections were dewaxed in xylene (2x4 minutes) and rehydrated through a series of alcohol (100 % (2x2minutes), 90 % (1x2minutes), 70 % (1x2minutes)) washes.

2.1.1.2 Antigen Retrieval

During the formalin fixation process, methylene bridges form which can cross link proteins and mask antigenic sites. By performing antigen retrieval, this allows the cross links to be broken exposing the antigen binding site. A heat mediated method was utilised for antigen retrieval. A TE buffer (1mM EDTA (Sigma), 5mM Tris (VWR) at pH 8 was utilised. This solution were pre-heated for 13.5 minutes to a temperature of 96°C prior to the tissue slides being placed for 5 minutes in the solution and pressure cooked followed by a cooling down period of 20 minutes.

2.1.1.3 Reducing Background Staining

Background staining results due to the presence of endogenous peroxidase activity in tissue. This endogenous activity is blocked by incubating the slides in 3% hydrogen peroxide (H₂O₂) for duration of 10 minutes followed by washing the slides in water.

Another cause of background staining is due to the formation of hydrophobic bonds between antibodies and the tissue resulting in non-specific staining between primary and secondary antibodies rather than the target protein. To reduce this non-specific staining, tissue slides were incubated in 5% horse serum with antibody diluent for 20 minutes.

2.1.1.4 Primary Antibody Incubation

Prior to staining for Ki-67 (a mouse monoclonal antibody (Dako, Cambridgeshire, UK)) at a concentration of 1:150, it was imperative to establish optimum conditions for antigen staining. This was done by performing a series of investigations on RCC tissue by varying various factors such as antigen retrieval, antibody solutions, incubation times, and temperatures allowing the strongest specific antigen staining with the lowest background staining. It was crucial to have both a positive and negative control in the chosen methodology. The positive control ensured that the methodology was not only working but there was limited variation between runs for the same antibody and the negative control ensured the specificity of the antibody.

2.1.1.5 Secondary Antibody Incubation

Following incubation with the primary antibody or the negative isotype matched control, the tissue slides were washed with TBS twice for 5 minutes. Following this, the DAKO Envision System was utilising allowing the detection of the protein of interest. The tissue slides were incubated with Envision at room temperature for 30 minutes following which they were again thoroughly washed with TBS twice for 5 minutes.

2.1.1.6 Detection and Visualisation

The chromagen used for staining the tissue sections was DAB (3,3'-diaminobenzidine) which was a combination of 5ml distilled water (dH₂O), 2 drops of DAB buffer solution, 4 drops of DAB substrate solution, and 2 drops of Hydrogen Peroxidase solution (Vector Laboratories). Slides were incubated with DAB for ten minutes to allow staining to develop and then washed in running water for ten minutes.

2.1.1.7 Counterstaining

Tissue sections were counterstained with haematoxylin and Scots Tap Water Substitute (S.T.W.S). Slides were submerged in the haematoxylin for approximately thirty seconds resulting in a red colour on the tissue section. The slides were then submerged in acid alcohol to remove excess colouring. Following this, slides were then submerged in S.T.W.S for another thirty seconds producing a blue colour which was in contrast to the brown positive staining at the site of the protein.

2.1.1.8 Dehydrating and mounting of slides

The last steps result in dehydrating the tissue through a series of alcohol washes, (70% (1x1minutes), 90% (1x1minutes), 100 % (2x1minutes)) and xylene (2x1minutes). The slides were then mounted onto coverslips using DPX mountant.

2.1.2 Apoptosis Assessment

The TUNEL method was utilised for the assessment of apoptosis. This method utilises the ability to label free 3'OH termini that are localised in apoptotic bodies.

2.1.2.1 Tissue Preparation

Tissue was prepared as described earlier for the proliferation.

2.1.2.2 Pre-treatment of tissue

To allow improvement of exposure, slides were incubated with Proteinase K (20µg/mL TBS) for 15 minutes thus allowing exposure of DNA by digesting DNA-binding proteins after which they were washed in dH₂O twice for 2 minutes.

2.1.2.3 Reducing Background Staining

This was performed as described earlier with 3% H₂O₂ for 5 minutes followed by two washes in water for 5 minutes.

2.1.2.4 Detection of 3'OH termini

Working strength TdT enzyme is applied to each slide (70% reaction buffer: 30% TdT enzyme) and the slides incubated at 37°C for one hour followed by incubation with the stop/wash buffer for 10 minutes, the negative control is not incubated with the TdT enzyme, only with equilibration buffer. Following this, the slides are washed three times in TBS for one minute.

2.1.2.5 Allowing visualisation of staining

The fragments which have been labelled now require binding to an anti-digoxigenin antibody that allows a permanent stain from the chromogenic substrates. The slides are incubated for 25°C for 30 minutes and then washed four times in TBS for 2 minutes.

2.1.2.6 Detection and Visualisation

This was performed as described earlier.

2.1.2.7 Counterstaining

This was performed as described earlier.

2.1.2.8 Dehydrating and mounting of slides

This was performed as described earlier.

2.2 Statistical Analysis

Statistical analysis was undertaken using a statistical software package SPSS (Chicago, IL, USA). Correlations with clinical and pathological parameters were performed using a chi square test (χ^2). The proliferation index (PI) and apoptotic index (AI) was obtained as a ratio of Ki-67 and TUNEL positive cells respectively relative to the total number of counted cells and calculated from observations of at least 1000 cells in each section. This

was performed by two independent observers (TQ and PM). Agreement between observers was excellent and measured in interclass correlation coefficient (ICCC). For the purpose of analysis, median values were utilised as a cut-off mark, values below this value were given a low score and those equal and above the median a high score. Disease specific survival rates were generated using the Kaplan-Meier method. The log rank test was utilised to compare significant differences between subset groups using univariate analysis.

Multivariate analysis was carried out based on the results of the univariate analysis.

Multivariate Cox regression analysis was performed to identify those factors that were independently associated with disease specific death. A stepwise backward procedure was utilised to ascertain which of the variables had a significant independent relationship with survival.

2.3 Results

2.3.1 Nuclear Grading

Two hundred and thirty seven patients were studied. The patient characteristics are shown in Table 2.2. The median follow up was 69 months (range 2.1-181). The median age was 60 years (range 23-86). Thirty three patients died of their disease. Within this cohort, the most common tumour stage was T1 (47%). The most common Fuhrman grades were II (36%) and III (41%).

Table 2.2: Relationship between clinicopathological characteristics and cancer specific survival.

<i>Variable</i>	<i>Numbers</i>	
		p-value
Age (<60/>60)	108/129	0.918
T Stage (1/2/3/4)	112/35/85/5	<0.001
Nuclear Grade (1/2/3/4)	25/86/97/29	0.005
Recurrence (No/Yes)	178/59	<0.001

Table 2.2 demonstrates that pathological characteristics were predictors of cancer specific survival.

Univariate analysis of potential predictors of cancer specific survival showed that the majority of the grading models were statistically significant predictors of cancer specific survival (Table 2.3). On multivariate analysis of those that were significant on univariate analysis, only model 5 which is a modified three tiered model combining grades 1 and 2 whilst grades 3 and 4 are kept as separate was found to be an independent prognostic factor in its association with cancer specific survival ($p=0.001$, HR 2.17, 95% CI 1.37-3.43, Table 2.3, Figure 2.1).

Table 2.3: Relationship between the various simplified nuclear grading systems and cancer specific survival.

<i>Variable</i>	<i>Numbers</i>	<i>Univariate Analysis</i>	<i>Multivariate Analysis</i>	
		p-value	p-value	HR
Nuclear Grade (1/2/3/4)	25/86/97/29	0.005		
Model 1 ((1+2)/(3+4))	111/126	0.008		
Model 2 ((1/(2+3+4))	25/212	0.237		
Model 3 ((1+2+3)/4)	208/29	0.002		
Model 4 (1/2/(3+4))	25/86/126	0.029		
Model 5 ((1+2)/3/4)	111/97/29	0.002	0.001	2.17 (1.37-3.43)

Table 2.2 demonstrates that the simplified grading system whereby grades 1 and 2 are combined and grades 3 and 4 are kept separate was the only independent predictor of cancer specific survival.

Figure 2.1: Kaplan Meier demonstrating Model 5 of the simplified system of classification for nuclear grading.

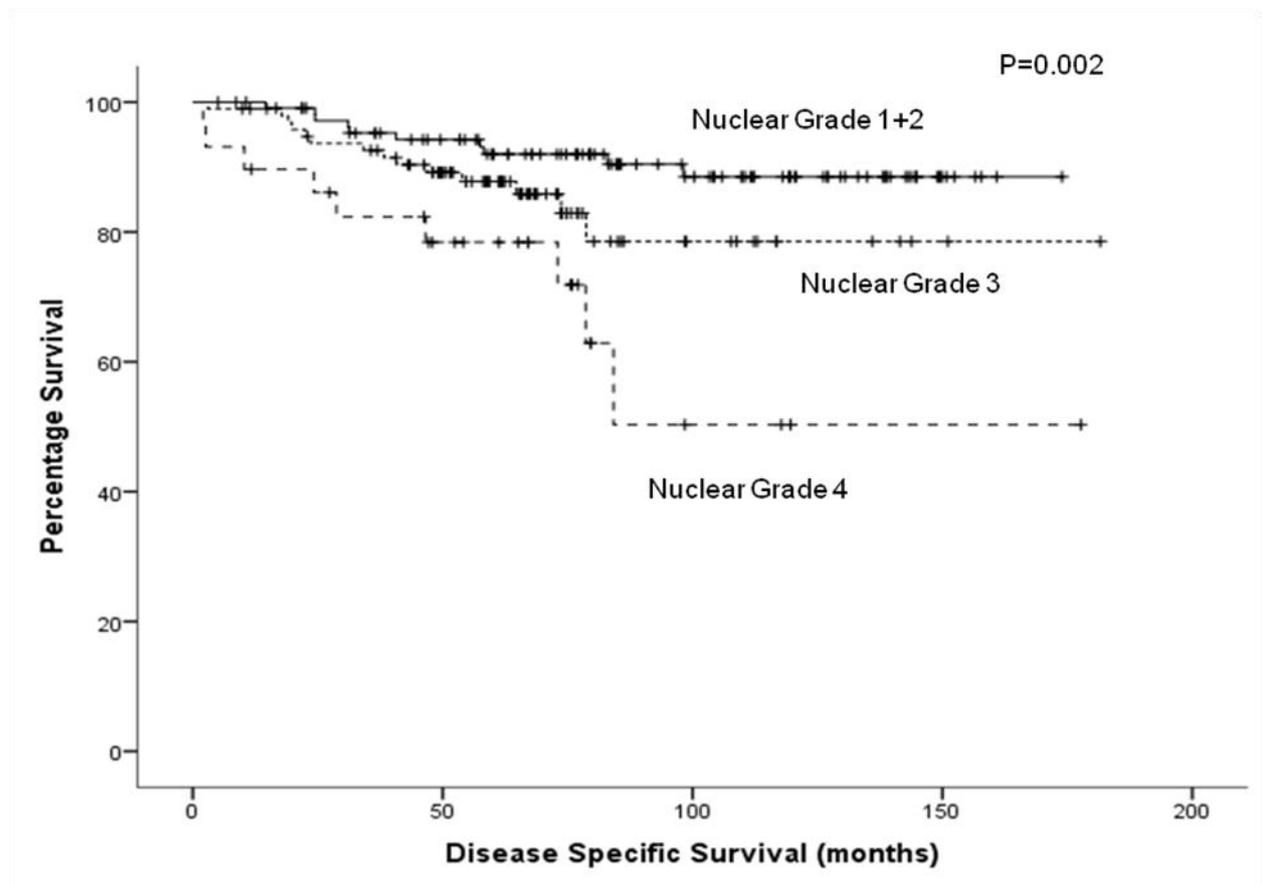


Figure 2.1 demonstrates the most prognostic simplified classification based on the original Fuhrman grading system ($p=0.002$).

On χ^2 analysis of the various simplified grading models, whilst majority of the grading models demonstrated a positive correlation with T Stage (Table 2.4), model 3, which is a two tiered model combining grades 1, 2 and 3 whilst grade 4 is kept separate demonstrated the strongest correlation to T Stage ($p<0.001$, Table 2.4). When analysing the grading models, the majority of these demonstrated a positive correlation to recurrence, whilst models 3 and 5 demonstrated the strongest correlation to this clinicopathological factor ($p<0.001$, Table 2.4).

Table 2.4: Interrelationships between clinicopathological characteristics of patients and various simplified nuclear grading systems.

Variable	Numbers	T Stage	Recurrence
Nuclear Grade (1/2/3/4)	25/86/97/29	0.001	0.001
Model 1 ((1+2)/(3+4))	111/126	0.029	0.01
Model 2 ((1/(2+3+4))	25/212	0.07	0.278
Model 3 ((1+2+3)/4)	208/29	<0.001	<0.001
Model 4 (1/2/(3+4))	25/86/126	0.015	0.016
Model 5 ((1+2)/3/4)	111/97/29	0.001	<0.001

Table 2.4 demonstrates that the various grading systems have relationships to T stage and evidence of recurrence at follow up.

2.3.2 Inflammation

Seventy nine patients were studied. Median age at diagnosis was 60 years (range 39-82).

Median follow up was 93 months (range 0.1-152). Nineteen patients died of their disease.

Forty six patients had T1/2 disease and thirty three patients had T3/4 disease. Forty

patients had evidence of recurrence on radiological imaging.

χ^2 demonstrated that mGPS positively correlated with tumour stage, grade and necrosis

($p=0.001$, $p=0.044$ and $p=0.042$ respectively, Table 2.5).

Table 2.5: Interrelationships between clinicopathological characteristics of patients with renal cancer.

<i>Variable</i>	<i>Numbers</i>	<i>Sex</i>	<i>Grade</i>	<i>T Stage</i>	<i>Tumour Necrosis</i>	<i>Local inflammatory cell infiltrate</i>	<i>mGPS</i>
Age (<65/>65)	40/39	0.316	0.954	0.423	0.291	0.831	0.054
Sex (male/female)	47/32		0.15	0.164	0.233	0.241	0.713
Grade (1/2/3/4)	9/37/21/12			0.005	0.315	0.59	0.044
T Stage (T1/T2/T3/T4)	32/14/29/4				0.166	0.595	0.001
Tumour Necrosis (absence/presence)	39/40					0.194	0.042
Local inflammatory cell infiltrate (low/high)	62/17						0.755
mGPS (0/1/2)	57/19/3						

Table 2.5 demonstrates that mGPS had a positive correlation with T stage and nuclear grade, both of which are recognised prognostic factors in renal cancer.

On univariate analysis, T Stage ($p < 0.001$), Grade ($p = 0.044$) and mGPS ($p < 0.001$, Figure 2.2) were significant predictors of disease specific survival whilst local inflammatory response and necrosis did not show significance ($p = 0.152$ and $p = 0.122$, Table 2.6). On multivariate analysis of the significant individual covariates, mGPS (HR 8.64, 95% CI 3.5-21.29, $p < 0.001$) was a significant independent predictor of disease specific survival (Table 2.6).

Figure 2.2: Kaplan Meier survival graph for mGPS against disease specific survival.

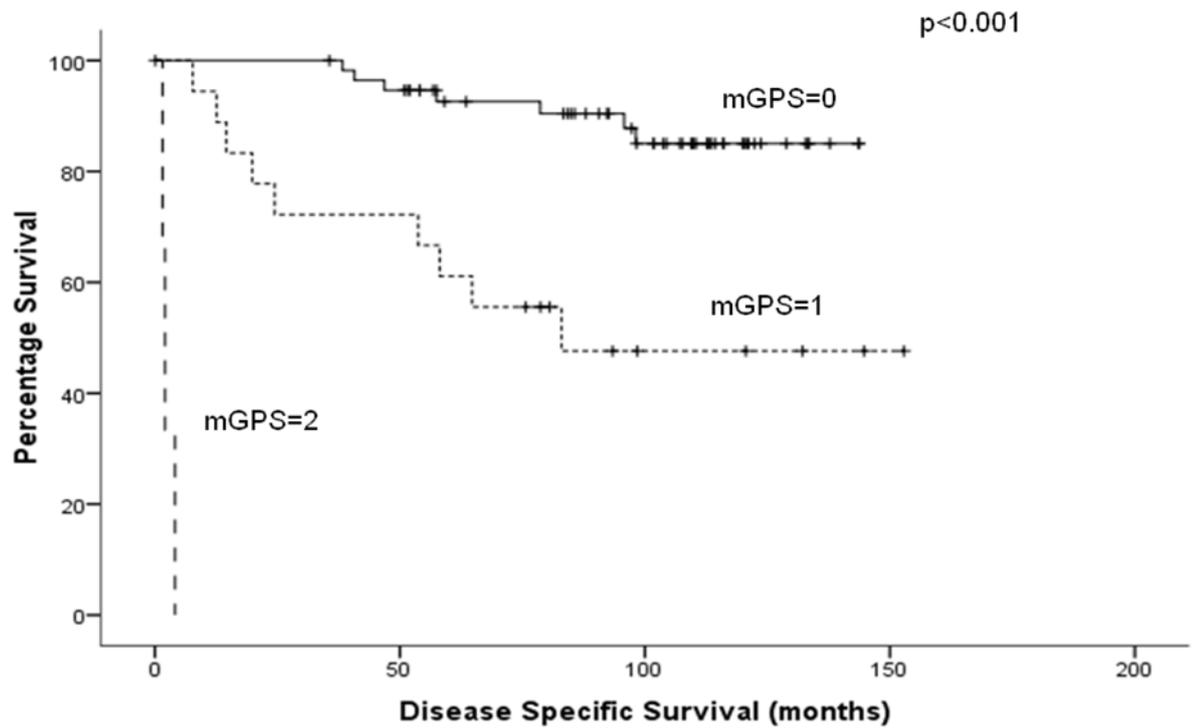


Figure 2.2 demonstrates that an elevated mGPS was significantly associated with disease specific survival ($p < 0.001$).

Table 2.6: Relationships between clinicopathological characteristics and disease specific survival.

<i>Variable</i>	<i>Numbers</i>	<i>Univariate Analysis</i>	<i>Multivariate Analysis</i>	
		p value	p value	HR
Age (<65/>65)	40/39	0.838		
Sex (male/female)	47/32	0.259		
Grade (1/2/3/4)	9/37/21/12	0.044		
T Stage (T1/T2/T3/T4)	32/14/29/4	<0.001		
Tumour Necrosis (absence/presence)	39/40	0.122		
Local inflammatory cell infiltrate (low/high)	62/17	0.152		
mGPS (0/1/2)	57/19/3	<0.001	<0.001	8.64 (3.5-21.29)

Table 2.6 demonstrates the relationships between clinicopathological characteristics, tumour necrosis, local inflammatory response and disease specific survival.

2.3.3 Tumour Necrosis

Analysis was based on forty seven patients. Median age at diagnosis was 59 years (range, 41-80 years). Median follow up was 98 months (range, 0.1-163.3 months). Twenty patients died of their disease. Thirty three patients had T1/2 disease and fourteen patients had T3/4 disease. Tumour volume was recorded for each case, median 10cm³ (range 0.5-30 cm³). Twenty one patients subsequently had evidence of recurrence on radiological imaging.

Necrosis was present in 27 cases (57%). On χ^2 analysis of absence/presence of necrosis, there was no correlation with T stage, nuclear grade, recurrence or tumour volume (Table 2.7). On univariate analysis, absence/presence of necrosis was associated with disease specific survival but failed to reach significance with those with necrosis present having a

mean survival of 90 months in comparison to 130 months for those for whom there was no evidence of necrosis ($p=0.052$, Table 2.8).

Table 2.7: Interrelationships between clinicopathological characteristics of patients with renal cancer and presence and absence of tumour necrosis.

<i>Variable</i>	<i>Numbers</i>	<i>T Stage</i>	<i>Nuclear Grade</i>	<i>Tumour Necrosis (negative/positive)</i>	<i>Recurrence</i>	<i>Tumour Volume</i>
Age (<60/>60)	28/19	0.282	0.353	0.96	0.141	0.372
T Stage (1/2/3/4)	22/11/12/2		0.001	0.446	0.003	<0.001
Nuclear Grade (1/2/3/4)	8/20/13/6			0.299	0.003	0.074
Tumour Necrosis (negative/positive)	20/27				0.256	0.226
Recurrence (No/Yes)	26/21					0.011
Tumour Volume (<10cm ³ / ^{>} 10cm ³)	21/26					

Table 2.7 demonstrates that no relationship was shown between the absence or presence of necrosis to other recognised pathological prognostic factors.

Table 2.8: Relationships between clinicopathological characteristics, various classifications of necrosis and disease specific survival.

<i>Variable</i>	<i>Numbers</i>	<i>Univariate Analysis</i>	<i>Multivariate Analysis</i>	
		p value	p value	HR
Age (<60/>60)	28/19	0.712		
T Stage (1/2/3/4)	22/11/12/2	<0.001	0.006	2.41 (1.28-4.53)
Nuclear Grade (1/2/3/4)	8/20/13/6	0.008	0.012	2.29 (1.20-4.36)
Tumour Necrosis (negative/positive)	20/27	0.052		
Tumour Necrosis (0/1/2/3)	20/13/10/4	<0.001		
Tumour Necrosis (<25%/>25%)	33/14	<0.001	<0.001	11.84 (3.81-36.75)

Table 2.8 demonstrates that the absence or presence of necrosis was associated with reduced disease specific survival but failed to reach significance. Classification of necrosis utilising a quantified based system and utilising a two tiered classification with 25% as a cut-off value, both were associated with reduced disease specific survival with a two tiered system of classification of necrosis being an independent predictor of disease specific survival.

When quantifying extent of involvement of necrosis, 43% of cases had no necrosis, 28% had <25% involvement of necrosis, 21% of cases had between 25-50% involvement and 8% had >50% involvement of necrosis. On χ^2 analysis (Table 2.9) using a quantitative based classification of necrosis, there was no correlation with T stage or nuclear grade. There was a positive correlation with recurrence ($p=0.009$) and tumour volume ($p=0.017$). On univariate analysis, this quantitative based assessment was associated with disease specific survival ($p<0.001$, Table 2.8, Figure 2.3).

Table 2.9: Interrelationships between clinicopathological characteristics of patients with renal cancer and quantifying the extent of necrosis.

<i>Variable</i>	<i>Numbers</i>	<i>T Stage</i>	<i>Nuclear Grade</i>	<i>Tumour Necrosis (0/1/2/3)</i>	<i>Recurrence</i>	<i>Tumour Volume</i>
Age (<60/>60)	28/19	0.282	0.353	0.514	0.141	0.372
T Stage (1/2/3/4)	22/11/12/2		0.001	0.371	0.003	<0.001
Nuclear Grade (1/2/3/4)	8/20/13/6			0.28	0.003	0.074
Tumour Necrosis (0/1/2/3)	20/13/10/4				0.009	0.017
Recurrence (No/Yes)	26/21					0.011
Tumour Volume (<10cm ³ / ^{>} 10cm ³)	21/26					

Table 2.9 demonstrates that a quantitative based assessment of necrosis had a positive correlation with recurrence and tumour volume.

Figure 2.3: Kaplan Meier survival graph for a quantitative based assessment of necrosis against disease specific survival.

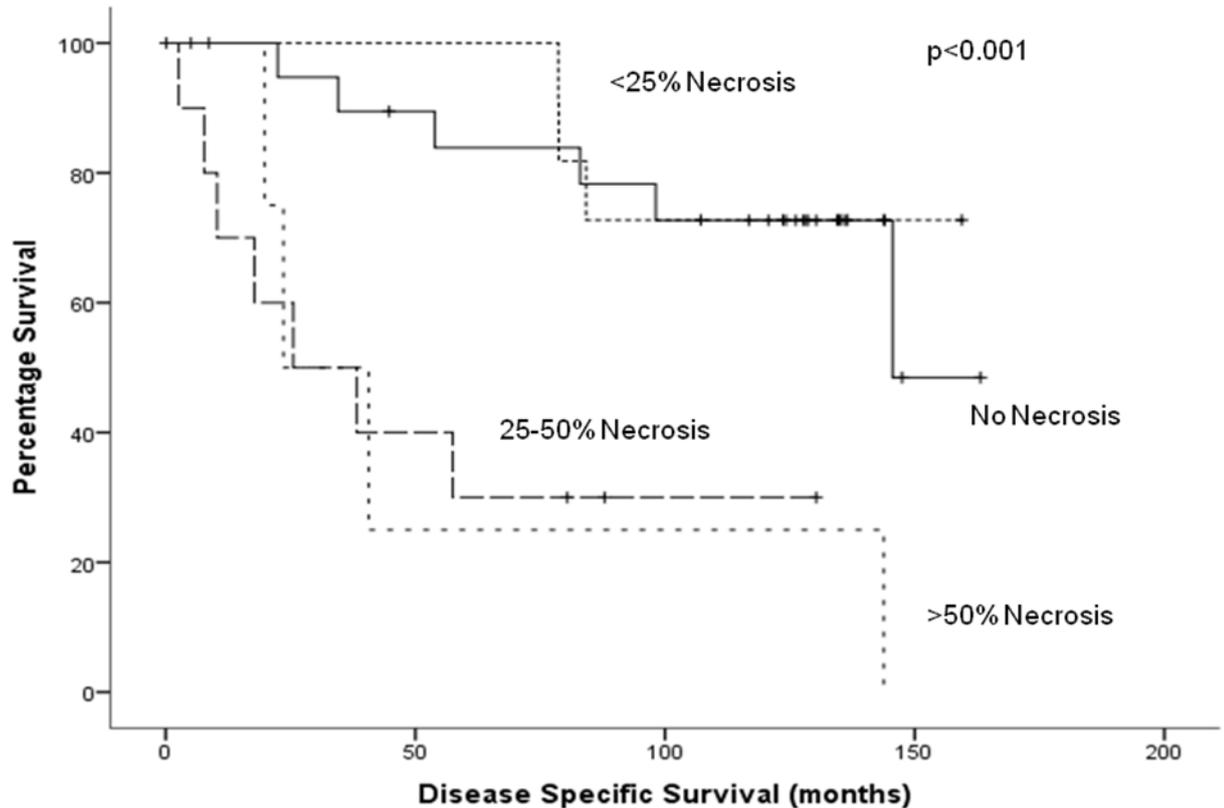


Figure 2.3 demonstrates that an extent based classification of necrosis was significantly associated with disease specific survival ($p < 0.001$).

Further analysis was performed on the quantitative assessment of necrosis to assess if it could be further refined into a simpler two tiered system. Analysis of this quantitative assessment into a two tiered scoring system, $<25\%$ and $>25\%$ involvement of necrosis showed 14 cases (30%) had more than 25% involvement of necrosis. On χ^2 analysis there was no correlation with T stage or nuclear grade (Table 2.10). There was a positive correlation with recurrence ($p = 0.003$) and tumour volume ($p = 0.007$). On univariate analysis, using the simpler 2 tiered assessment of $<25\%$ and $>25\%$ involvement, this was associated with disease specific survival ($p < 0.001$, Table 2.8, Figure 2.4). This significance

was maintained on multivariate analysis (HR 11.84, 95% CI 3.81-36.75, $p < 0.001$) (Table 2.8).

Table 2.10: Interrelationships between clinicopathological characteristics of patients with renal cancer and an extent based classification of necrosis with a 25% cut off.

<i>Variable</i>	<i>Numbers</i>	<i>T Stage</i>	<i>Nuclear Grade</i>	<i>Tumour Necrosis (<25%/>25%)</i>	<i>Recurrence</i>	<i>Tumour Volume</i>
Age (<60/>60)	28/19	0.282	0.353	0.96	0.141	0.372
T Stage (1/2/3/4)	22/11/12/2		0.001	0.446	0.003	<0.001
Nuclear Grade (1/2/3/4)	8/20/13/6			0.299	0.003	0.074
Tumour Necrosis (<25%/>25%)	33/14				0.003	0.007
Recurrence (No/Yes)	26/21					0.011
Tumour Volume (<10cm ³ />10cm ³)	21/26					

Table 2.10 demonstrates that when utilising a two tiered system for scoring necrosis with a 25% cut off, this had a positive correlation with recurrence and tumour volume.

Figure 2.4: Kaplan Meier survival graph using a 25% cut off for necrosis against disease specific survival.

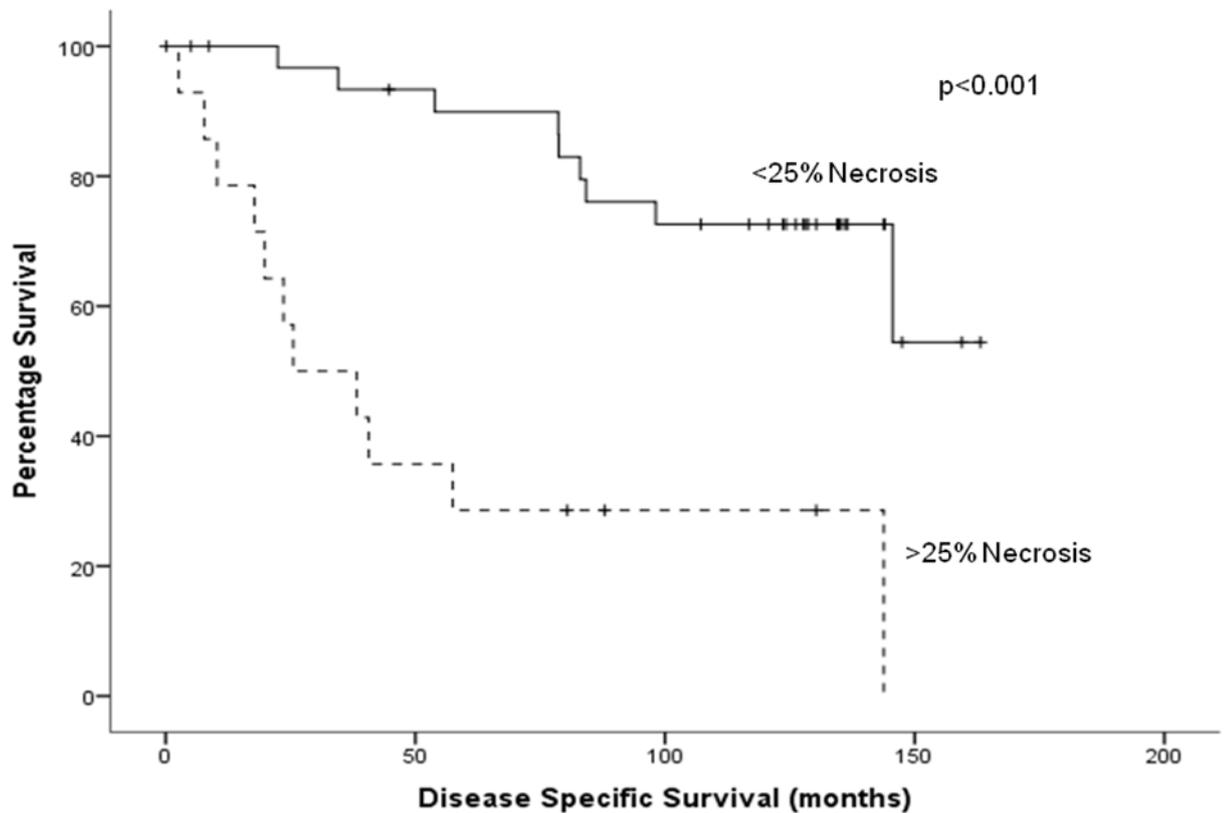


Figure 2.4 shows that using a 25% cut off for classification of necrosis into a two tiered system was significantly associated with disease specific survival ($p < 0.001$).

2.3.4 Molecular Markers

Analysis was based on fifty seven patients. Median age at diagnosis was 60 years (range 41-80). Median follow up was 107 months (range 0.1-163). Twenty two patients died of their disease. Thirty eight patients had T1/2 disease and nineteen patients had T3/4 disease. Tumour volume was recorded for each case; median tumour volume was 9.8cm^3 (range 1-30 cm^3). Twenty five patients subsequently had evidence of recurrence on radiological imaging.

When assessing proliferation of these tumours, 29 cases (51%) had a high proliferation index. On χ^2 analysis, the proliferation index demonstrated a positive correlation with T Stage and recurrence ($p=0.068$, $p=0.007$ respectively, Table 2.11) but failed to demonstrate significance when correlated to grade. On univariate analysis, the proliferation index was associated with disease specific survival ($p=0.012$, Table 2.12, Figure 2.5) but was not an independent predictor of survival on multivariate analysis.

Table 2.11: Interrelationships between clinicopathological characteristics, the proliferation and apoptotic indices of patients with renal cancer.

<i>Variable</i>	<i>Numbers</i>	<i>T Stage</i>	<i>Grade</i>	<i>Tumour Necrosis</i>	<i>PI</i>	<i>AI</i>	<i>Recurrence</i>	<i>Tumour Volume</i>
Age (<60/>60)	29/28	0.335	0.316	0.227	0.513	0.675	0.021*	0.692
T Stage (T1/T2/T3/T4)	26/12/17/2		0.001	0.957	0.068	0.801	0.002	<0.001
Grade (1/2/3/4)	10/26/15/6			0.443	0.193	0.726	0.01	0.091
Tumour Necrosis (absence/presence)	32/25				0.083	0.778	0.602	0.15
PI (low/high)	28/29					0.675	0.007	0.692
AI (low/high)	33/24						0.19	0.911
Recurrence (No/Yes)	32/25							0.064
Tumour Volume (<10cm ³ / ^{>} 10cm ³)	29/28							

Table 2.11 demonstrates that the proliferation index had a positive correlation with recurrence and a relationship was demonstrated with T Stage but this failed to reach significance.

Table 2.12: Relationships between clinicopathological characteristics and disease specific survival.

<i>Variable</i>	<i>Numbers</i>	<i>Univariate Analysis p value</i>	<i>Multivariate Analysis p value</i>	<i>HR</i>
Age (<60/>60)	29/28	0.201		
T stage (T1/T2/T3/T4)	26/12/17/2	0.001	0.005	2.097 (1.256-3.501)
Grade (1/2/3/4)	10/26/15/6	0.012		
Tumour Necrosis (absence/presence)	32/25	0.092		
PI (low/high)	28/29	0.012		
AI (low/high)	33/24	0.108		

Table 2.12 demonstrates that the proliferation index was associated with disease specific survival but was an independent factor.

Figure 2.5: Kaplan Meier for the proliferation index against disease specific survival.

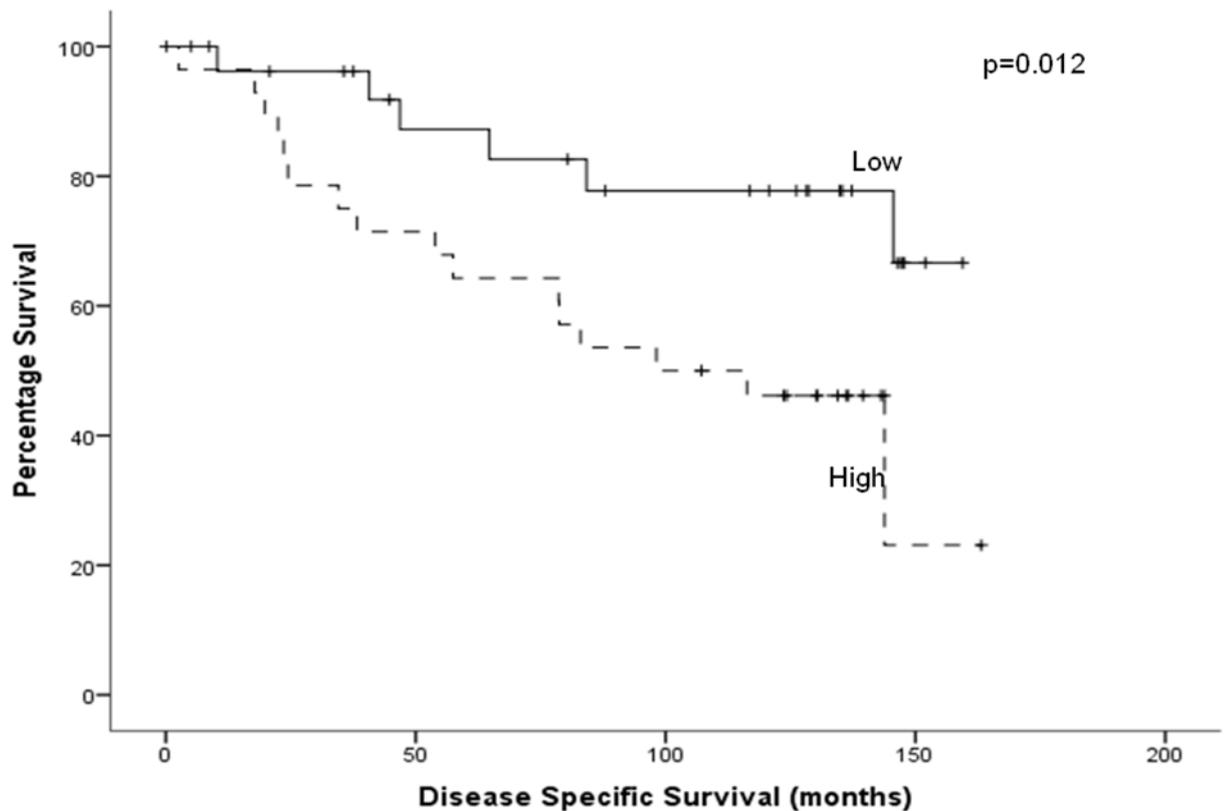


Figure 2.5 demonstrates that the proliferation index was associated with disease specific survival ($p=0.012$).

When assessing apoptosis of these tumours, 24 cases (42%) had a high apoptotic index. On χ^2 analysis, the apoptotic index failed to demonstrate any correlations to other clinicopathological parameters (Table 2.11). On univariate analysis, the apoptotic index was associated with disease specific survival but failed to reach significance with those who had a high apoptotic index having a mean survival of 130 months in comparison to those with low apoptotic index who had a mean survival of 100 months ($p=0.108$, Table 2.12).

2.4 Discussion

In these cohorts of those with RCC, a simplified 3-tiered model of nuclear grading where grades 1 and 2 are combined whilst grades 3 and 4 were kept separate (model 5) was an independent predictor of cancer specific survival on multivariate analysis. Furthermore, this modified model was also one of only two to correlate to disease recurrence. It has also been demonstrated that an elevated mGPS independently correlates to a poor disease specific survival in those undergoing potentially curative treatment for renal cancer. We have also demonstrated that an elevated mGPS is directly associated with tumour stage, grade and necrosis. We have demonstrated that the prognostic information provided by a quantitative based assessment of tumour necrosis classification is superior to a simple absence/presence response. Furthermore, when utilising this quantitative assessment in a two tiered system, $<25\%$ and $>25\%$ involvement of tumour necrosis, this was retained as an independent prognostic factor. We have demonstrated that prognostic information is provided by assessing molecular markers such as the tumour proliferation and apoptotic indices. In this study, the proliferation index of tumour cells was associated with disease specific survival but was not retained as an independent predictor of survival.

Several studies have demonstrated that the Fuhrman grading system is capable of predicting cancer specific survival independent of pathological stage [85, 145]. Studies however have demonstrated that the conventional Fuhrman grading system is complex [142, 154] and that a simplified system improves inter-observer agreement [148, 149]. It has previously been demonstrated that combining grades 1 and 2 improves the prognostic ability of the Fuhrman grading system and a three tiered system combining grades 1 and 2 whilst keeping grades 3 and 4 separate is an independent predictor of survival [154, 155], a finding similar to that reported in this study. There have been reports that this simplified three tiered model has a similar performance in multivariate models predicting outcome to the conventional 4 tiered Fuhrman system [152, 153]. In terms of cancer specific survival, the gap between grades 3 and 4 was more evident than the gap between grades 1 and 2. This result is similar to that demonstrated by several other studies [152-155] where a three tiered model was proposed [154, 155]. This further shows that grades 3 and 4 would be less suitable for combining than grades 1 and 2 and strengthens the argument for a three tiered model.

As with most studies examining the modification of the Fuhrman grading system, this is also limited due its retrospective nature with no analysis of inter or intra observer variability when assigning the Fuhrman grade and no external review of nuclear grade. In our analysis, a simplified version of the Fuhrman grading system whereby grades 1 and 2 are combined and grades 3 and 4 were kept separate was shown to be an independent predictor of cancer specific survival and demonstrated a positive correlation to disease recurrence suggesting that this modified model can be considered an option for the purposes of prognosis in those with clear cell renal cancer.

Previous studies have demonstrated that the local inflammatory response plays a prognostic role in various malignancies [187, 198]. It has been reported that quantifying the degree of infiltration by lymphocyte subsets provides prognostic information in renal

cancer [186]. It was therefore of interest that a simplified assessment of the local inflammatory cell infiltrate [187] which is not as time consuming as quantifying lymphocyte subsets has been shown to prognostic in colorectal cancer [187, 198]. In this study the local inflammatory response was not a significant predictor of disease specific survival. One of the reasons could be the small cohort study.

mGPS is a well-established marker of systemic inflammation. The variables used are common ones and offer the benefit of being objective and obtainable. We have demonstrated an independent association between preoperative systemic inflammation and disease specific survival. A limitation of this study is small cohort size, only 79 patients were available for analysis and from these 79 only 19 patients had died of their disease.

Historically, tumour necrosis has been associated with more aggressive tumour activity. Studies have produced conflicting results regarding the prognostic significance of tumour necrosis in renal cancer, with some showing an association with poor survival [145, 147, 166-168] whilst others have shown no relationship to outcome [137, 169, 170]. It has been shown that an extent based classification of necrosis is superior to a simple absence/presence response and is retained as an independent prognostic factor [173]. We have also demonstrated that a quantitative assessment of necrosis is superior to an absence/presence response but is not retained as an independent prognostic factor. Klatte et al suggested a cut off of 20% and a 3-tiered system and we used a similar figure of 25% but utilised a 2-tiered system. When using the cut off of 25% and only having two groups, less than 25% and more than 25% involvement of necrosis, we have shown that this was associated as an independent prognostic factor. We felt that using a cut off of 25% and maintaining to use a two-tiered system would allow simpler refinement of the necrosis parameter in prognostic algorithms where a two tiered system is already utilised and to examine if this were to increase the predictive accuracy of the entire model but this is yet to be tested.

Various studies have reported that proliferation and apoptosis are associated with known prognostic factors and predictors of survival [216, 218, 242, 243, 246-251, 258-261] whilst others have reported no correlation or independence to survival [236, 252-255, 262-265]. Other molecular markers have also been reported to offer prognostic information in renal cell cancer but these often utilise tumour proliferation and/or apoptosis in the cohorts to assess if the molecular markers being investigated offer further prognostic value to proliferation and/or apoptosis which are established [216, 236, 243, 251]. Some have reported that tumour proliferation when combined with other pathological factors confers improved prognostic accuracy in comparison to traditional prognostic algorithms [216, 218, 242, 247].

Tumour proliferation and apoptosis are well recognised prognostic factors for disease specific survival. We have demonstrated that although apoptosis was not a significant predictor of survival, both tumour proliferation and apoptosis are prognostic to disease specific survival which is in keeping with literature.

Despite limitations to this work such as limited numbers for parts of the analysis and the retrospective nature of the work, we have demonstrated that the reclassification of the Fuhrman grading system, quantitative assessment of tumour necrosis, the role of inflammation and molecular markers are prognostic for clear cell cancer. Despite these findings, not all are routinely utilised in the assessment of patients and furthermore and more importantly they cannot be used as a guide to which patients may benefit from treatment with the various therapeutic agents available when and if recurrence occurs.

Chapter 3-mRNA expression of Src kinase family members in RCC

3.1 Introduction

There is much evidence showing that levels of SFK are elevated in various malignancies such as prostate, breast, colon, lung and haematological malignancies [327, 328, 348, 364, 516-518]. Specifically, elevated levels of Src and Lyn have been observed in breast cancer and expression of Src was associated with decreased survival [516] whilst Lyn has been implicated in various other malignancies such as colon, prostate and breast cancer [329-332]. Src expression does not always correlate with poor survival, it has been reported that Src expression and activity decreases with bladder tumour stage [357-359, 361, 362] and grade [360]. Expression of Lyn has also been associated with malignancies including breast, colon and prostate [329, 332]. There is little evidence of the expression of SFK's in RCC and furthermore if there is a correlation between expression and pathological parameters. The aim of this study was to determine, via real time PCR, if Src and other Src family kinase members were at all expressed in RCC tissue specimens and to investigate if these expression levels were associated with clinical parameters.

3.2 Materials and Methods

Nineteen clinical specimens were utilised for Real time quantitative PCR (RT-PCR) (Table 3.1). This consisted of malignant tissue taken from RCC patients at the time of resection. Those specimens were utilised where all tissue was removed at time of surgery and radiological imaging prior to surgery showed no evidence of metastatic spread of disease. The Research Ethics Committee of West of Scotland has approved the study.

RT-PCR was utilised to determine mRNA expression of the SFK members. At the time of resection, representative parts of malignant tissue were identified, snap frozen and stored in liquid nitrogen. Total mRNA was extracted from 5-10mg of renal cell cancer tissue using the TRIZOL method according to manufacturer's protocol (Invitrogen, Paisley UK). RNA quality and quantity was examined by UV spectrometry (GeneQuant analyser, GE Healthcare, Little Chalfont, UK).

To ensure no DNA was contaminating the extracted RNA, DNA-free DNase treatment and removal reagent kit was added (Applera, Warrington, UK). Prior to incubation for 30 minutes at 37°C, to ensure that the same amount of template was being utilised, a starting concentration of 1000ng of RNA was applied for each sample. Random hexamer primers (50ng) were used for First Strand cDNA synthesis using SuperScript II RT according to manufacturer's instructions (Invitrogen). Before using cDNA for PCR amplification, 2 units of RNase H were added to samples and incubated for 20 minutes at 37°C to destroy any RNA template so that it was only cDNA that is being amplified.

RT-PCR was performed using an ABI Prism 7900 Sequence Detection System (Applied Biosystems, UK) and TaqMan® Gene Expression Assays. For the TaqMan® Gene Expression Assays, the manufacturer's protocol with recommended 40 rounds of amplification was applied. Thermal cycler condition were 50°C for 2 min, 95°C for 10 min followed by 40x 95°C for 15 sec and 60°C for 1 min.

Quantitative values were obtained from the threshold cycle (Ct value) at which the increase TaqMan® probe fluorescent signal associated with an exponential increase of each individual PCR product reaching a fixed threshold value. Each individual primer had a fixed threshold Ct value (Table 3.2). These fixed threshold values were used for every cDNA sample. Negative controls for each primer were included in each run on a 96 well plate.

To enable the comparison of different mRNA expression levels, their relation to the average expression level of two housekeeping genes (*GAPDH*, glyceraldehydes-3-

phosphate dehydrogenase and *HPRT*, hypoxanthine-guanine phosphoribosyl-transferase) were evaluated. The housekeeping gene with the lowest standard deviation (*GAPDH*) was used for evaluation of the mRNA expression levels. Data was analysed using the Sequence Detection Software, this calculated the threshold cycle (Ct) value. The expression of the target assay was normalised by subtracting the Ct value of the housekeeping gene from the Ct value of the relevant target assay. The fold increase, relative to the control, was obtained by using the formula $2^{-\Delta Ct}$, and then expressed as a percentage ($\times 100$). All samples were measured in triplicates.

3.3 Statistical Analysis

Differences in expression levels were analysed using the Mann-Whitney U test or Kruskal-Wallis test, including a Wilcoxon-type test for trends, when appropriate.

3.4 Results

The cohort for RT-PCR analysis consisted of nineteen renal cell cancers. Median age of diagnosis was 60 years (range 42-72). Table 3.1 demonstrates clinicopathological parameters of these patients.

Table 3.1: Clinicopathological characteristics of patients utilised for RTPCR.

<i>Variable</i>	<i>Numbers</i>
Age (<60/>60)	10/9
Sex (M/F)	9/10
T Stage (T1/T2/T3/T4)	5/3/9/2
Grade (1/2/3/4)	4/5/6/4
Tumour Necrosis (absence/presence)	8/11
Recurrence (No/Yes)	12/7
Tumour Volume (<10cm ³ / ^{>} 10cm ³)	7/12

Table 3.1 demonstrates the clinicopathological characteristics of patients.

The most highly expressed SFK member in renal cell cancer tissue was Src followed by Lyn, Hck, Fgr and Fyn (Table 3.2, Figure 3.1). The least expressed SFK member was Blk.

Table 3.2: Intron-skipping primers used for RTPCR, their fixed threshold Ct values and median expression levels in renal cell cancer tissue.

<i>Gene</i>	<i>Gene Expression Assay ID</i>	<i>Threshold (Ct)Value</i>	<i>Expression Levels</i>
SRC	Hs00178494_m1	0.23	404.9
LCK	Hs00178427_m1	0.17	13.5
LYN	Hs00176719_m1	0.25	233.3
FYN	Hs00176628_m1	0.2	53.2
FGR	Hs00178340_m1	0.2	58.1
HCK	Hs00176654_m1	0.26	75
BLK	Hs00176441_m1	0.19	2.5
YES	Hs00736972_m1	0.26	9.7
GAPDH	N/A	0.2	N/A

Table 3.2 demonstrates the threshold values of the SFK's with Src the highest expressed.

Figure 3.1: Graph showing expression of mRNA levels of SFK members in renal cancer tissue.

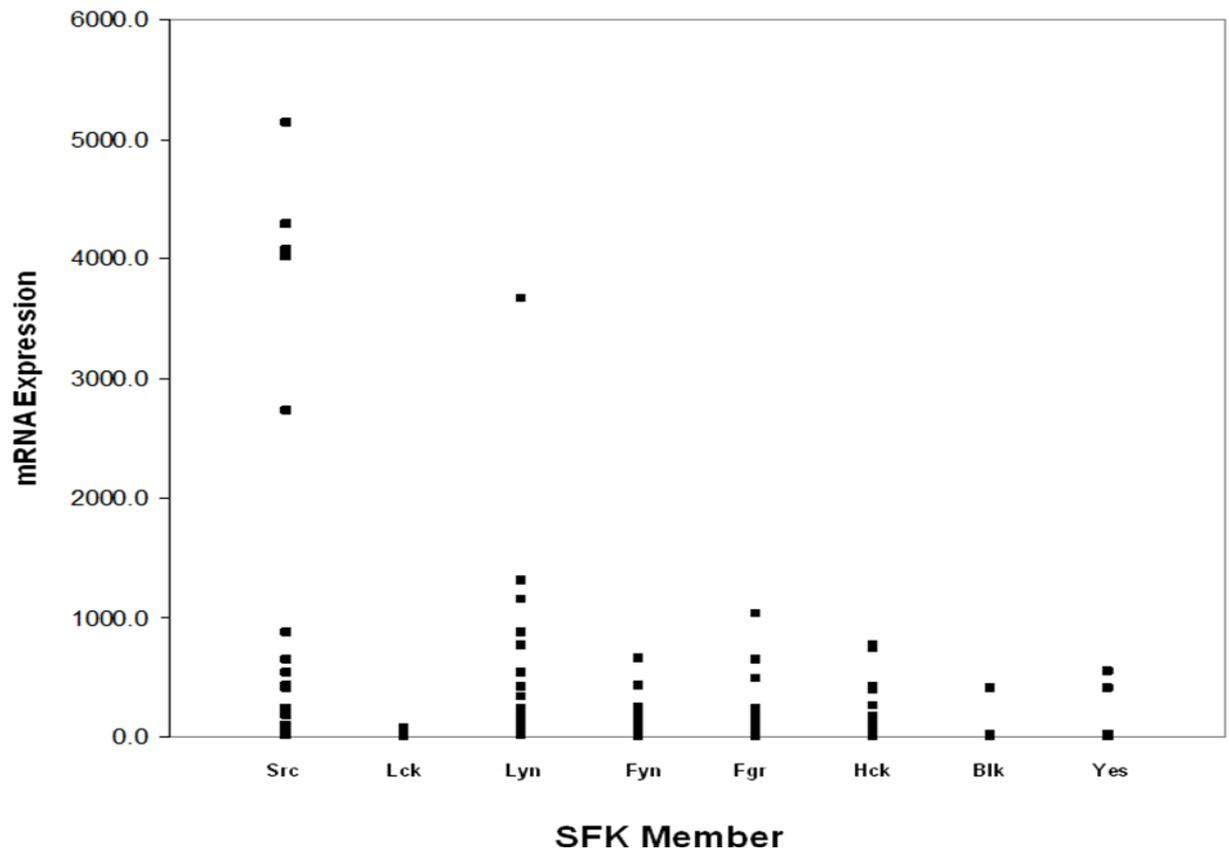


Figure 3.1 demonstrates that Src was the highest SFK member expressed in RCC.

When analysing SFK members, expression of Yes and Blk were significantly different across different T stages ($p=0.039$, $p=0.045$, Figure 3.2). Interestingly, higher expression of all SFK members was observed in T2 stage disease when compared to T3 disease and this was observed as significant for Lck, Lyn, Fyn, Blk and Yes ($p=0.032$, $p=0.032$, $p=0.032$, $p=0.032$, $p=0.032$, Figure 3.2).

Figure 3.2: Graphs showing expression of mRNA levels of each SFK member according to T stage.

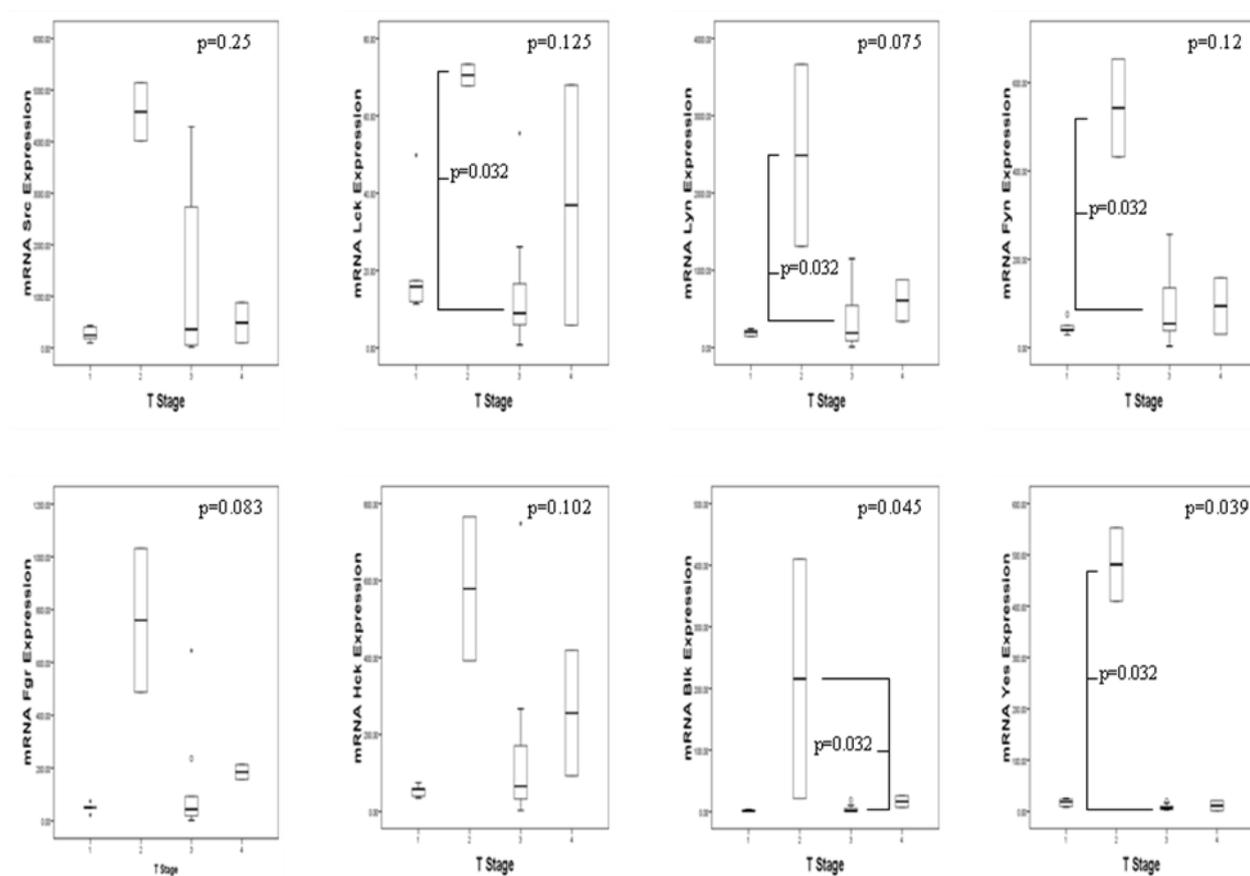


Figure 3.2 demonstrates that expression of Btk and Yes were significantly different across all T stages whilst a significant difference was demonstrated between T2 and T3 disease for Lck, Lyn, Fyn, Btk and Yes.

When analysing SFK member expression in correlation with tumour grade, no significant difference was observed (Figure 3.3).

Figure 3.3: Graphs showing expression of mRNA levels of each SFK member according to tumour grade.

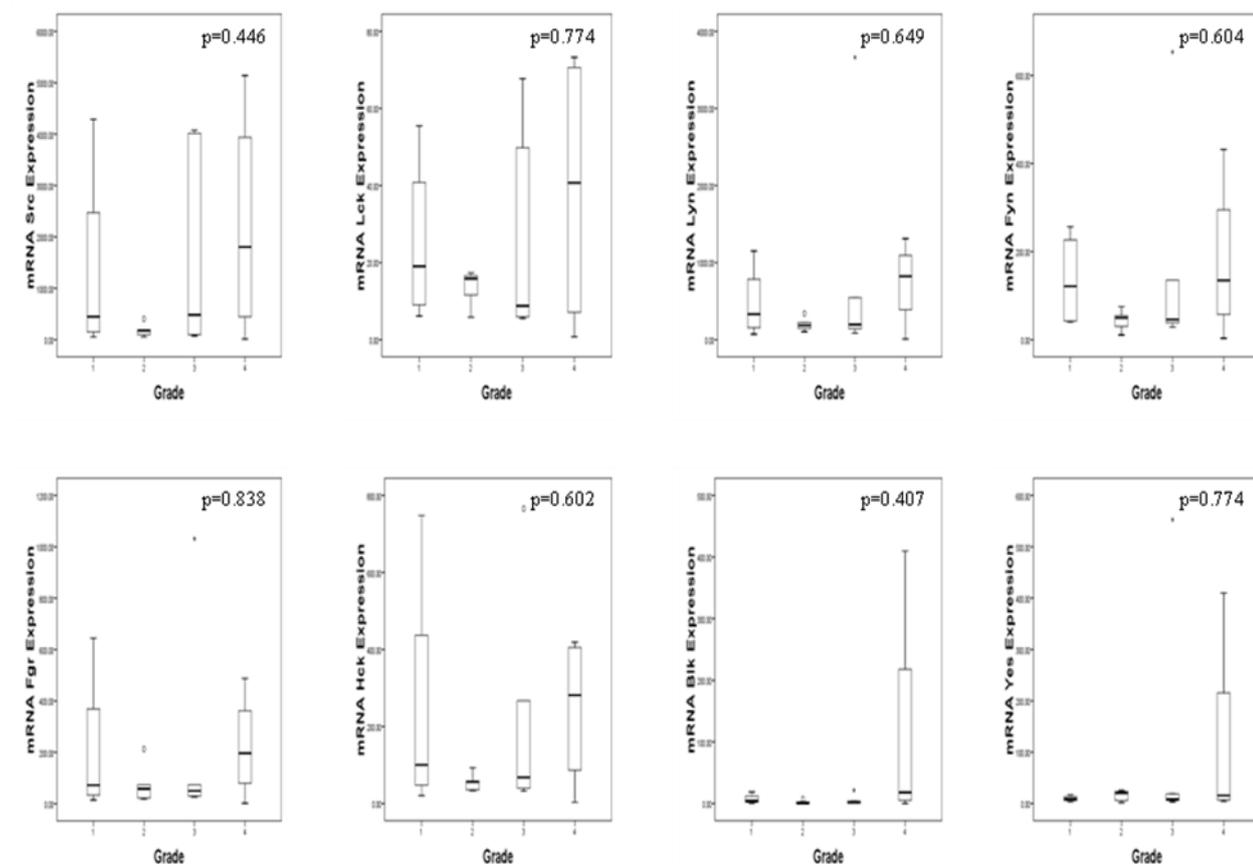


Figure 3.2 demonstrates that no correlation was observed between SFK member expression and nuclear grade in RCC.

3.5 Discussion

In this pilot study, SFK members are not only expressed in renal cell carcinoma but are expressed to varying degrees. Of the SFK members, Src is the most highly expressed followed by Lyn, Hck, Fgr and Fyn. Src has a five-fold higher expression than the least SFK member expressed. We have also reported that there is a significant fall in expression of Lck, Lyn, Fyn, Blk and Yes between T2 and T3 disease.

Despite having a limited cohort group, we have shown that there is a significant difference in expression of several SFK members between T2 and T3 disease. In renal cancer, T2

disease is still organ confined with T3 disease invading perinephric fascia and is therefore no longer organ confined. Src kinase has a role in signal transduction of multiple oncogenic cellular processes including migration and invasion. We hypothesise that SFK members are being up-regulated at a time when the cancer is going beyond being organ confined and starting to metastasise after which up-regulation is no longer required as the cancer has invaded surrounding tissue.

Expressions of SFK members have been demonstrated in a variety of tumours [348, 364, 516-518]. Specifically, expression of Src and Lyn has been demonstrated to be elevated in breast and colon cancer [329, 332, 516]. When assessing activity of SFK members, it has been demonstrated that increased expression of Src is associated with poor survival in breast cancer [336, 516] whereas expression of Src is associated with improved clinical outcome in bladder cancer [358, 360, 361].

Limitations of this study include the method of RT-PCR and cohort size. Tissue that is utilised for RT-PCR should be instantly fresh frozen after retrieval to prevent mRNA degrading leading to a distortion in mRNA quality and results. We believe that this was kept to a minimum given that tissue was frozen at the earliest time possible and UV spectrometry demonstrated high quality RNA. Given that the cohort size was limited, significant correlations could not be observed between expression of a particular member and survival. Even if a SFK member is expressed, this does not necessarily constitute activity. Furthermore, mRNA expression does not necessarily correlate with protein synthesis and protein expression. Further work is required to assess not only if Src is expressed in RCC but also to assess if the downstream marker FAK is expressed and if expression/activity correlates to clinicopathological parameters. Therefore to gain a better understanding of Src and SFK activation we established a cohort of patients samples to allow us to assess expression and establish correlations with clinical parameters.

Chapter 4-Expression and prognostic significance of SFK members in RCC

4.1 Introduction

Having established mRNA expression levels of SFK members in RCC and the differing expression levels, it was further important to investigate whether Src expression/activity, SFK activity or FAK expression/activity correlated to clinicopathological parameters in those with RCC.

As mentioned earlier, Src kinase is composed of a C-terminal tail, kinase domain, two protein-protein interaction domains (SH2, SH3) and a unique amino-terminal domain that varies between Src family members. Classical activation of Src kinase occurs by an initial dephosphorylation of a conserved tyrosine residue in the C-terminal domain known as the negative regulatory region (Y⁵³⁰) and followed by a subsequent autophosphorylation of the Y⁴¹⁹ site in the kinase domain [383, 384]. Both these events are required to occur before the kinase can be considered fully activated. Consequently antibodies to phosphorylated Src kinase at the Y⁴¹⁹ site can be used as a marker for activated Src kinase and related family members [519]. In addition, when SFK's are activated, several downstream markers such as FAK are phosphorylated and could therefore act as biomarkers for SFK activation [459]. Src phosphorylates FAK at several other sites as well as including Y³⁹⁷, Y⁴⁰⁷, Y⁵⁷⁶, Y⁵⁷⁷, Y⁸⁶¹ and Y⁹²⁵ [452-456] but it has been reported that the Y⁸⁶¹ is the major site on the FAK domain associated with activation [455, 457, 458].

Therefore within this chapter we aim to utilise paraffin embedded clinical samples to establish if Src expression, SFK activity and FAK are associated with pathological parameters and survival.

4.2 Materials and Methods

IHC staining was utilised on full tissue sections in a cohort of 57 patients diagnosed with RCC. These patients had undergone complete resection of the tumour at time of nephrectomy and pre-operative CT scans showed no evidence of regional or metastatic spread. Patients were staged pathologically and graded according to the TNM classification and Fuhrman grading respectively. Cancer specific survival rate was the time from diagnosis until time of death or last follow up. The cause of death was determined by linkage through the Scottish Cancer Registry. In those who were deceased, if the primary cause of death was of renal cancer, these were classed as cancer specific and all other causes were non-cancer specific deaths. The Research Ethics Committee of West of Scotland has approved the study.

4.2.1 Western Blotting

Prior to IHC, it was necessary to confirm antibody specificity. This allows the detection and quantifies the amount of protein in cells.

4.2.1.1 Culture of renal cell cancer cells

Cells were routinely maintained in RPMI 1640 (Invitrogen, UK) containing phenol red and supplemented with 10% bovine serum albumin (BSA) (Invitrogen, UK), 2mM L-glutamine (Invitrogen, UK), and penicillin/streptomycin (50 units/ml, 50g/ml (Invitrogen, UK)) in 5% CO₂ at 37°C. 786-O (ATCC) and 769-P (ATCC) RCC cell lines were seeded in conical flasks and left to adhere until 80% confluent.

4.2.1.2 Cell trypsinisation and lysis

Once cells were 80% confluent, excess media was removed and the cells gently washed twice with hanks balanced salt solution (HBSS) to allow elimination of serum as it contains trypsin inhibitors. Cells were then incubated in 3 mls of trypsin for 5 minutes in

5% CO₂ at 37°C, in order to detach the cells from the flask after which 3mls of RPMI was added to neutralise the trypsin. Following this, the cell suspension was transferred to a 15ml centrifuge tube and centrifuged for 2 minutes at 1200rpm. The supernatant was poured off and the resulting cell pellet washed twice in ice-cold HBSS followed by further centrifugation for 2 minutes at 1200rpm. The cell pellet were re-suspended and lysed by adding 200µl of RIPA buffer (Tris 50mM; NaCl 150mM; SDS 0.1%; Na. Deoxycholate 0.5%; NP40 1% v/v, protease inhibitor (Roche, UK)) in an eppendorf and the solution sheared with a needle. This was performed on ice. This re-suspension was then spun at 14000rpm for 15 minutes at -4°C. The resulting supernatant was poured off and the pellet discarded.

4.2.1.3 Quantification of protein

It was necessary to determine the protein concentration of the cell lysates prior to performing Western blot analysis. This ensures that the same concentration of protein is utilised in these experiments allowing accuracy.

The method used to determine protein concentration of cell lysates was the Bio-Rad protein assay, which is based on the Bradford dye-binding procedure (Bradford 1976) involving a colorimetric assay for measuring the total protein concentration.

Samples were prepared in triplicates in disposable cuvettes. A standard solution of 200µl of Bio-Rad protein assay reagent (Bio-Rad) and 795µl of dH₂O to which 5µl of protein sample was included to the mix. The solution was thoroughly mixed with a pipette to ensure even distribution of the protein for an accurate concentration reading. To allow analysis of the protein concentrations, protein standards were prepared using Bovine Serum Albumin (BSA). BSA was diluted with dH₂O to 1 mg/ml. A reference sample consisting of only dH₂O and serial dilutions for protein standards from 1-50 µg/ml were made. These were utilised to calibrate the spectrophotometer utilising the protein 595 programme.

Optical density for the reference and the seven protein standard samples were measured at 595 nm. Optical density at 595 nm (O.D. 595) was then read for all other protein samples and the concentration of protein present generated from the standards concentration. The spectrophotometer calculated at that time the amount of protein ($\mu\text{g/ml}$) present in the sample, plotting a graph of absorbance at 595 nm against the protein concentration of the standards that were being utilised. It is this standard curve that is used to determine the protein concentration of the measured sample from its O.D. 595 value. The initial protein concentration ($\mu\text{l/ml}$) is calculated from a diluted protein sample (1:200). To determine the final protein concentration in mg/ml following formula was used:

$$\text{Protein reading } (\mu\text{l/ml}) \times 0.2 = \text{Final protein concentration } (\text{mg/ml})$$

For western blot analysis, $20\mu\text{g}$ of protein was utilised with the volume calculated from the final concentration.

4.2.1.4 Freezing/Storing cells

By performing this, it allowed the storage of cells in a manner which would allow them to be utilised at a later date. Following trypsinisation, the cell suspension was transferred to a 15ml centrifuge tube and this spun at 1200rpm for 5 minutes. The supernatant was discarded and the pellet re-suspended in 1ml of RPMI and 10% dimethyl sulphoxide (DMSO) which serves as a cryoprotectant. The cells were then transferred in an alcohol bath at -80°C for 24 hours prior to be stored in liquid nitrogen at -180°C . When cells were required, these aliquots were warmed for 1 minute at 37°C in a water bath before being transferred into 10mls of RPMI otherwise with the DMSO being toxic would result in cell death.

4.2.1.5 Protein denaturation

To allow the primary antibody access and bind to its epitope, it is necessary to denature the protein. Denaturing the protein allows it to unfold and enabling easier access for the antibody to the epitope and therefore run more affectively through the gel. Having previously measured the concentration of protein in each sample, $20\mu\text{g}$ of protein was

removed for each sample and transferred to a new eppendorf tube and stored on ice. The cell lysate of each sample was diluted (1:1 v/v) with Laemli buffer and β -mercaptoethanol and boiled for 4 minutes at 100°C. The Laemmli buffer aids in the unfolding process. The detergent within the buffer has a negative charge which binds with the protein causing unfolding of the protein. The β -mercaptoethanol prevents the reformation of disulphide bonds helping maintain the protein in a denatured state. Boiling the samples aids in the unfolding process of the proteins. The molecular weight marker (Biotinylated Protein Ladder –Cell Signalling Technology) was also boiled. This is utilised to determine the size of the detected protein. Once the samples were boiled, they were kept on ice.

4.2.1.6 Gel electrophoresis

The principle behind this method is that the proteins move through the polyacrylamide gel due to the electrical charge that is utilised. The detergent in the Laemmli buffer results in the proteins being negatively charged and is thus attracted to the positive node.

Electrophoresis separates the proteins by molecular weight. Smaller proteins travel at a faster rate through the acrylamide pores of the gel resulting in them being found further down the gel. The gel was placed into the electrode assembly and the mini buffer tank was filled with running buffer (200mM Tris, 2M Glycine, 1% SDS (For 1* dilute in dH₂O)). 25 μ g of denatured protein and 10 μ l of the molecular weight marker was carefully loaded into dedicated wells. Once all samples were loaded, the process of electrophoresis was undertaken at 120Volts (V) for 90 minutes.

4.2.1.7 Protein transfer

To enable detection by antibody exposure, the protein needs to be transferred from the polyacrylamide gel to a PVDF (polyvinylidene difluoride) membrane. This transfer was utilised using the Mini-Trans Blot Cell tank (Bio-Rad Laboratories).

Fibre pads and 3M Whatmann paper with the PVDF membrane was utilised. The PVDF membrane was pre-treated in 100% methanol for five minutes prior to all three constituents being treated in transfer buffer (248mM Tris, 1.3M Glycine, 20% Methanol (For 1* dilute

in dH₂O)). Following this, the gel plates were removed and the stacking gel discarded and the remaining resolving gel treated in transfer buffer for five minutes. The 'transfer sandwich' was then constituted utilising the fibre pads, 3M Whatmann paper, PVDF membrane and the resolving gel (Figure 4.1).

Figure 4.1: Representation of Western Blot sandwich.

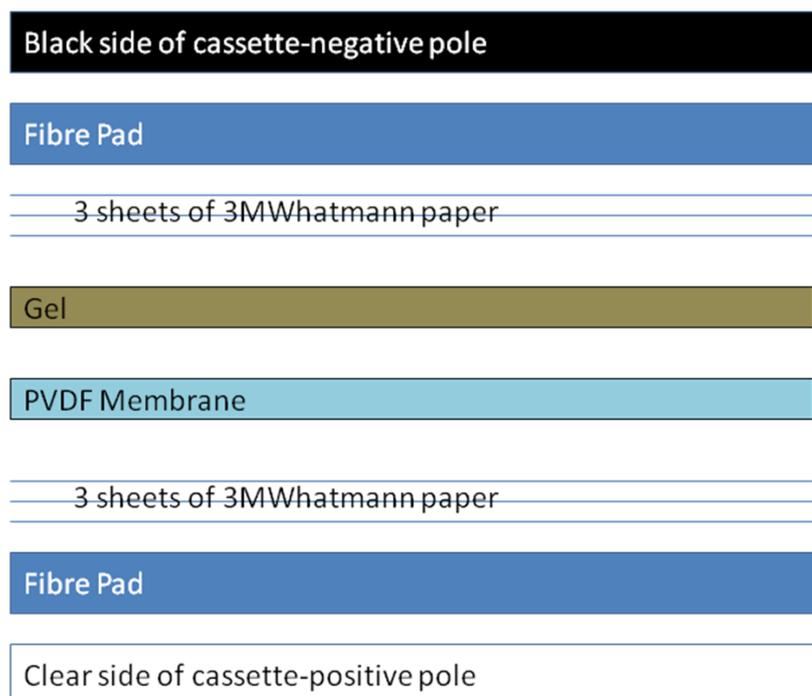


Figure 4.1 demonstrates a schematic representation of the various layers for the Western Blot.

Whilst constructing these various layers it was important that no air bubbles were present between the layers as this would prevent efficient transfer. These were removed by gently rolling over each layer with a glass rod. The constructed sandwich was then placed in the electrode assembly and placed in the tank which was filled with the transfer buffer. To maintain buffer temperature ion distribution, the Bio-Ice cooling unit (Bio-Rad Laboratories) and a magnetic stirrer were utilised. The protein transfer process from gel (negative/cathode) to the membrane was completed after running for sixty minutes at 100V.

4.2.1.8 Blocking of Membrane

The primary antibody can bind non-specifically to the membrane resulting in non-specific staining as well increased background staining. This was prevented by incubating the membrane in 2% BSA which was prepared in TBS-Tween (TBST) for one hour at room temperature. This step and all future steps were performed on an orbital shaker at 70 rpm.

4.2.1.9 Incubating of membrane with primary antibody

Following membrane blocking, the membrane was incubated with the primary antibody (1:500). All antibodies were prepared in 10mls of 0.2% BSA to further reduce non-specific binding. Membranes were incubated with the primary antibody overnight at 4°C.

4.2.1.10 Incubating of membrane with secondary antibody

Following incubation with the primary antibody and prior to incubating with the secondary antibody, the membrane was washed in TBST three times for ten minutes duration allowing the removal any excess antibody. Detection of the primary antibody requires a secondary antibody which is bound to either biotin or an enzyme conjugate such as horseradish peroxidase (HRP) which is species specific to the primary antibody. The secondary HRP linked antibody utilised was anti-rabbit IgG (1:2000, Cell Signalling Technology). Anti-biotin HRP linked antibody was added to detect the biotinylated ladder (1:20,000, Cell Signalling Technology). As with the primary antibodies, these were prepared in 10mls of 0.2% BSA. The membrane was incubated for one hour at room temperature.

4.2.1.11 Protein Visualisation

A chemiluminescent method was utilised to detect the protein of interest. This involves the emission of light due to the dissipation of energy from a substance in an excited state. HRP catalyses oxidation of luminol, a chemiluminescent substrate in alkaline conditions. The resulting oxidation causes the luminol to be in an excited state which then decays to a ground state via emitting light. ECL Plus (Amersham) was utilised for this. The peroxidase

conjugated with the antibody oxidises the chemiluminescent component of the ECL Plus substrate (Lumigen PS-3 Acridan). This results in the production of thousands of acridinium ester intermediates per minute. These intermediates react with the peroxidase producing a sustained, high intensity chemiluminescent with a maximum emission at 430nm. This light was detected on autoradiography film. Following incubation with the secondary antibody, membranes were washed again in TBST three times for ten minutes duration. During this process of the membranes being washed in TBST, the ECL Plus reagents (Solution A and B) were decanted and allowed to warm to room temperature. They were mixed in a ration 40:1 (A:B) and given that they are sensitive to light, this was performed in semi-darkness. 3mls of solution was utilised per membrane. Membranes were placed protein side up on a sheet of cellophane and incubated with the ECL Plus mixture for five minutes. Following this, the membrane was transferred into a film cassette. In complete darkness, autoradiography films (Kodak Medical X-ray films) were exposed for various times (1-15 minutes). The film was then developed using the Kodak X-OMAT x-ray processor allowing both marker and protein bands to be visualised.

4.2.1.12 Stripping of membrane

This process allowed the membrane for further use. Antibodies were removed from the membrane by washing the membrane in TBST three times for ten minutes duration. Following this the membrane was incubated in 20mls of re-Blot stripping buffer (Chemicon) for 15 minutes at 37°C followed by a further wash in TBST three times for ten minutes. The membrane was then blocked as described earlier and the steps repeated with the primary and secondary antibodies. To confirm equal sample lading, the membranes after being stripped were further probed with anti- β Tubulin HRP linked antibody (1:1000, Abcam) and the membrane visualised as described earlier.

4.2.2 Immunohistochemistry

IHC is a method that allows the detection and visualisation of cellular proteins *in situ* utilising an antibody specific for that particular protein investigated. There are two

methods for IHC. Direct IHC allows the direct attachment of the primary antibody to the protein investigated but in general does not yield a high level of sensitivity. Indirect IHC allows a higher level of sensitivity. In this method a second or secondary antibody which is labelled with a marker binds to the primary antibody already bound to the protein allowing signal amplification and visualisation. The method utilised here involved rabbit and mouse monoclonal antibodies and the DAKO Envision System, staining and visualising antigenic sites with peroxidase and DAB (diaminobenzidine chromagen). The primary antibody recognises the expressed protein, Envision attaches itself as the secondary antibody which has built on dextran polymer technology consisting of peroxidase. This secondary antibody has been raised in goats and reacts equally well with both rabbit and mouse antibodies. Following incubation with the secondary antibody, the tissue is incubated with a substrate solution which consists of DAB and H₂O₂. The peroxidase molecules on the Envision interact with this substrate solution producing an insoluble crisp brown precipitate at the site of the protein which is then visualised utilising a light microscope.

c-Src kinase and activated Src kinase expression (Src Y⁴¹⁹) was investigated using antibodies for c-Src kinase (36D10, Cell Signalling Technology, Beverly MA, USA) and Src Y⁴¹⁶ (Cell Signalling Technology). Dephosphorylated Src and FAK were investigated using antibodies for Src Y⁵²⁷ and FAK Y⁸⁶¹ respectively (Invitrogen, Paisley, UK). In humans the phosphorylation sites that were investigated in the current study are amino acids Y⁵³⁰ and Y⁴¹⁹. Antibodies used relate to the rabbit sequence and not the human sequence.

4.2.2.1 Tissue Preparation

This was performed as described earlier in Chapter 2.

4.2.2.2 Antigen Retrieval

This was performed as described earlier in Chapter 2.

Two solutions were utilised, the first solution was 10mM Citrate buffer (1:10 dilution of pre-made Epitope Retrieval Buffer at pH 6.0) and the second was TE buffer (1mM EDTA

(Sigma), 5mM Tris (VWR) at pH 9. Both solutions were pre-heated for 13.5 minutes to a temperature of 96°C prior to the tissue slides being placed in the solution under pressure for 5 minutes followed by a cooling down period of 20 minutes.

4.2.2.3 Reducing Background Staining

This was performed as described earlier in Chapter 2.

4.2.2.4 Primary Antibody Incubation

Prior to staining for the various proteins, it was imperative to establish optimum conditions for antigen staining. This was done by performing a series of investigations on RCC tissue by varying various factors such as antigen retrieval, antibody solutions, incubation times, and temperatures allowing the strongest specific antigen staining with the lowest background staining (Table 4.1). It was crucial to have both a positive and negative control in the chosen methodology. The positive control ensured that the methodology was not only working but there was limited variation between runs for the same antibody and the negative control ensured the specificity of the antibody.

4.2.2.5 Secondary Antibody Incubation

This was performed as described earlier in Chapter 2.

4.2.2.6 Detection and Visualisation

This was performed as described earlier in Chapter 2.

4.2.2.7 Counterstaining

This was performed as described earlier in Chapter 2.

4.2.2.8 Dehydrating and mounting of slides

This was performed as described earlier in Chapter 2.

Table 4.1: Overview of IHC staining methods.

<i>Protein</i>	<i>Antibody</i>	<i>Antigen Retrieval</i>	<i>H₂O₂</i>	<i>Horse Serum Conc.</i>	<i>Antibody Conc.</i>	<i>Incubation Time and Temp</i>
c-Src	Rabbit Cell Signalling	Citrate Buffer pH 6	3%	5%	1:200	60 minutes, room temp
Y ⁵²⁷ Src	Mouse Invitrogen	Citrate Buffer pH 6	3%	5%	1:3000	Overnight, 4°C
Y ⁴¹⁶ Src	Rabbit Cell Signalling	EDTA Buffer pH 9	3%	5%	1:25	Overnight, 4°C
Y ⁸⁶¹ FAK	Rabbit Invitrogen	Citrate Buffer pH 6	3%	5%	1:200	Overnight, 4°C

Table 4.1 demonstrates an overview of the staining methodologies for the various proteins.

4.2.3 Weighted Histoscore

Protein expression for tumour cells was assessed over the full tissue specimen using the weighted histoscore method [520]. The weighted histoscore grades staining intensity as negative (0), weak (1), moderate (2), and strong (3), then multiplication of the percentage of tumour cells within each category. This is calculated using the following formula, 0 * the % of negative tumour cells + 1 * the % of cells staining weakly positive + 2 * the % of cells staining moderately positive + 3 * the % of cells staining strongly positive.

Two observers (TQ and PM) independently scored tissue sections. Results ranged from 0 to 300. Results were considered discordant if there was a difference of more than 50 between the two scores. In this case the tissue section was re-evaluated by both observers. Each cellular location was independently assessed for any evidence of expression (Figure 4.2). Bland-Altman plots were constructed to ensure no bias between observers existed (Figures 4.3-4.6) and ICC's calculated to demonstrate consistency (Table 4.2).

Tumours were then divided into those with high histoscore (above median) or low histoscore expression (below or equal to the median) for the purposes of this analysis as has previously been reported [336, 360, 521]. ICC was utilised to assess agreement

between observers and this was excellent (>0.8) for all proteins investigated as demonstrated in Table 4.2. Given the excellent ICCC for the protein Src when all tissues were independently scored at the various cellular locations, it was felt that for the remaining proteins that the second observer score performed by PM was only required for a third of the patients.

Figure 4.2: Demonstrating the different cellular locations that were assessed.

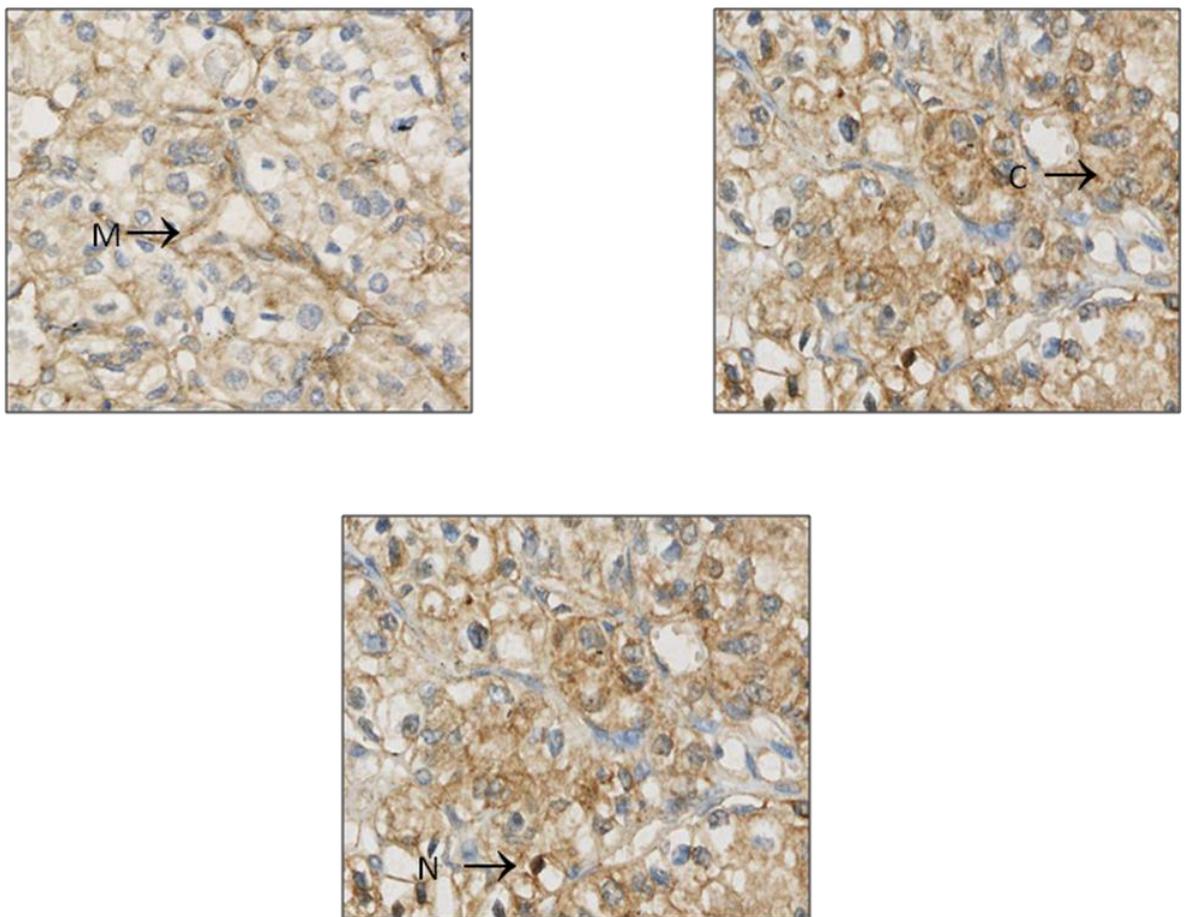


Figure 4.2 demonstrates positive staining of the various cellular locations that were assessed for any evidence of expression.

Table 4.2: ICC scores for each protein expression analysis.

ICC	Nuclear	Cytoplasmic	Membrane
c- <i>Src</i>	0.94	0.95	0.95
Y ⁵²⁷ <i>Src</i>	0.96	0.94	0.93
Y ⁴¹⁶ <i>Src</i>	0.94	0.91	0.95
Y ⁸⁶¹ <i>FAK</i>	0.89	0.88	0.92

Table 4.2 demonstrates excellent agreement between observers as demonstrated by ICC scores >0.8.

To further ensure the agreement between the two observers, Bland-Altman plot were constructed for the different proteins at the various cellular locations (Figures 4.3-4.6).

Figure 4.3: Bland-Altman plots for c-Src antibody at the various cellular locations.

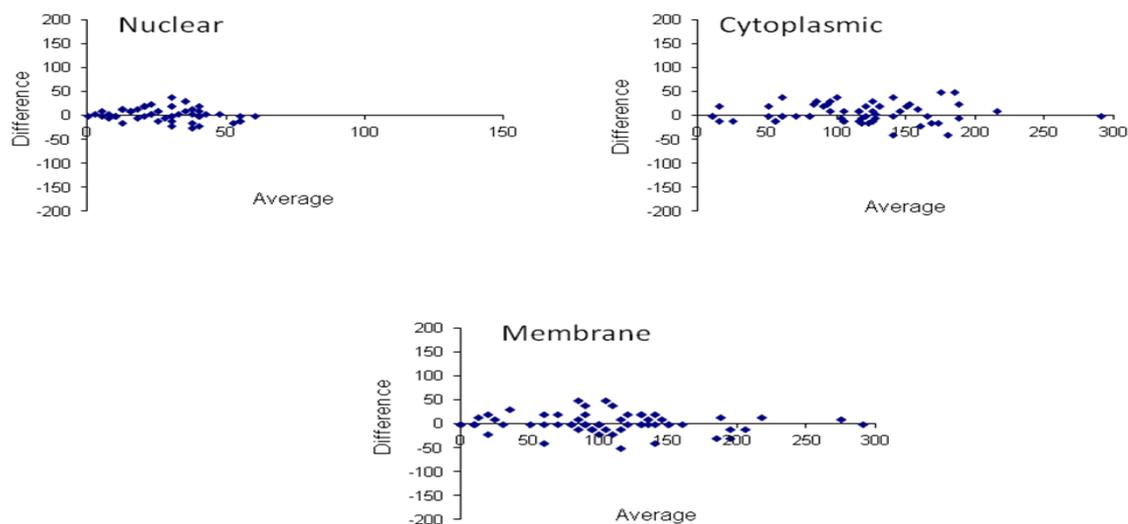


Figure 4.3 demonstrates excellent concordance between observers for c-Src scoring.

Figure 4.4: Bland-Altman plots for Y⁵²⁷ Src antibody at the various cellular locations.

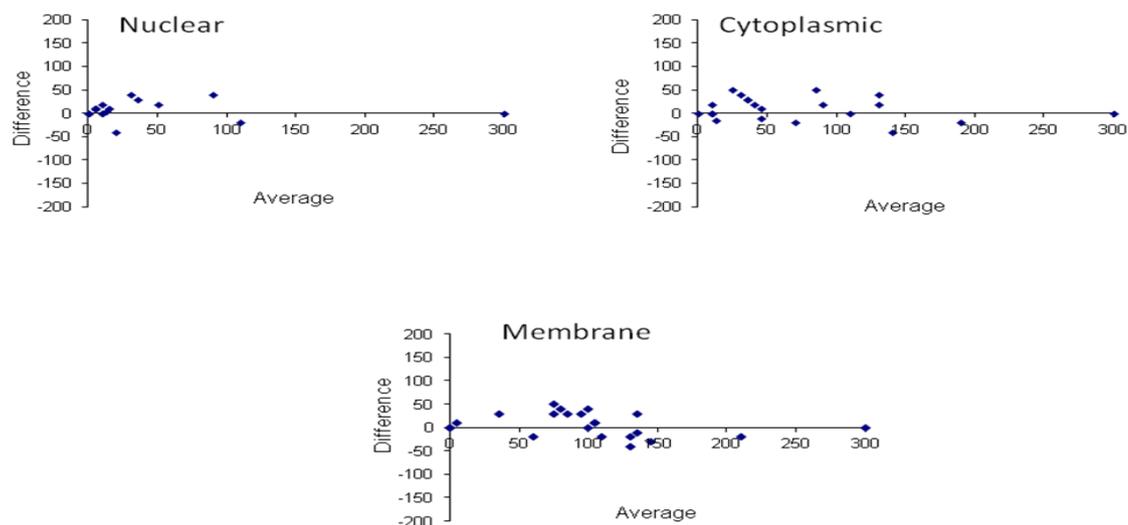


Figure 4.4 demonstrates excellent concordance between observers for Y⁵²⁷ Src scoring.

Figure 4.5: Bland-Altman plots for Y⁴¹⁶ Src antibody at the various cellular locations.

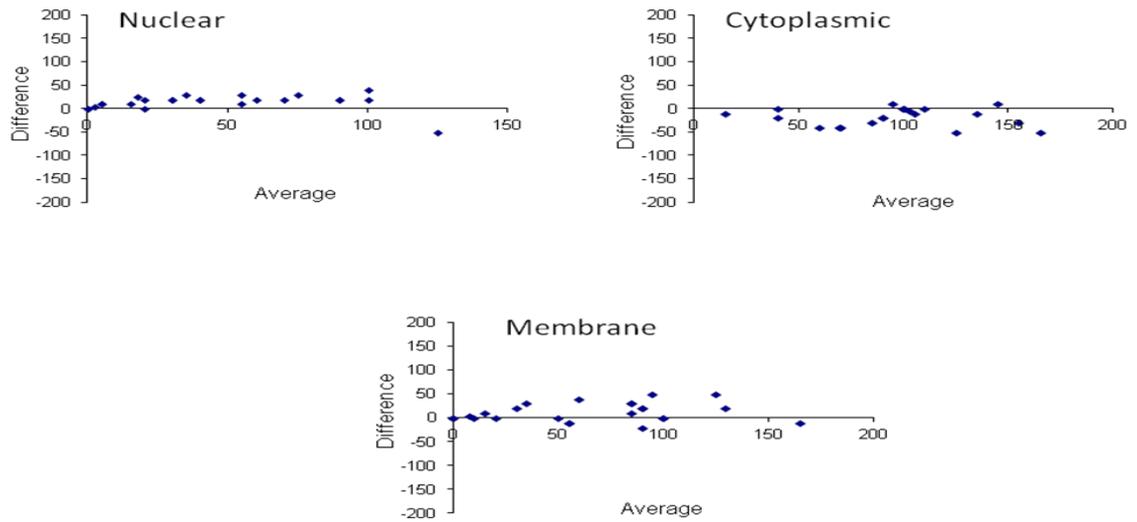


Figure 4.5 demonstrates excellent concordance between observers for Y⁴¹⁶ Src scoring.

Figure 4.6: Bland-Altman plots for Y⁸⁶¹ FAK antibody at the various cellular locations.

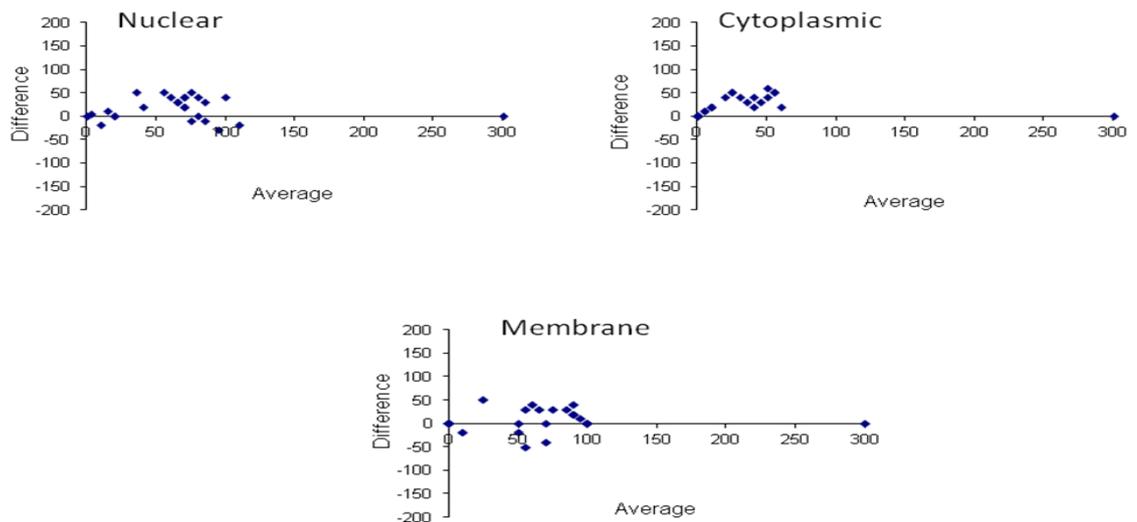


Figure 4.6 demonstrates excellent concordance between observers for Y⁸⁶¹ FAK scoring.

4.3 Statistical Analysis

This was undertaken using SPSS (Chicago, IL, USA). Cancer specific survival rates were generated using the Kaplan-Meier method. The log rank test was utilised to compare

significant differences between subset groups using univariate analysis. Multivariate analysis was carried out based on the results of the univariate analysis. Multivariate Cox regression analysis was performed to identify those factors that were independently associated with cancer specific death. A stepwise backward procedure was utilised to ascertain which of the variables had a significant independent relationship with survival. Chi squared (χ^2) analysis was utilised to assess relationships between pathological parameters and the biomarkers at the various locations. Pearson correlation was utilised to assess if relationships could be identified between the various proteins at the various cellular locations. p-values < 0.004 were deemed significant according to Bonferroni's correction.

4.4 Results

As mentioned earlier, antibody validation was performed prior to performing immunohistochemistry.

Figure 4.7: Western Blot specificity for c-Src.

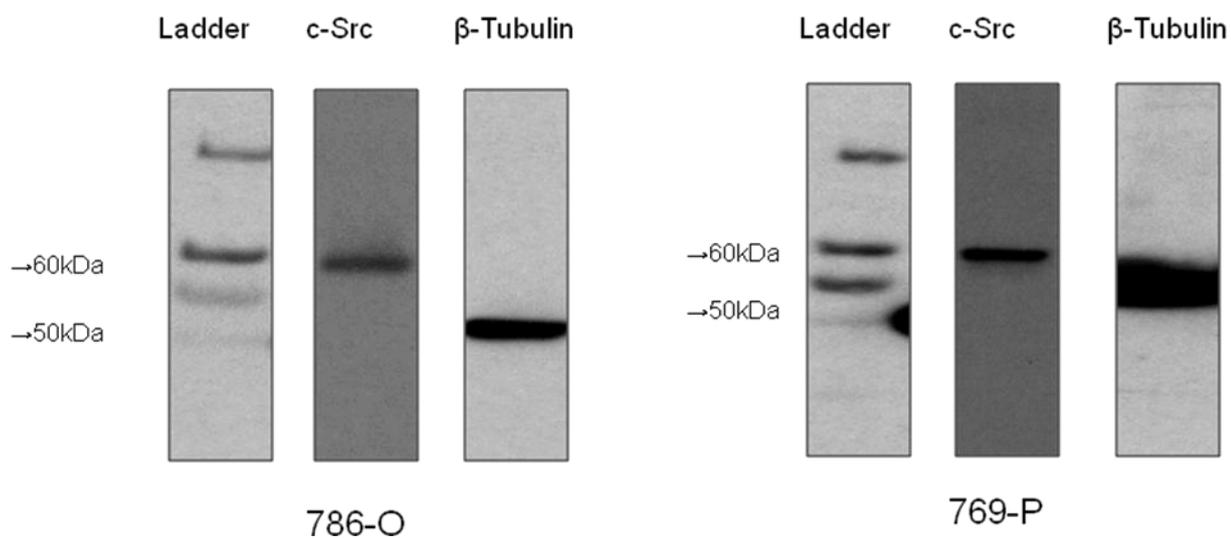


Figure 4.7 demonstrates Western Blot for c-Src (60kDa) and β -Tubulin (50kDa) as a protein loading control which demonstrates the specificity for the c-Src antibody on renal cell cancer cell line lysates.

Figure 4.8: Western Blot specificity for Y⁵²⁷.

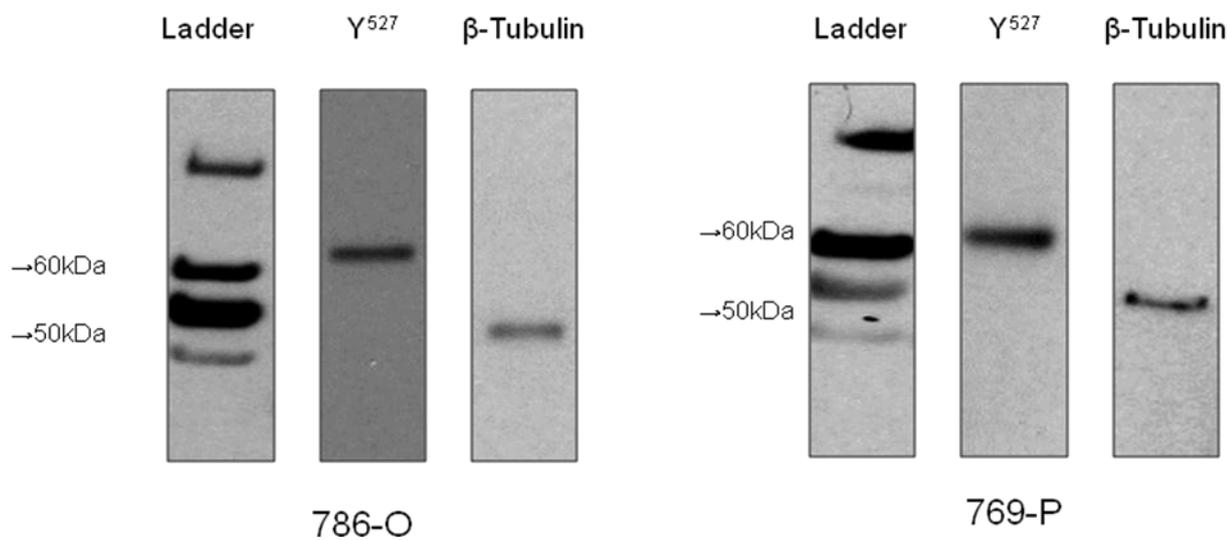


Figure 4.8 demonstrates Western Blot for Y⁵²⁷ (60kDa) and β -Tubulin (50kDa) as a protein loading control which demonstrates the specificity for the Y⁵²⁷ antibody on renal cell cancer cell line lysates.

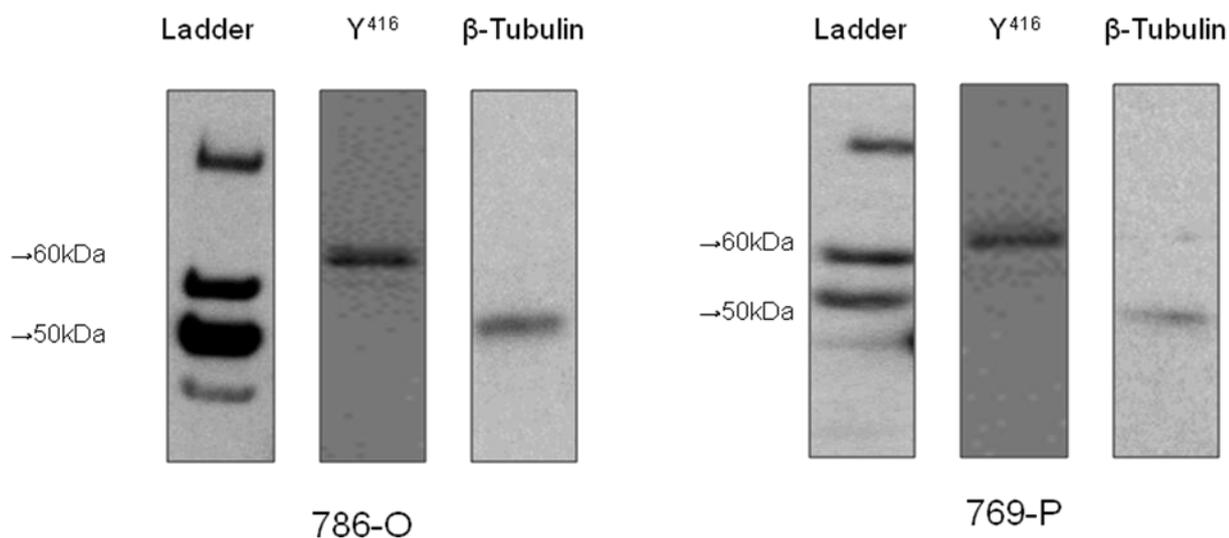
Figure 4.9: Western Blot specificity for Y⁴¹⁶.

Figure 4.9 demonstrates Western Blot for Y⁴¹⁶ (60kDa) and β-Tubulin (50kDa) as a protein loading control which demonstrates the specificity for the Y⁴¹⁶ antibody on renal cell cancer cell line lysates.

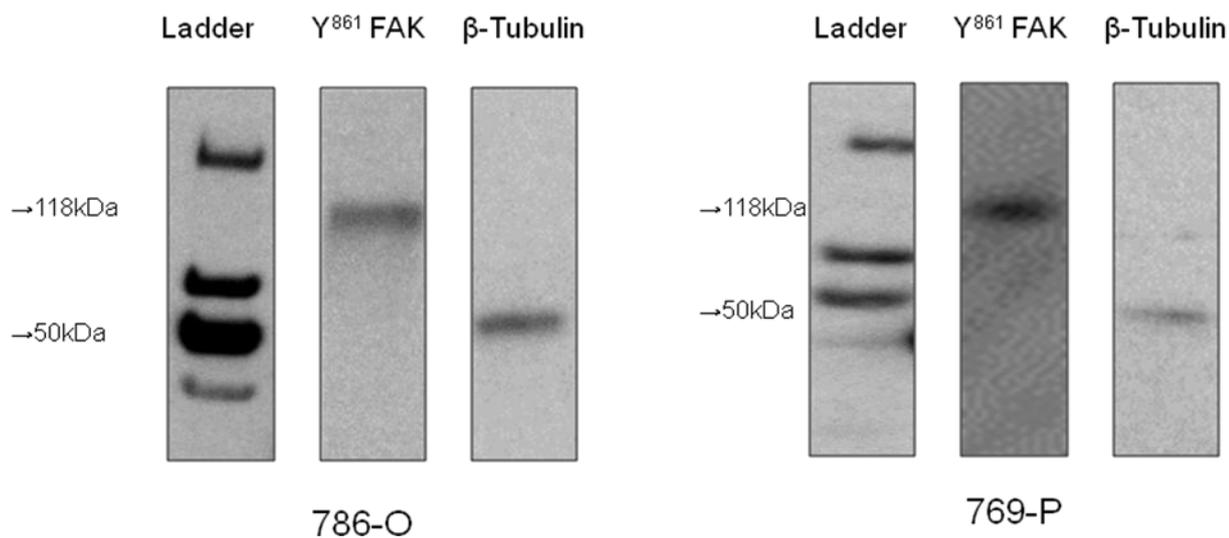
Figure 4.10: Western Blot specificity for Y⁸⁶¹ FAK.

Figure 4.10 demonstrates Western Blot for Y⁸⁶¹ FAK (118kDa) and β-Tubulin (50kDa) as a protein loading control which demonstrates the specificity for the Y⁸⁶¹ FAK antibody on renal cell cancer cell line lysates.

IHC analysis was based on 57 clear cell renal cancer patients with full clinical follow up. Table 4.3 demonstrates clinicopathological parameters of these patients. Median age at diagnosis was 60 years (range 41-80). Median follow up was 107 months (range 0.1-163). Twenty two patients died of their disease. Thirty eight patients had T1/2 disease and nineteen patients had T3/4 disease. Tumour volume was recorded for each case; median tumour volume was 9.8cm³ (range 1-30 cm³). Twenty five patients subsequently had evidence of recurrence on radiological imaging.

Table 4.3: Clinicopathological characteristics of patients utilised for IHC.

<i>Variable</i>	<i>IHC Cohort</i>
Age (<60/>60)	29/28
Sex (M/F)	32/25
T Stage (T1/T2/T3/T4)	26/12/17/2
Grade (1/2/3/4)	10/26/15/6
Tumour Necrosis (absence/presence)	32/25
Recurrence (No/Yes)	32/25
Tumour Volume (<10cm ³ / ^{>} 10cm ³)	29/28

Table 4.3 demonstrates the clinicopathological characteristics of the cohort.

Initial analysis was performed on clinicopathological features which are known prognostic indicators for survival in renal cancer. T stage and nuclear grading were significantly associated with poor prognosis, thus demonstrating that this cohort was associated with classical clinical parameters and validating it for use in a biomarker study (Table 4.4).

Table 4.4: Interrelationships between clinicopathological characteristics of patients and protein expression/activation to cancer specific survival.

<i>Variable</i>	<i>Univariate Analysis</i>		<i>Multivariate Analysis</i>	
	Numbers	p value	p value	HR
Age (<60/>60)	29/28	0.201		
T Stage (T1/T2/T3/T4)	26/12/17/2	0.001	0.001	2.35 (1.41-3.91)
Grade (1/2/3/4)	10/26/15/6	0.012		
Tumour Necrosis (absence/presence)	32/25	0.092		
c-Src nuc (negative/positive)	31/26	0.437		
c-Src cyto (negative/positive)	32/25	0.95		
c-Src mem (negative/positive)	29/28	0.097		
Y527 Src nuc (negative/positive)	34/23	0.72		
Y527 Src cyto (negative/positive)	31/26	0.968		
Y527 Src mem (negative/positive)	29/28	0.329		
Y416 Src nuc (negative/positive)	29/28	0.799		
Y416 Src cyto (negative/positive)	32/25	0.311		
Y416 Src mem (negative/positive)	29/28	0.79		
Y861 Fak nuc (negative/positive)	29/28	0.489		
Y861 Fak cyto (negative/positive)	36/21	0.001	0.006	3.35 (1.40-7.98)
Y861 Fak mem (negative/positive)	30/27	0.678		

Table 4.4 demonstrates the association of known clinicopathological parameters and protein expression/activation to cancer specific survival.

c-Src kinase

As c-Src was the highest expressed of all the family members at the mRNA level, expression at the protein level was further investigated. Specificity of the antibody was tested and confirmed by Western Blot analysis on renal cell cancer cell lines 786-O and 769-P as described earlier (Figure 4.7).

Of the tumours investigated, 98% showed some degree of nuclear expression, 100% showed some degree of cytoplasmic expression and 97% showed some degree of membrane expression. The cellular distribution of c-Src is demonstrated as histograms (Figure 4.11).

Figure 4.11: Histograms for c-Src expression.

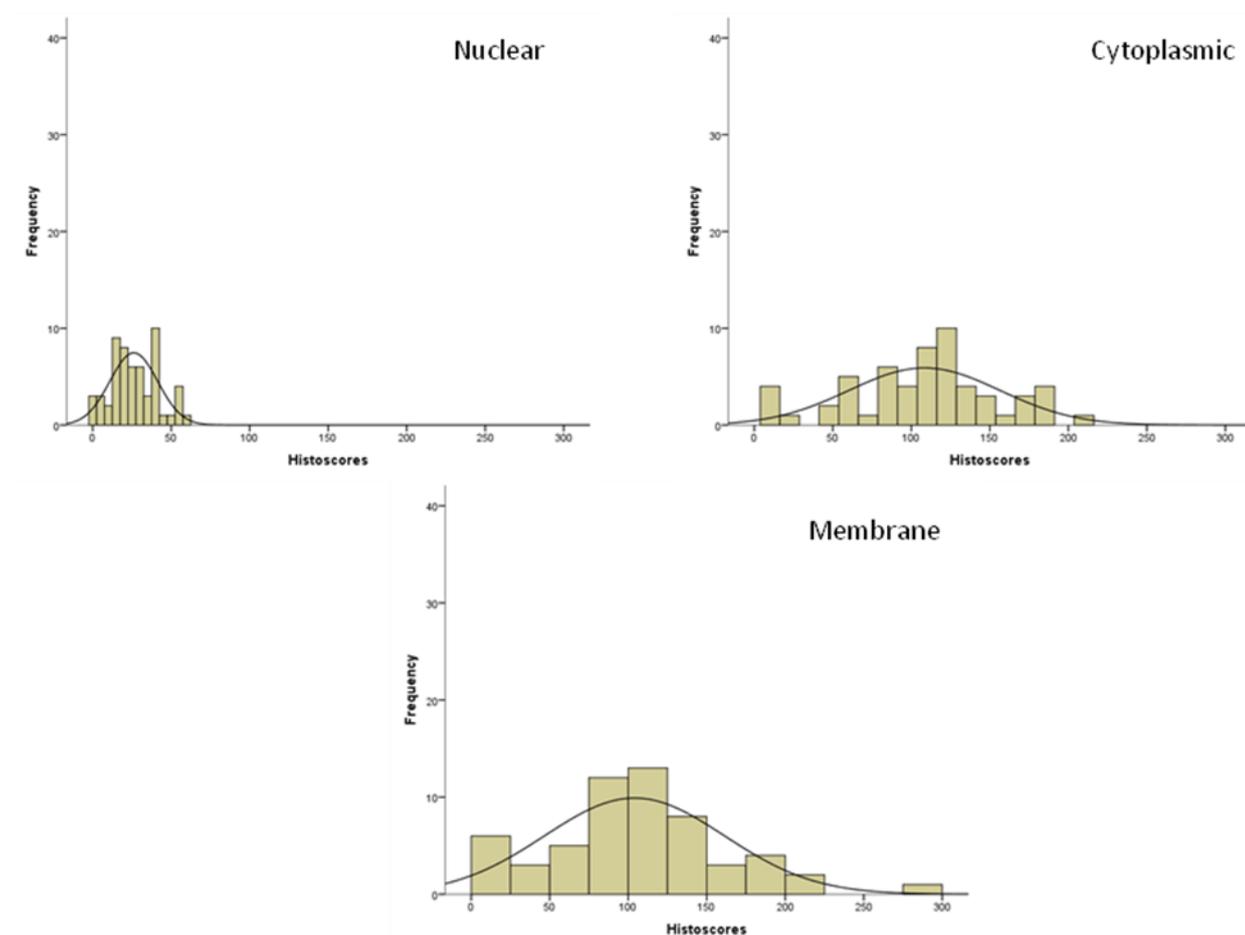


Figure 4.11 demonstrates histograms for nuclear, cytoplasmic and membrane c-Src expression demonstrating the intensity of staining.

For the purposes of analysis, the median cut off values of expression at the different cellular locations was utilised, those below and equal to the median score were given a low expression and those above the median were given a score of high as mentioned in the methodology. For nuclear expression this value was 25, cytoplasmic expression was 115 and membrane 100. χ^2 analysis demonstrated that cytoplasmic c-Src kinase expression positively correlated with nuclear grade ($p=0.023$, Table 4.5) but no correlation was demonstrated with age, T stage, tumour necrosis, recurrence or tumour volume. Membrane c-Src kinase protein expression negatively correlated with recurrence ($p=0.021$, Table 4.5)

but no relationship was demonstrated with age, T stage, nuclear grade, tumour necrosis or tumour volume. These results suggest that membrane c-Src confers good prognosis, however on univariate analysis expression of c-Src at the different cellular locations did not show significance (Table 4.4). However a trend was observed between high membrane c-Src kinase expression and improved disease specific survival, those patients expressing high membrane c-Src kinase had a median survival of 130 months compared to 97 months for those with low expression, this did not however reach significance ($p=0.097$, Figure 4.12). Pearson correlation demonstrated that cytoplasmic c-Src kinase expression demonstrated a positive correlation with nuclear c-Src kinase expression ($p=0.0022$, Table 4.6).

Table 4.5: Interrelationships between clinicopathological characteristics of patients and protein expression/activation using χ^2 (chi squared) analysis.

<i>Variable</i>	<i>Numbers</i>	<i>Age</i>	<i>T Stage</i>	<i>Grade</i>	<i>Tumour Necrosis(absence/presence)</i>	<i>Recurrence (No/Yes)</i>	<i>Tumour Volume (<10cm3/>10cm3)</i>
c-Src nuc (negative/positive)	31/26	0.350	0.187	0.500	0.830	0.196	0.350
c-Src cyto (negative/positive)	32/25	0.703	0.538	0.023	0.985	0.985	0.703
c-Src mem (negative/positive)	29/28	0.148	0.205	0.916	0.363	0.021*	0.692
Y527 Src nuc (negative/positive)	34/23	0.487	0.157	0.793	0.623	0.962	0.487
Y527 Src cyto (negative/positive)	31/26	0.144	0.447	0.708	0.751	0.749	0.684
Y527 Src mem (negative/positive)	29/28	0.026	0.335	0.916	0.882	0.358	0.513
Y416 Src nuc (negative/positive)	29/28	0.692	0.889	0.916	0.227	0.701	0.513
Y416 Src cyto (negative/positive)	32/25	0.227	0.056	0.443	0.985	0.289	0.083
Y416 Src mem (negative/positive)	29/28	0.026	0.667	0.686	0.703	0.222	0.897
Y861 Fak nuc (negative/positive)	29/28	0.897	0.478	0.482	0.703	0.701	0.692
Y861 Fak cyto (negative/positive)	36/21	0.207	0.023	0.001	0.326	0.036	0.045
Y861 Fak mem (negative/positive)	30/27	0.150	0.702	0.753	0.540	0.933	0.361

Table 4.5 demonstrates the interrelationships between clinicopathological characteristics of patients and protein expression/activation (* denotes inverse relationship).

Figure 4.12: Kaplan Meier plotted for membrane c-Src expression.

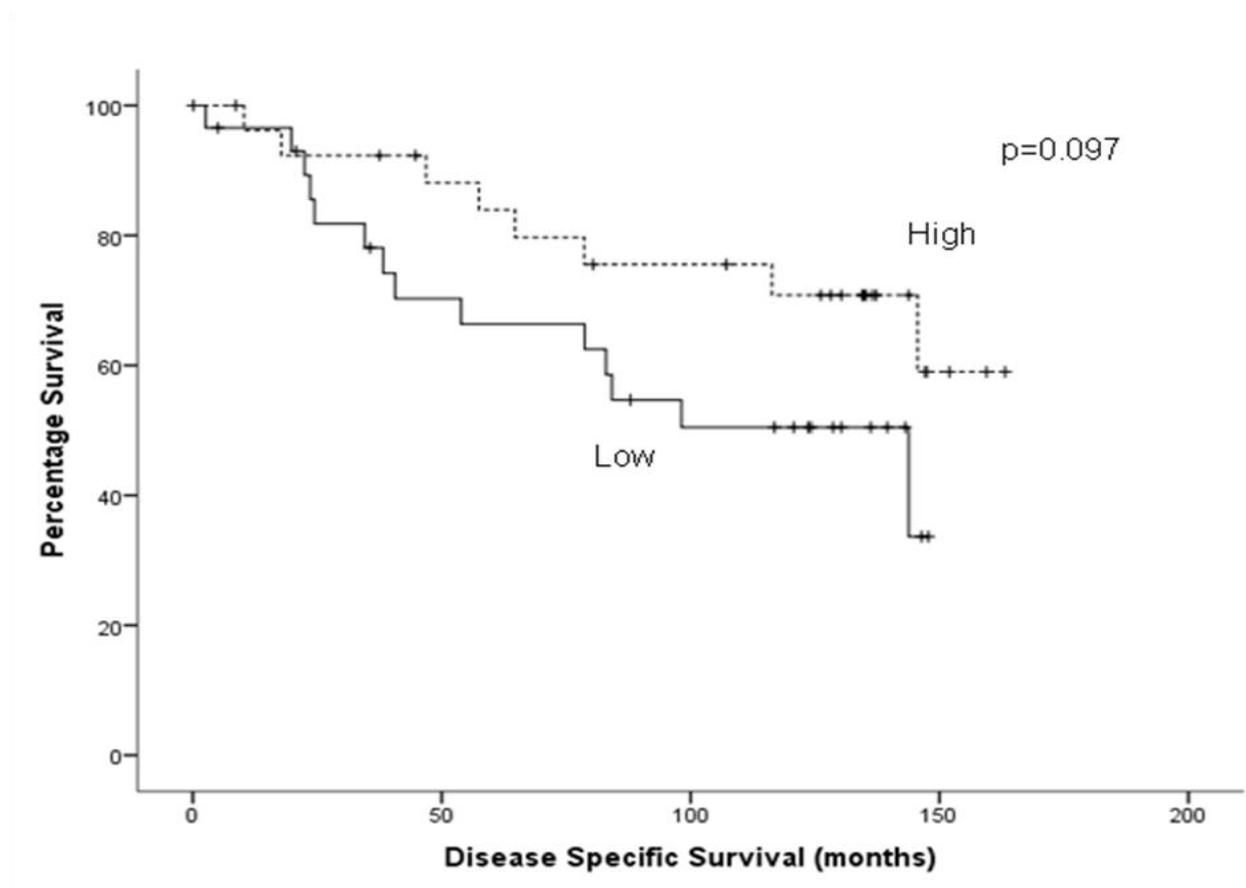


Figure 4.12 demonstrates that those with high expression of membrane c-Src were associated with increased disease specific survival but did not reach significance.

Table 4.6 demonstrates the interrelationships between the various cellular locations of protein expression/activation. PC-Pearson Correlations, Sig-Significance (2-tailed), ns-non significant p-values (figures in bold denote significant p values as denoted by Bonferronis correction ($p < 0.004$)).

Y⁵²⁷ Src Kinase

Phosphorylated Src Y⁵²⁷ was investigated. Specificity of the antibody was tested and confirmed by Western Blot analysis on renal cell cancer cell lines 786-O and 769-P as described earlier (Figure 4.8).

Of the tumours investigated 47% showed some degree of nuclear expression, 96% showed some degree of cytoplasmic expression and 93% showed some degree of membrane expression. The cellular distribution of Y⁵²⁷ Src is demonstrated as histograms (Figure 4.13).

Figure 4.13: Histograms for Y⁵²⁷ Src expression.

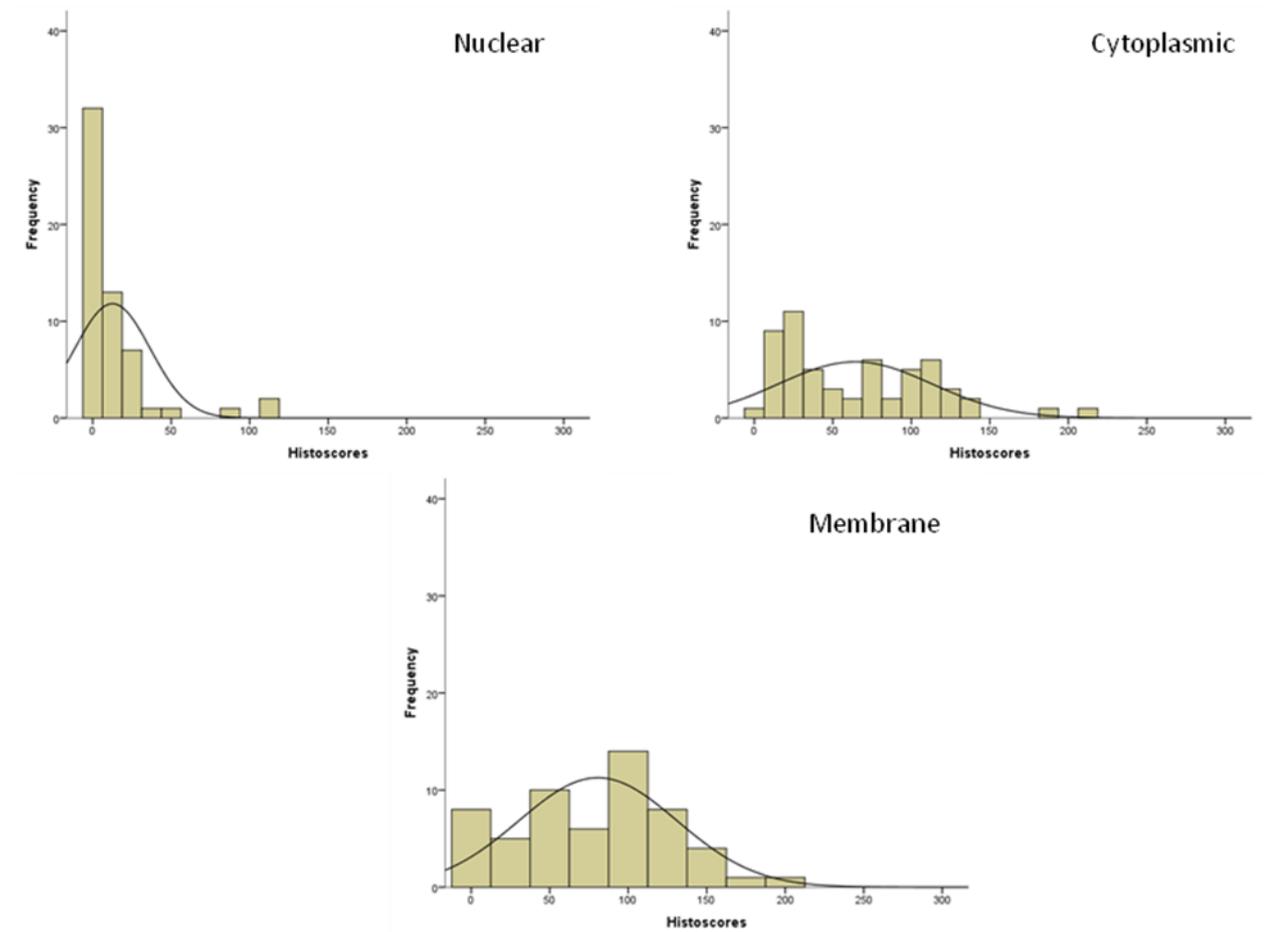


Figure 4.13 demonstrates histograms for nuclear, cytoplasmic and membrane Y⁵²⁷ Src expression demonstrating the intensity of staining.

There was a difference in the expression of Y⁵²⁷ at the different cellular locations in comparison to c-Src expression with this difference possible as a result of tissue heterogeneity and therefore tissue expression variability as well as c-Src expression encompassing total expression of c-Src in its various phosphorylated forms. The median cut off values for the purposes of analysis were 0 for nuclear expression, 50 for cytoplasmic and for membrane 85. χ^2 analysis demonstrated that phosphorylated membrane Y⁵²⁷ expression positively correlated with age ($p=0.026$, Table 4.5) but no correlation was demonstrated with T stage, nuclear grade, tumour necrosis, recurrence or

tumour volume. On univariate analysis, expression of Y⁵²⁷ at any of the cellular locations investigated was not significantly associated with disease specific survival (Table 4.4). Pearson correlation demonstrated that phosphorylated cytoplasmic Y⁵²⁷ expression demonstrated a positive correlation with cytoplasmic c-Src kinase expression (p=0.0022, Table 4.6).

Y⁴¹⁶ Src Kinase

The autophosphorylated status at Y⁴¹⁶ was investigated. Specificity of the antibody was tested and confirmed by Western Blot analysis on renal cell cancer cell lines 786-O and 769-P as described earlier (Figure 4.9).

Of the tumours investigated 84% showed some degree of nuclear expression, 100% showed some degree of cytoplasmic expression and 81% showed some degree of membrane expression. The cellular distribution of Y⁵²⁷ Src is demonstrated as histograms (Figure 4.14).

Figure 4.14: Histograms for Y⁴¹⁶ Src expression.

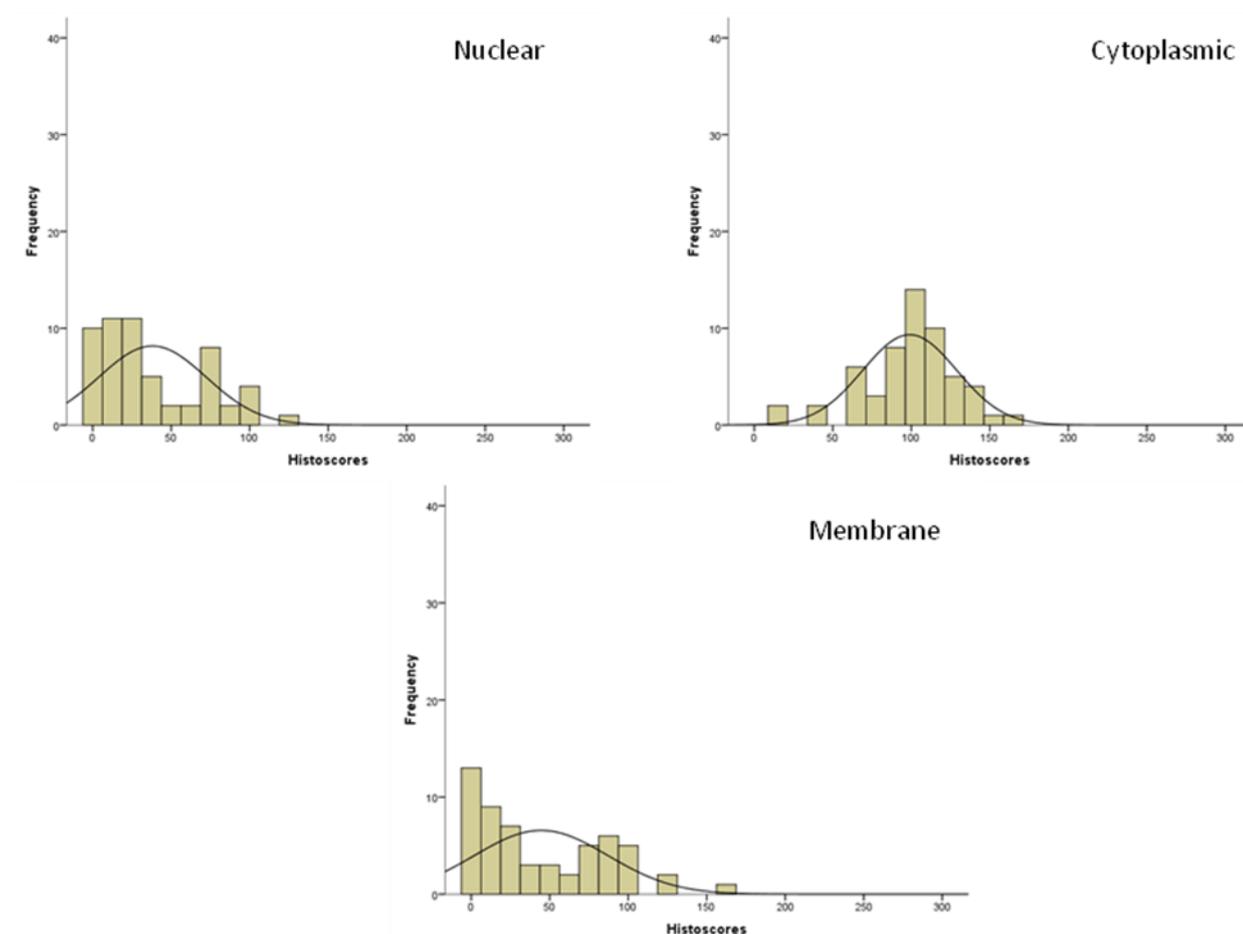


Figure 4.14 demonstrates histograms for nuclear, cytoplasmic and membrane Y⁴¹⁶ Src expression demonstrating the intensity of staining.

The median cut off values for the purposes of analysis were 20 for nuclear expression, 100 for cytoplasmic and for membrane 20. χ^2 analysis demonstrated that membrane Y⁴¹⁶ expression positively correlated with age ($p=0.026$, Table 4.5) but no correlation was demonstrated with T stage, nuclear grade, tumour necrosis, recurrence or tumour volume. On univariate analysis, expression of Y⁴¹⁶ at any of the cellular locations investigated was not associated with disease specific survival (Table 4.4). Pearson correlation demonstrated that membrane Y⁴¹⁶ expression demonstrated a negative correlation with cytoplasmic c-Src

kinase expression ($p=0.0005$, Table 4.6) and a positive correlation was demonstrated with phosphorylated membrane Y^{527} expression ($p=0.0001$, Table 4.6).

Y^{861} FAK

The autophosphorylated status at Y^{861} FAK was investigated. Specificity of the antibody was tested and confirmed by Western Blot analysis on renal cell cancer cell lines 786-O and 769-P as described earlier (Figure 4.10).

Of the tumours investigated 98% showed some degree of nuclear expression, 39% showed some degree of cytoplasmic expression and 100% showed some degree of membrane expression. The cellular distribution of Y^{861} FAK is demonstrated as histograms (Figure 4.15).

Figure 4.15: Histograms for Y⁸⁶¹ FAK expression.

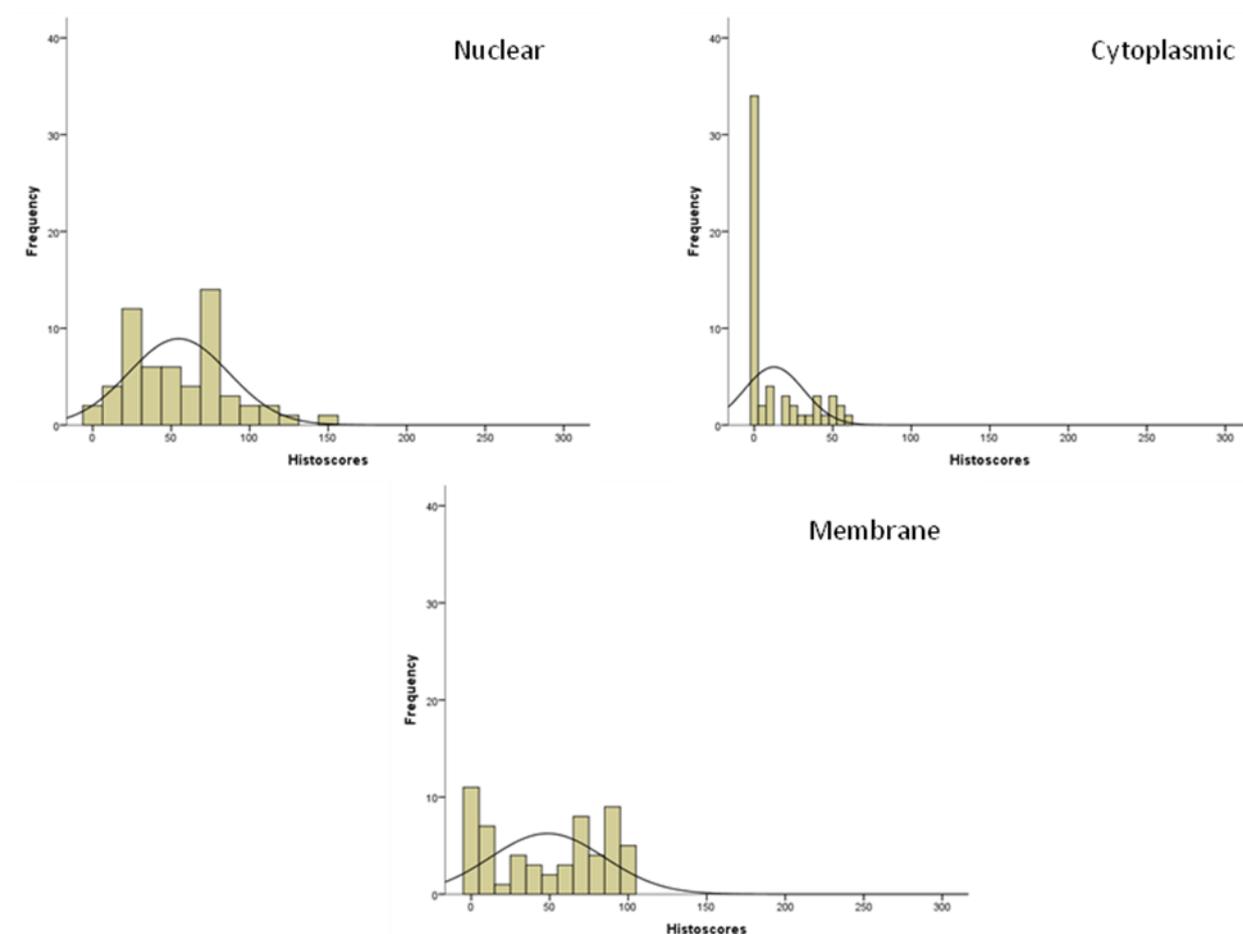


Figure 4.15 demonstrates histograms for nuclear, cytoplasmic and membrane Y⁸⁶¹ FAK expression demonstrating the intensity of staining.

The median cut off values for the purposes of analysis were 50 for nuclear expression, 0 for cytoplasmic and for membrane 50. χ^2 analysis demonstrated that expression of cytoplasmic FAK Y⁸⁶¹ demonstrated a positive relationship with T stage, nuclear grade, recurrence and tumour volume ($p=0.023$, $p=0.001$, $p=0.036$ and $p=0.045$ respectively, Table 4.5) but no relationship was demonstrated with age or tumour necrosis. On univariate analysis, high expression of cytoplasmic FAK Y⁸⁶¹ was associated with decreased cancer specific survival, ($p=0.001$, Table 4.4, Figure 4.16). This association was also independent on multivariate analysis (HR 3.35, 95% CI 1.40-7.98, $p=0.006$, Table

4.4). Pearson correlation demonstrated that membrane FAK Y⁸⁶¹ expression demonstrated a positive correlation with membrane Y⁵²⁷ and membrane Y⁴¹⁶ expression (p=0.0002 and p=0.0002, Table 4.6).

Figure 4.16: Kaplan Meier plotted for cytoplasmic Y⁸⁶¹ FAK expression.

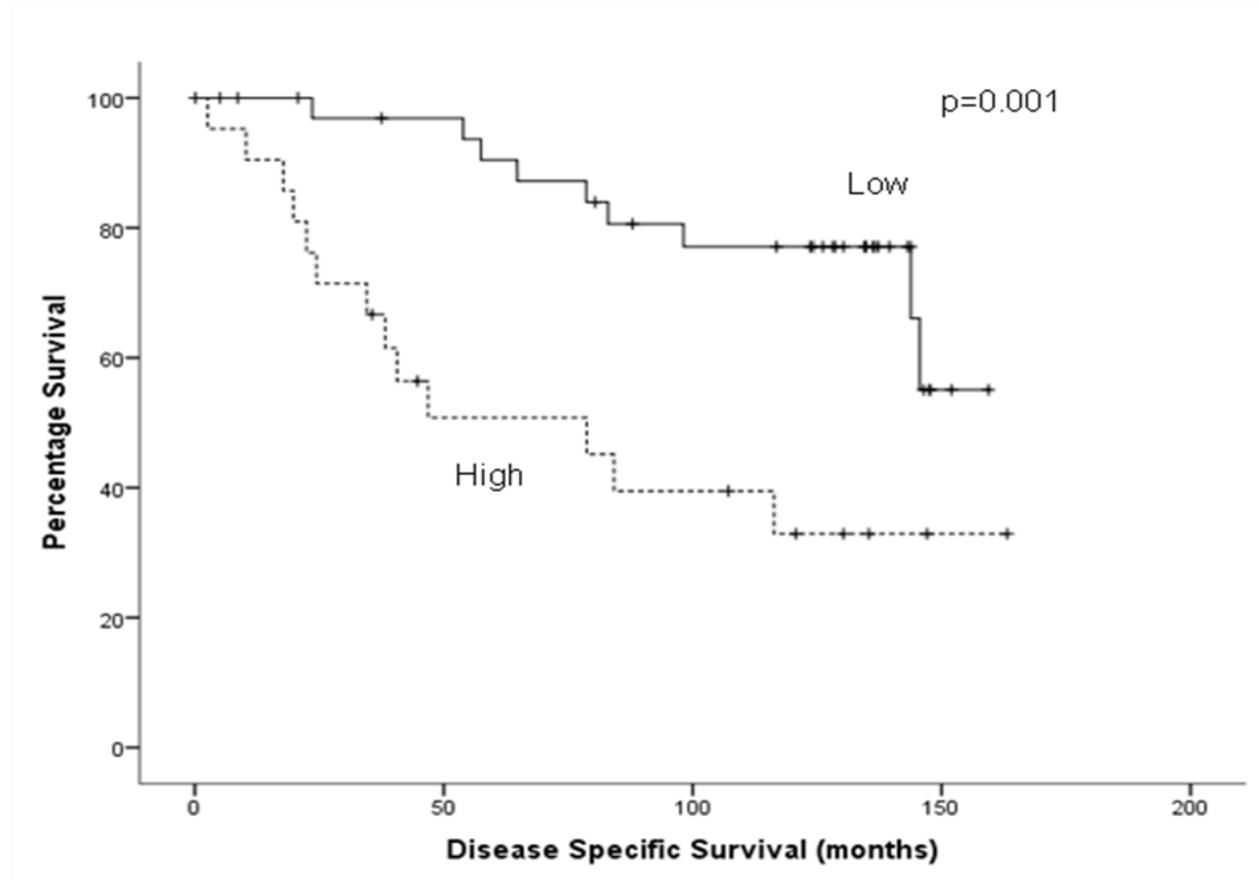


Figure 4.16 demonstrates that those with high expression of cytoplasmic Y861 FAK were associated with decreased disease specific survival.

4.5 Discussion

SFK can be localised at different cellular locations with the inactive form localised at the perinuclear region of cells [399]. Much work has been conducted in breast cancer which has reported high cytoplasmic Src kinase levels being associated with shorter disease specific survival [339] and high expression of activated Src kinase being associated with

increased risk of recurrence [336]. In addition, prostate cancer studies have demonstrated that Src kinase expression and activation is associated in quicker time to relapse and shorter disease specific survival [348]. Therefore current literature supports a role for Src as a negative prognostic marker in solid tumours.

In contrast in the current study, χ^2 analysis, demonstrates that membrane Src kinase expression was negatively associated with recurrence and a trend towards improved survival was also observed. These results require confirming in a larger patient cohort; however the combination of reduced recurrence and improved survival being associated with Src kinase expression suggests that Src kinase might not be responsible for driving progression in renal cancer. Although Src expression itself might not be involved with renal cancer progression, activation of the Src or other Src family members might be involved.

It has been suggested that a biomarker for prediction of Src kinase activity would be to measure phosphorylation of the protein at a site associated with activity [363, 522].

Currently there are two sites within Src known to be associated with activation.

Phosphorylation of the Y⁵³⁰ on the c-terminal tail by Csk tyrosine kinase acts as a negative regulatory protein-binding site, keeping Src kinase in a closed conformation [523]. Upon dephosphorylation this allows Src kinase to undergo a transformational change allowing the second site of activation, Y⁴¹⁹, to be accessible which requires to be autophosphorylated for full activation of Src kinase. Y⁴¹⁹ is referred to as the classical site and is the most commonly used in cell line studies investigating the functional relevance of Src kinase activation [475]. In this study, membrane Y⁴¹⁶ expression demonstrated a negative correlation with cytoplasmic Src kinase and a positive correlation with membrane Y⁵²⁷, confirming validity of results regarding Src kinase needing to undergo dephosphorylation at the Y⁵³⁰ before autophosphorylation at the Y⁴¹⁹ can proceed.

Activated phosphorylated Src kinase at the classical site Y⁴¹⁶ when assessed at individual locations was shown to have no correlation to disease specific survival. This antibody is

not specific for one particularly Src family member as this part of the sequence is highly conserved between Src family members making it impossible to produce an antibody specific for only one member. Therefore as the antibody cross reacts with all family members that are phosphorylated at this site, this could account for the lack of correlation with disease specific survival.

Autophosphorylation of FAK at the Y³⁹⁷ site occurs as a result of many stimuli thereby creating a high affinity binding site for the Src homology 2 domain of several proteins including Src kinase [453, 454]. Src phosphorylates FAK at several sites but it has been reported that the Y⁸⁶¹ is the major site on the FAK domain associated with activation [455], and therefore maybe used as a surrogate marker not only for SFK activation but also for SFK function [459].

Expression of cytoplasmic Y⁸⁶¹ was associated with reduced disease specific survival and positively correlated with T stage, nuclear grade, recurrence and tumour volume suggesting that the phosphorylation of Y⁸⁶¹ may be associated with more aggressive renal cancers. Expression of membrane Y⁸⁶¹ positively correlated with membrane Y⁴¹⁶ further suggesting that of the Src kinase family members, c-Src kinase may not be responsible for the phosphorylation at the Y⁸⁶¹ site in clear cell renal cancer, therefore implicating a role for an alternative SFK member in renal cancer progression.

We have demonstrated that activation of c-Src kinase itself is associated with improved survival but the presence of the downstream marker FAK Y⁸⁶¹, a surrogate marker for SFK member activation, is associated with decreased cancer specific survival suggesting that another of the SFK members is responsible for poor survival in RCC.

Given the introduction of SFK inhibitors in metastatic/recurrent RCC and the findings that c-Src is the most highly expressed SFK in RCC and furthermore expression may confer improved survival, it is important to assess the effects of these inhibitors in RCC to ensure that they are utilised in a safe clinical manner if at all. To assess this, further work is required investigating the functional effect of these inhibitors on renal cell carcinoma cells

and establish if they are eliciting their effects via a Src kinase specific mechanism or via others SFK's or signalling pathways. In the next chapter we will begin to try and unpick the mechanisms by which Src kinase inhibitors are currently being employed in renal cancer clinical trials to elicit their effects.

Chapter 5-Effects of SFK inhibitor Dasatinib on Src expression, SFK activity, FAK expression and cellular metabolic activity/apoptosis on RCC cell lines

5.1 Introduction

The previous chapters have demonstrated that SFK members are not only expressed in varying degrees but also that activation correlated to survival. It was demonstrated that expression of membrane c-Src was associated with improved survival and expression of cytoplasmic FAK Y⁸⁶¹ was associated with poor survival. This would suggest that another SFK member besides c-Src is responsible for the phosphorylation of FAK at Y⁸⁶¹ and therefore associated with poor survival.

With the introduction of SFK inhibitors being clinically trialled in those with metastatic renal cancer, it is important to assess which SFK they should target as c-Src inhibitors could be potentially harmful to those with high expression as this may confer improved survival. One of the SFK inhibitors is Dasatinib, this drug has been developed and utilised in clinical trials by Bristol Myer Squib. Dasatinib is a tyrosine kinase inhibitor of Abl, Src and SFKs and is a small molecule ATP competitive multikinase inhibitor [378]. It causes cell cycle arrest, prevents cell proliferation as well as inducing apoptosis and inhibiting metastases. Dasatinib binds to the SH1 domain of Src and inhibits its activity. Dasatinib is not specific to a particular SFK member. Dasatinib has been previously been demonstrated to inhibit SFK activity of c-Src, Lyn and FAK in prostate cancer [348] as well as inhibiting growth in breast cancer cell lines [475] and inducing cell cycle arrest and apoptosis in hepatocellular carcinoma cell lines [480].

To assess SFK inhibitors further, we aim to utilise RCC cancer cell lines (786-O, 769-P) to investigate the effects of Dasatinib on cellular metabolic activity and apoptosis as well as the impact on levels of c-Src, Y⁴¹⁶ Src and FAK Y⁸⁶¹ expression.

5.2 Materials and Methods

5.2.1 Culture of renal cancer cells

As described earlier in Chapter 4.

5.2.2 Counting of cells

Once cells were 80% confluent, they were harvested by trypsinisation as described earlier in Chapter 4. These cells were then counted utilising a haemocytometer and seeded according to assay that was to be performed. For Western blots, cells were seeded in 6-well plates with 2 mls of RPMI (5×10^4 cells/ml) and left to adhere overnight whilst for proliferation and apoptotic assays, 96-well plates were utilised where cells were seeded in 100 μ l RPMI (5×10^3 cells/well). Western blots were repeated in triplicates whilst proliferation and apoptosis were performed in triplicates and the experiment repeated four times.

5.2.3 Drug treatment of cells

Following overnight cell adherence, the media was replaced with increasing concentration of Dasatinib (Sigma Aldrich). Cells in which the medium alone was replaced and medium plus the drug vehicle (DMSO) in which the Dasatinib was reconstituted were utilised as controls. These cells were incubated for 48 hours.

5.2.4 Assessment of cellular metabolic activity

Cellular metabolic activity was assessed using the WST-1 assay (Water Soluble Tetrazolium Salts, Millipore, UK) following the manufacturer's protocol. Following

treatment with increasing concentrations of Dasatinib for 48 hours, 10 μ l of WST-1 was added to each well. This measures the metabolic activity of viable cells. Proliferating cells cleave the tetrazolium salt to formazan by cellular mitochondrial dehydrogenases. This increasing activity of mitochondrial dehydrogenases corresponds to an increase in formazan dye metabolism. This colouration is measured at an absorbance of 440nm using a standard multiwell spectrophotometer (UVSpectramax Molecular Devices).

5.2.5 Assessment of cell apoptosis

Following treatment with Dasatinib for 48 hours, apoptosis was assessed utilising the Cell Death ELISA^{PLUS} kit (Roche, UK) based on manufacturer's protocol. Cells were centrifuged for ten minutes at 1200rpm. The supernatant was transferred and lysed with lysis buffer for 30 minutes at 37°C. 200 μ l was then transferred to a streptavidin coated microplate. The immunoreagent was then added to the samples and incubated for two hours at 37°C on an orbital shaker at 200rpm. This immunoreagent consisted of biotinylated anti-histone and peroxidase-coupled anti-DNA antibodies. This allows the detection of mono and oligonucleosomes in the cytoplasmic fractions of cell lysates using biotinylated anti-histone and peroxidase-coupled anti-DNA antibodies. The wells are streptavidin coated with anti-histone and bind to the biotin coated histone antibodies forming the anti-histone and peroxidase complex. This complex only forms if apoptosis has occurred. The solution was gently removed from each well and the wells washed three times with incubation buffer. 100 μ l of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) was added to each well for fifteen minutes and absorbance measured at 405nm and 490nm using a standard multiwell spectrophotometer (UVSpectramax Molecular Devices). The enrichment factor was calculated as the absorbance of the dead-dying cells/absorbance of corresponding negative control.

5.2.6 Western Blot Analysis

This was performed as described earlier in Chapter 4.

5.3 Statistical Analysis

Cellular metabolic activity and apoptosis were assessed using a 2 way ANOVA followed by Bonferroni's post hoc test to assess for significant differences and to correct for multiple conditions. For the purposes of analysis Western blots, ImageJ software was utilised to assess the density of the bands following which a 2 way ANOVA was utilised to assess this difference.

5.4 Results

5.4.1 Cellular Metabolic Activity and Apoptosis

As mentioned earlier, two RCC cell lines were utilised, 786-O and 769-P. Metabolic activity and apoptotic assays were performed in triplicates and repeated in four independent experiments.

Figure 5.1: Demonstrating the effect of increasing concentrations of Dasatinib on cellular metabolic activity on 769-P and 786-O cell lines.

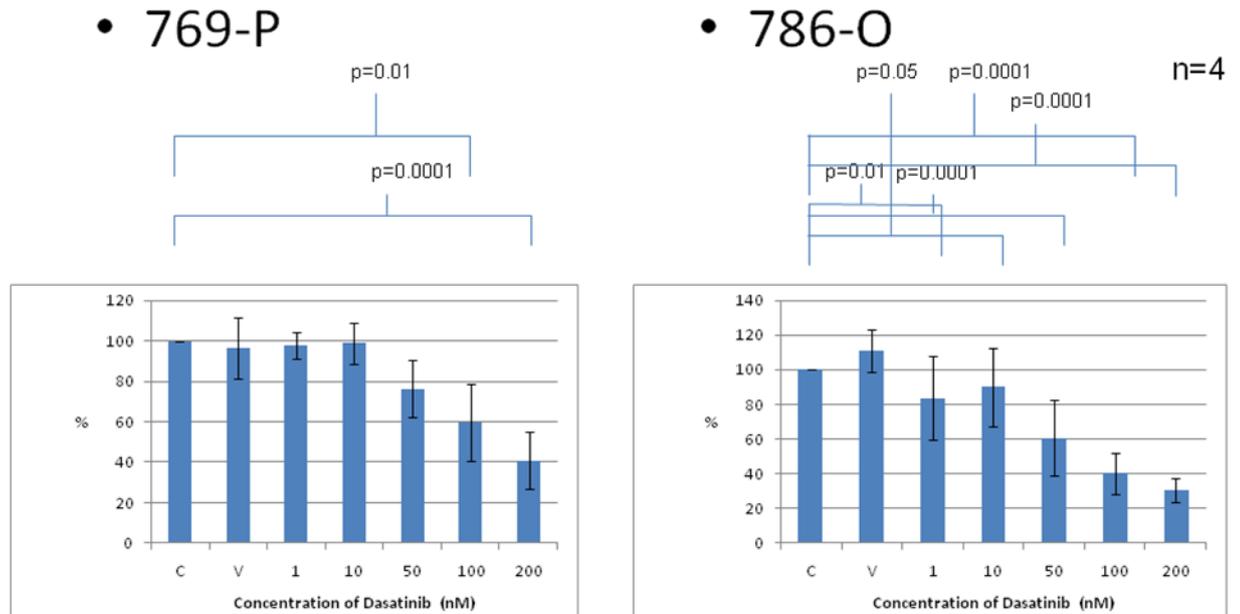


Figure 5.1 demonstrates that cellular metabolic activity is inhibited with increasing doses of Dasatinib in both RCC cell lines and the p-values for those concentration where a significant difference was demonstrated between the control and the concentration of Dasatinib.

Figure 5.2: Demonstrating the effect of increasing concentrations of Dasatinib on cellular apoptosis on 769-P and 786-O cell lines.

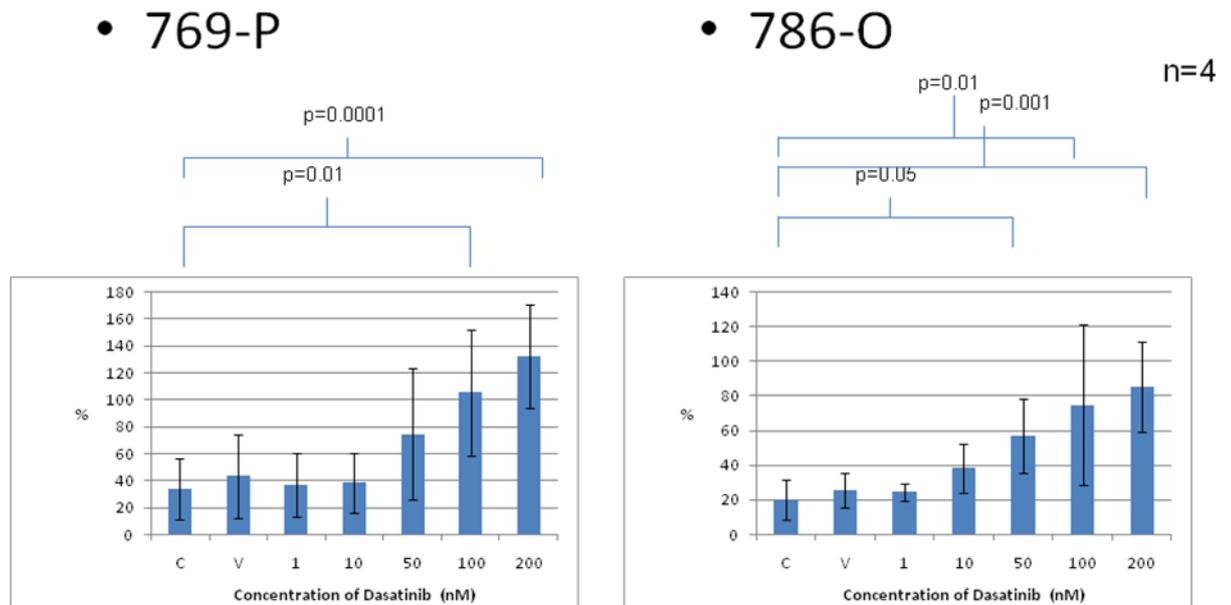


Figure 5.2 demonstrates that cellular apoptosis increases with increasing doses of Dasatinib in both RCC cell lines and the p-values for those concentration where a significant difference was demonstrated between the control and the concentration of Dasatinib.

It is demonstrated that not only does the SFK inhibitor, Dasatinib, have an effect on the metabolic activity and apoptosis on RCC cell lines but furthermore this is a dose dependant relationship. This would suggest that SFK inhibitors may have a role to play in those with RCC. Various doses of Dasatinib were demonstrated to have a significant impact on metabolic activity and apoptosis when comparisons were made to the control (C). Of the various doses, 100nM Dasatinib was demonstrated to have a significant impact on both cell lines for cellular metabolic activity and apoptosis. At concentrations above this the effect appeared to be toxic and was causing the cells to die.

5.4.2 Western Blot Analysis

Figure 5.3: Western Blots for 769-P RCC cell line with increasing concentration of Dasatinib assessing c-Src, Src Y⁴¹⁶ and FAK Y⁸⁶¹.

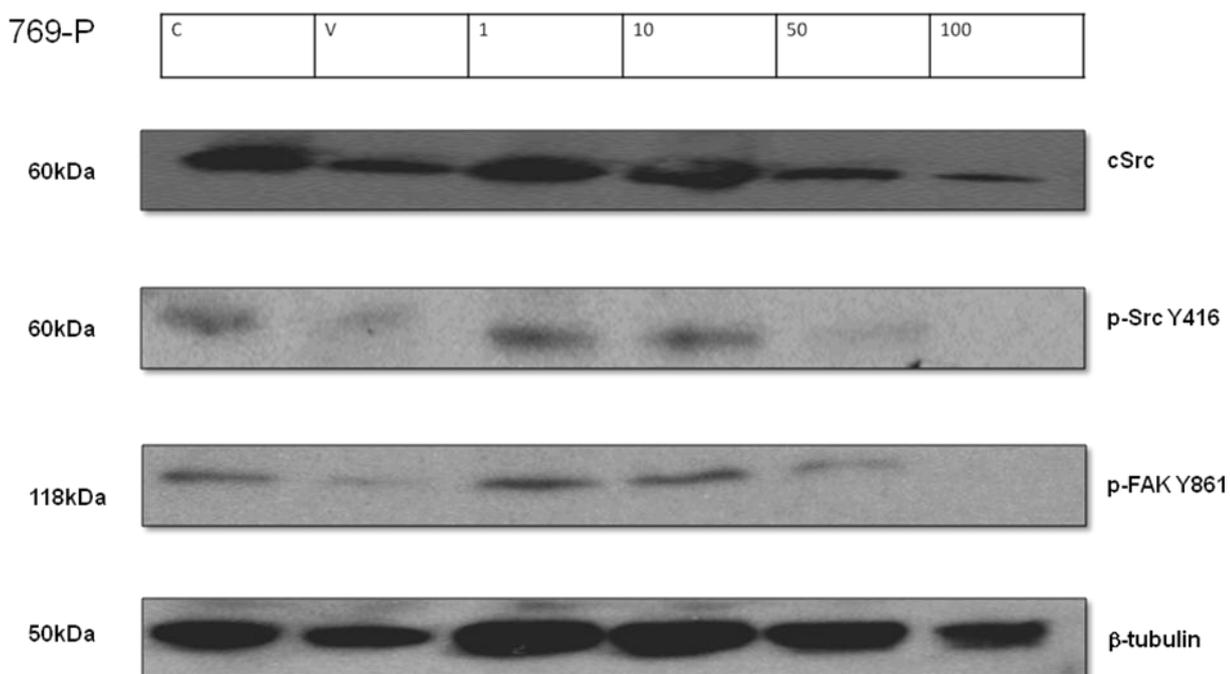


Figure 5.3 demonstrates that increasing the concentration of Dasatinib results in a decrease in expression of c-Src, Src Y⁴¹⁶ and FAK Y⁸⁶¹.

Figure 5.4: Western Blots for 786-O RCC cell line with increasing concentration of Dasatinib assessing c-Src, Src Y⁴¹⁶ and FAK Y⁸⁶¹.

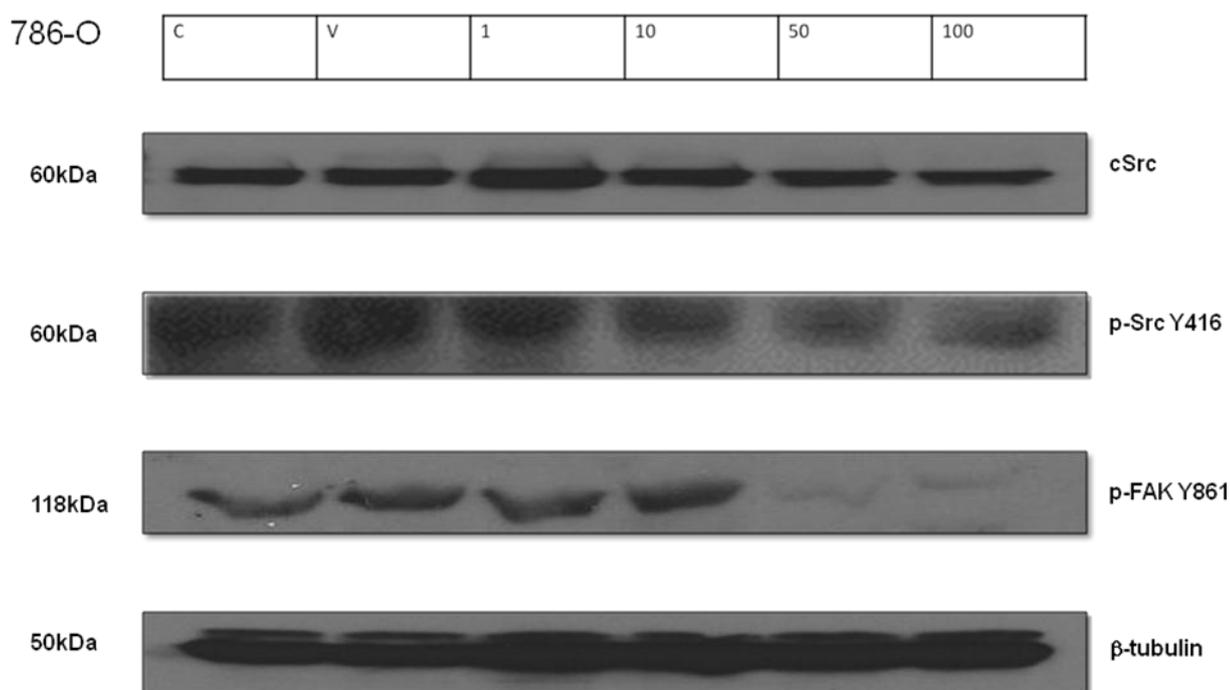


Figure 5.4 demonstrates that increasing the concentration of Dasatinib results in decreasing expression of c-Src, SrcY⁴¹⁶ and FAK Y⁸⁶¹.

Western Blots demonstrated increasing the concentration of Dasatinib had an impact on expression of Src Y⁴¹⁶ and FAK Y⁸⁶¹ with this expression dose dependant. Western Blots were performed in triplicates and ImageJ software was utilised to quantify the difference in signal, all proteins investigated were normalised to B-tubulin control and compared to control to assess if expression was altered following treatment using a 2-way ANOVA test.

Figure 5.5: Demonstrating the effect of increasing concentration of Dasatinib on c-Src expression.

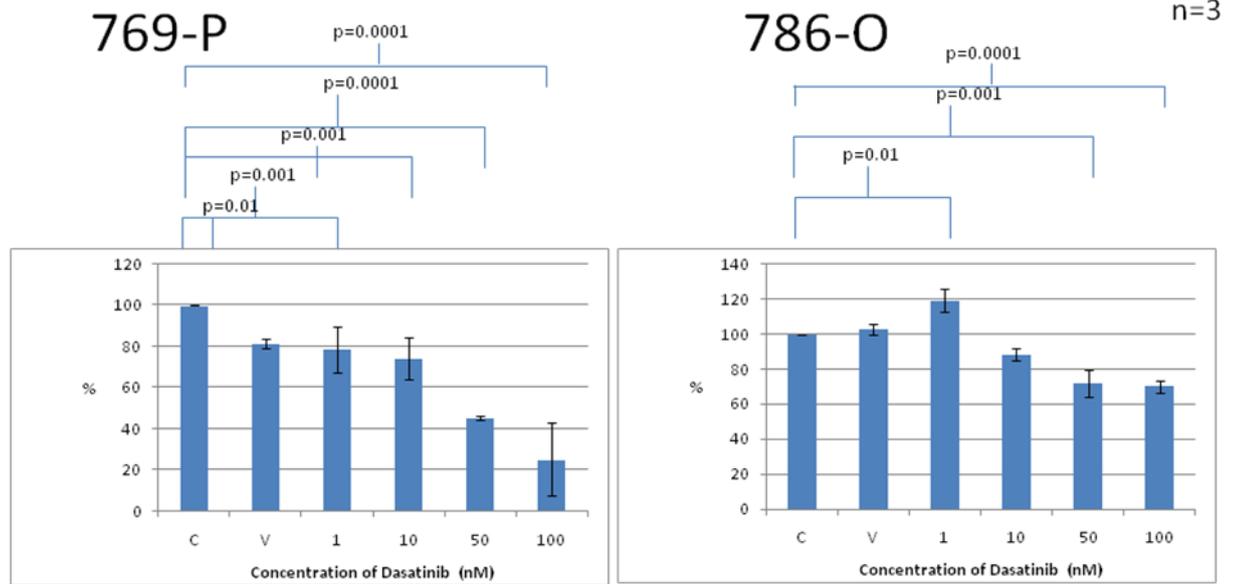


Figure 5.5 demonstrates that at increasing concentrations of Dasatinib results in a significant impact on c-Src expression.

Figure 5.6: Demonstrating the effect of increasing concentration of Dasatinib on Src Y⁴¹⁶ expression.

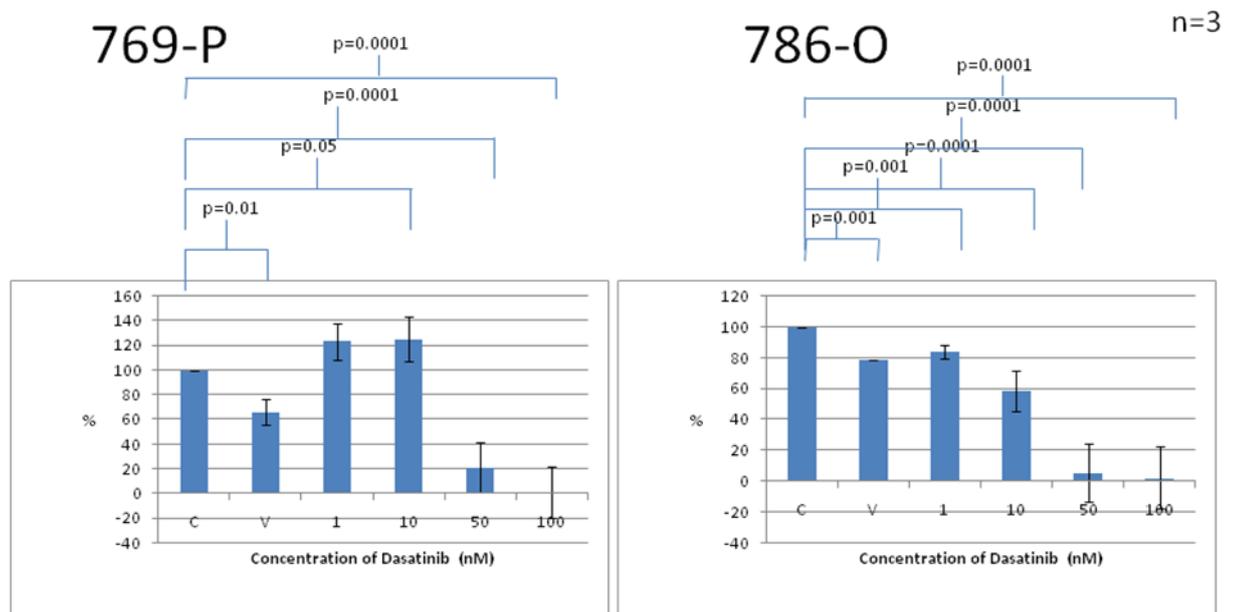


Figure 5.6 demonstrates that at increasing concentrations of Dasatinib results in a significant impact on Src Y⁴¹⁶ expression.

Figure 5.7: Demonstrating the effect of increasing concentration of Dasatinib on FAK Y⁸⁶¹ expression.

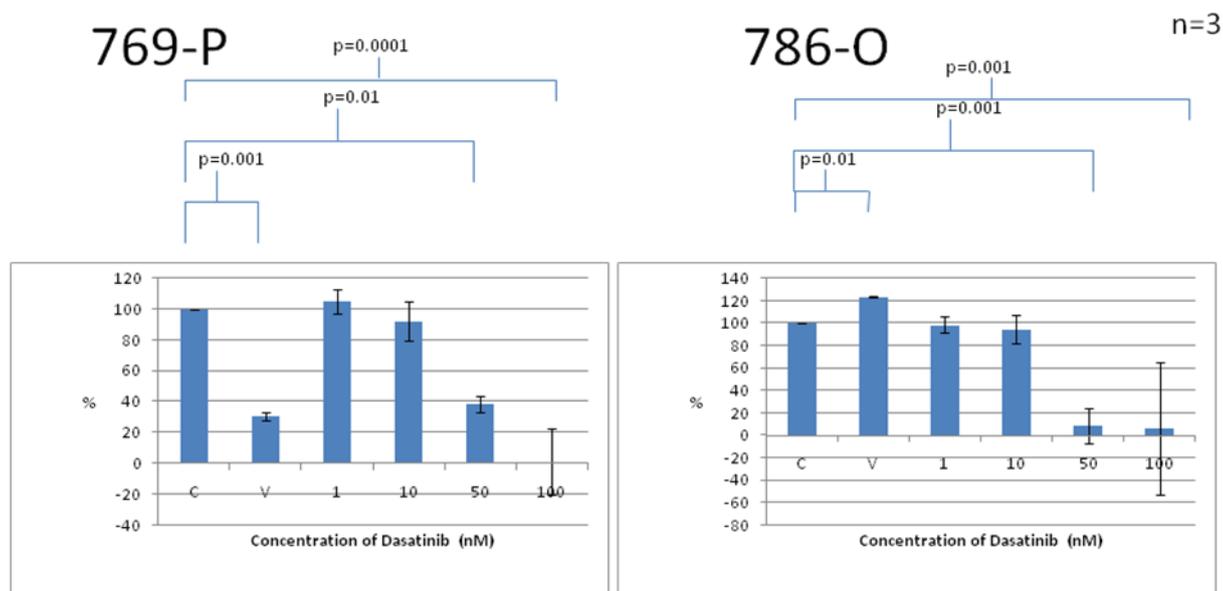


Figure 5.7 demonstrates that at increasing concentrations of Dasatinib results in a significant impact on FAK Y⁸⁶¹ expression.

5.5 Discussion

Dasatinib has been previously demonstrated to inhibit cell cycle growth in prostate, breast and hepatocellular cancer cell lines [348, 475, 480]. To determine the effect of these inhibitors, reliable biomarkers need to be distinguished allowing activity to be determined. Active Src results in the phosphorylation of downstream markers such as FAK and paxillin which play a key role in the regulation of proliferation and migration of normal and tumour cells [416-420]. As previously stated, the most reliable marker for SFK activation which is utilised in cell line studies is Y⁴¹⁹ [475] which has been demonstrated to be a biomarker of activity for Dasatinib [469]. It has been demonstrated that prostate cancer cell lines demonstrated reduced expression of Y⁴¹⁹ and FAK Y⁸⁶¹ when exposed to Dasatinib [348]. When assessing both RCC cell lines, it was demonstrated that Dasatinib reduced the rate of cellular metabolic activity and increased the rate of apoptosis in a dose dependant manner.

This effect was significant at various concentrations but in general was effective at 50nM and 100nM and toxic at 200nM. It has been demonstrated that SFK activity is completely inhibited in prostate [348, 459] and colorectal cancer cell lines [469] at a similar concentration of Dasatinib with the suggestion that concentrations of 100nM and above inhibits multiple targets and therefore exerts a non-specific target response [378].

We have previously demonstrated that c-Src, Src Y⁴¹⁶ and FAK Y⁸⁶¹ are present in both cell lines when antibody specificity was checked prior to immunohistochemistry. Western Blot analysis by way of ImageJ analysis has demonstrated that c-Src expression does decrease significantly at various concentrations of Dasatinib but it is important to remember that expression does not constitute SFK activity. Expression of SFK activity (Src Y⁴¹⁶) which is a reliable biomarker of activity, following treatment with Dasatinib was demonstrated to be inhibited. Furthermore, Dasatinib was demonstrated to inhibit phosphorylation of FAK Y⁸⁶¹ which is a downstream marker of SFK activity.

Dasatinib which is a non-specific SFK inhibitor has been demonstrated to inhibit expression of c-Src. We have previously demonstrated that membrane c-Src expression correlates to improved survival. Furthermore, Dasatinib inhibits phosphorylation of SFK activity and FAK Y⁸⁶¹ with high expression of the latter having been demonstrated to be an independent predictor of poor survival.

These findings further reinforce that SFK inhibition may play a role in the treatment of RCC and that another SFK member and not c-Src itself be responsible for phosphorylation of FAK Y⁸⁶¹. To further investigate this, in the next chapter we aim to silence c-Src and to assess the effects of cellular metabolic activity and apoptosis following exposure to Dasatinib. We will also assess expression of previously examined markers of activity on cell lysates by way of immunohistochemistry and Western Blot analysis following c-Src silencing and to see if this affects SFK activity and expression of the downstream marker FAK Y⁸⁶¹.

Chapter 6-Effects of SFK inhibitor Dasatinib on Src expression, SFK activity, FAK expression and cellular metabolic activity/apoptosis on c-Src silenced RCC cell lines

6.1 Introduction

The previous chapters have demonstrated of the SFK members, c-Src is the highest expressed in RCC and high expression is associated with improved survival. This is in contrast to FAK Y⁸⁶¹ (downstream marker of SFK activity) which is associated with a poor prognosis. Furthermore, treatment with the SFK inhibitor, Dasatinib, inhibits cellular metabolic activity, promotes apoptosis and inhibits phosphorylation of SFK activity and FAK Y⁸⁶¹.

In the current chapter we aim to investigate the impact of Dasatinib on SFK signalling and downstream events in the absence of Src kinase by silencing Src.

6.2 Materials and Methods

6.2.1 *Culture of renal cancer cells*

This was performed as described earlier in Chapter 4.

6.2.2 *Counting of cells*

This was performed as described in Chapter 5.

6.2.3 *Silencing of cells*

Once cells were 50% confluent, transfection was performed using Lipofectamine (Invitrogen, UK) according to manufacturer's protocols. Lipofectamine binds the DNA and passes the cell membrane by endocytosis. 10µl of Lipofectamine per well was added to 240µl of Optimem (Invitrogen, UK) and 200nM siRNA (10ul of 20nmol ON TargetPlus smartpool Src or a Non-Targeting control; Thermo Fisher Scientific, UK) was added to another 240µl of Optimem and incubated at room temperature for 10 minutes. These two mixtures were then gently mixed together and left to incubate at room temperature for a further 20 minutes. During this incubation period, the RPMI media was removed from the cells and replaced with Optimem. For those cells in 6-well plates, 1.5 mls of Optimem was utilised and for 96-well plates, 60µl of Optimem was used then cells incubated at 37°C with 5% CO₂ in air for 20 minutes. Following incubation, the siRNA was added to the cells to give the total volume in 6-well plates of 2mls and 100µl for 96-well plates. Plates were then incubated at 37°C with 5% CO₂ in air for 8 hours then Optimem replaced with RPMI media. Cells were then incubated for a further 16 hours prior to incubation with Dasatinib for 48 hours. As for earlier experiments, cells in which no drug was added and medium plus drug vehicle (DMSO) were utilised as controls.

6.2.4 *Assessment of cellular metabolic activity*

This was performed as described in Chapter 5.

6.2.5 *Assessment of cell apoptosis*

This was performed as described in Chapter 5.

6.2.6 *Western Blot Analysis*

This was performed as described earlier in Chapter 4.

6.2.7 *Formation of cell line pellets*

For the purposes of construction of the cell pellets, a concentration of 50nM of Dasatinib was utilised. This concentration was used as it was observed in the previous chapter that at 50nM, Dasatinib inhibited phosphorylation of Src Y⁴¹⁶ and FAK Y⁸⁶¹ and concentration of Dasatinib at 100nM and above hits multiple targets resulting in off target effect [378].

Following incubation with Dasatinib, the excess solution was removed and the cells gently washed twice with HBBS. Cells were then incubated in 3 mls of trypsin for 5 minutes in 5% CO₂ at 37°C, in order to detach the cells from the flask after which 3mls of RPMI was added to neutralise the trypsin. This suspension was then gently pipetted to ensure that all cells had been dislodged. Following this, the cell suspension was transferred to a 15 mls centrifuge tube and centrifuged for 2 minutes at 1200rpm. The supernatant was then discarded and the cell pellet re-suspended in 10 mls of RPMI. Each centrifuge was then placed on ice and transferred to the in house pathology department for formalin fixation, imbedding in individual paraffin wax blocks and cut into sections onto salinised glass ready for IHC. This experiment was repeated in triplicates.

6.2.8 *Clot formation of cell lines for immunohistochemistry*

The drug treated solution containing the neutralised trypsin was transferred into a 25 mls container to which 15 mls of normal saline was added allowing the cells to be washed. Following this, the suspension was placed into a centrifuge for 5 minutes at 1500 rpm. The supernatant was discarded leaving the cell pellet to which 2-3 drops of fridge stored human plasma was added and the solution gently mixed. 1-3 drops of thrombin working solution was then applied gently allowing a clot to form. Formalin was then added slowly to prevent clot fragmentation. Fixation of the clot in formalin then occurred overnight before being taken to the in house pathology department for imbedding into a paraffin wax block.

6.2.9 *Immunohistochemistry on cell pellets*

This was performed as described in Chapters 2 and 4 for c-Src, Src Y⁴¹⁶ and FAK Y⁸⁶¹.

6.3 Statistical Analysis

Cellular metabolic activity and apoptosis were assessed using a 2 way ANOVA followed by Bonferroni's post hoc test to assess for significant differences and to correct for multiple conditions. For the purposes of Western blot analysis, ImageJ software was utilised to assess the density of the bands following which a 2 way ANOVA was utilised to assess this difference.

6.4 Results

6.4.1 *Silencing of cell lines*

Immunohistochemistry and Western blotting were employed to assess the level of c-Src silencing. Figure 6.1 demonstrated that in both 769-P and 786-O cell lines, a fall in c-Src expression was observed in the silenced cells compared to the control cells, however there was still low levels of expression remaining in both cell lines.

Figure 6-1: Immunohistochemistry of cell line pellets for c-Src following silencing.

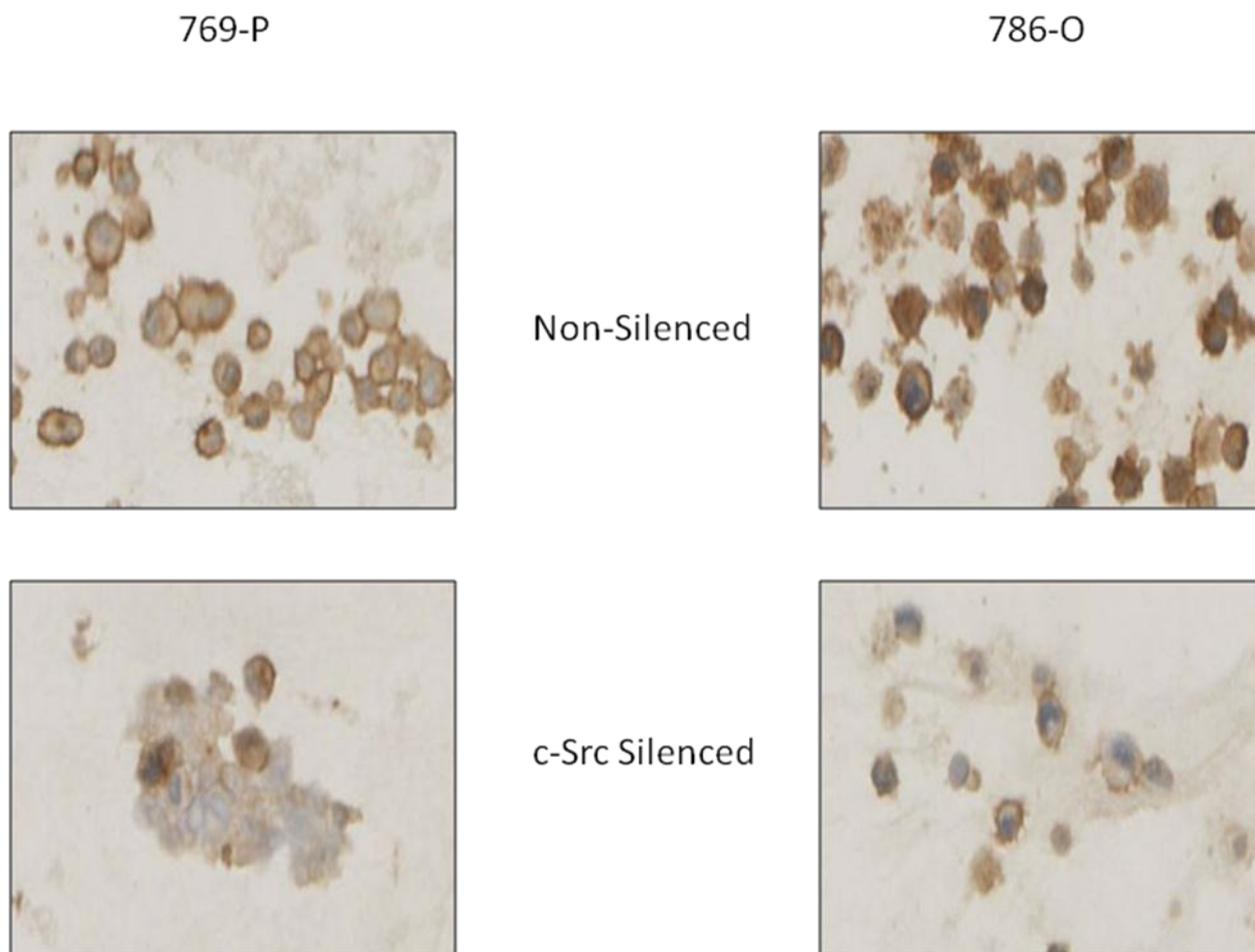


Figure 6.1 demonstrates that c-Src expression decreased in both cell lines following transfection.

Similarly by Western blot analysis although levels of c-Src were significantly lower in the transfected cells (Figure 6.2), low levels of c-Src expression remained. These experiments were performed in triplicates.

Figure 6-2: Western blot analysis of cell lines for c-Src following silencing.

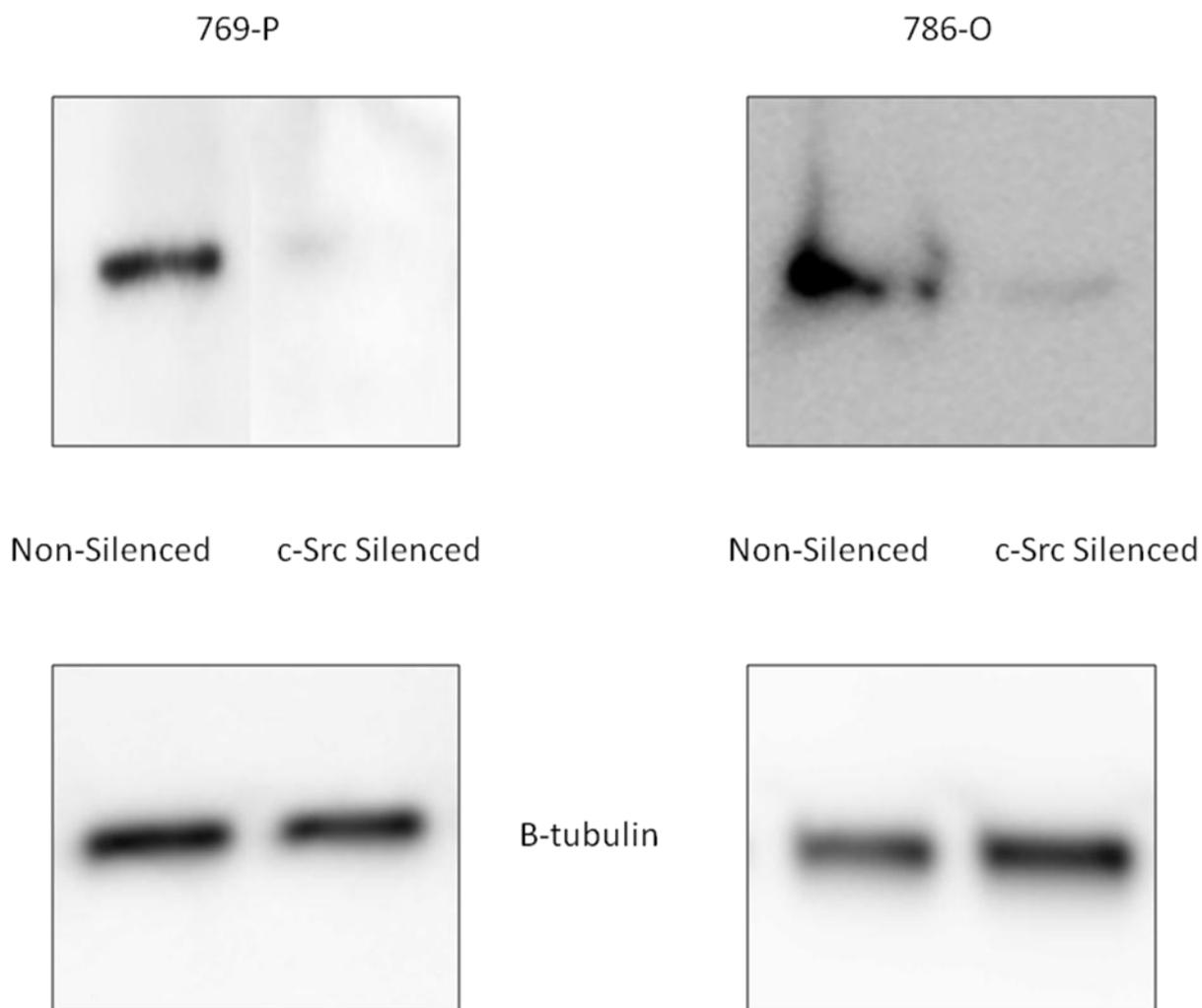


Figure 6.2 demonstrates that c-Src was silenced in both cell lines following transfection as expression for c-Src decreased in both cell lines.

Figures 6.1 and 6.2 demonstrate that c-Src expression was reduced as staining intensity and expression decreased in the cell pellets and Western blots respectively. As in Chapter 5, Western Blots were performed in triplicates and ImageJ software was utilised to quantify the difference in signal, all proteins investigated were normalised to B-tubulin control to assess if expression was altered following silencing using a 2-way ANOVA test (Figure 6.3). In 769-P, 60-70% knockdown was achieved, however this was less in 786-O cells where only 30-40% knockdown was achieved. If time had permitted, this would have been further optimised to improve the level of knockdown.

Figure 6-3: Demonstrates the effect of silencing on c-Src expression.

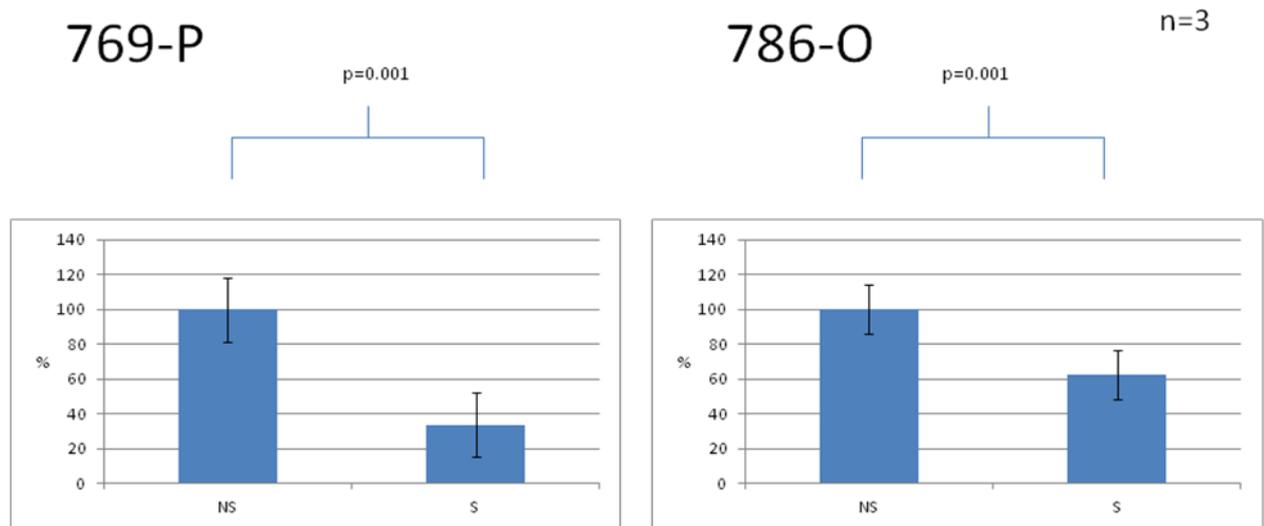


Figure 6.3 demonstrates expression of c-Src decreased following silencing and there was a significant decrease in expression of c-Src in both cell lines ($p=0.001$).

Silencing was then performed to assess the effect of reduced c-Src expression +/- Dasatinib on proliferation and apoptosis in our RCC cell lines.

6.4.2 Cellular Metabolic Activity and Apoptosis

As described in Chapter 5, assessment of cellular metabolic activity and apoptosis was performed in triplicates and repeated in four independent experiments.

Figure 6-4: Demonstrating the effect of increasing concentrations of Dasatinib on cellular metabolic activity on 769-P and 786-O non-silenced and c-Src silenced cell lines.

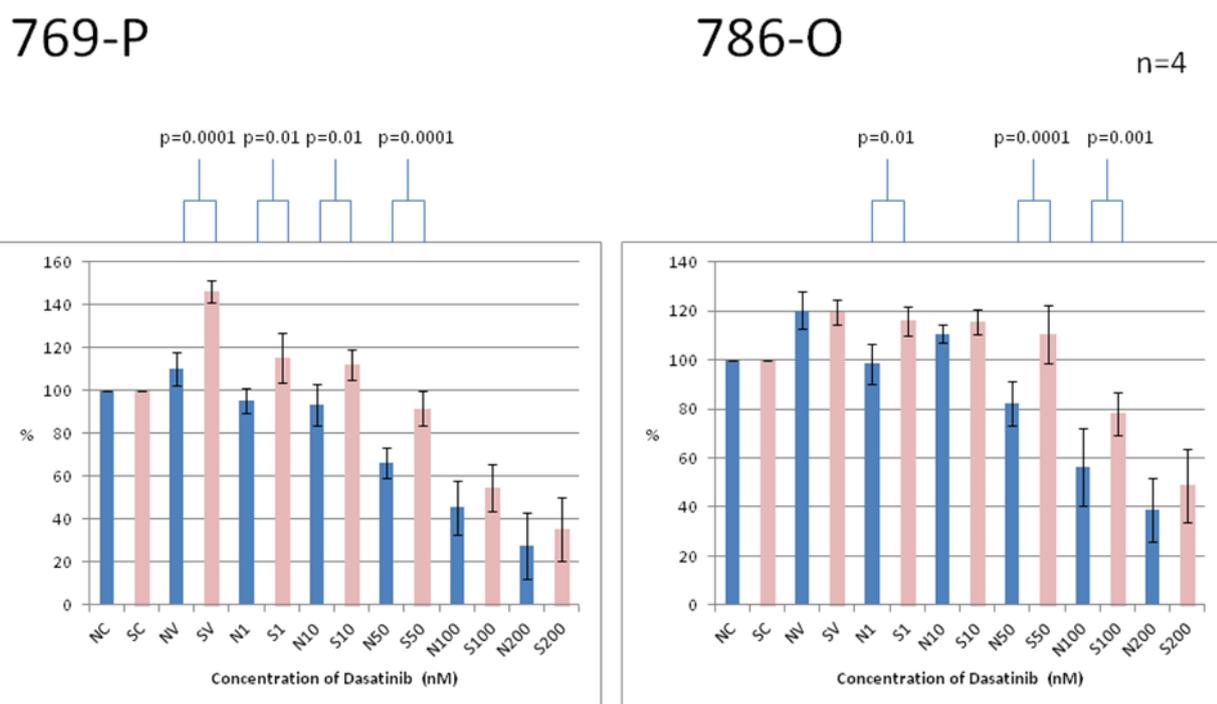


Figure 6.4 demonstrates that cellular metabolic activity is increased in the c-Src silenced cells with increasing doses of Dasatinib in both RCC cell lines.

Figure 6.4 demonstrates that cellular metabolic activity is inhibited with increasing doses of Dasatinib in both RCC cell lines as demonstrated in Chapter 5. Furthermore, there is a significant difference in non-silenced and silenced metabolic activity rates. When c-Src expression was reduced in the 769-P cells, metabolic activity rates were consistently higher in c-Src silenced cells compared to the non-silenced cells, although Dasatinib still inhibited metabolic activity under both conditions. This would suggest that Dasatinib was not functioning via c-Src. In 786-O cells, no significant difference in metabolic activity rate was observed between the silenced and non-silenced cells in the absence of Dasatinib and Dasatinib was observed to inhibit metabolic activity in both silenced and non-silenced cells. However, following treatment with 50nM Dasatinib and above, the silenced cells, although responding to Dasatinib consistently had higher metabolic activity rates than those of the non-silenced cells. It was noted that in the untreated cells containing the

vehicle, metabolic activity rates were higher in both silenced and non-silenced cells whilst no change was observed in the control groups.

Figure 6-5: Demonstrating the effect of increasing concentrations of Dasatinib on cellular apoptosis on 769-P and 786-O non-silenced and c-Src silenced cell lines.

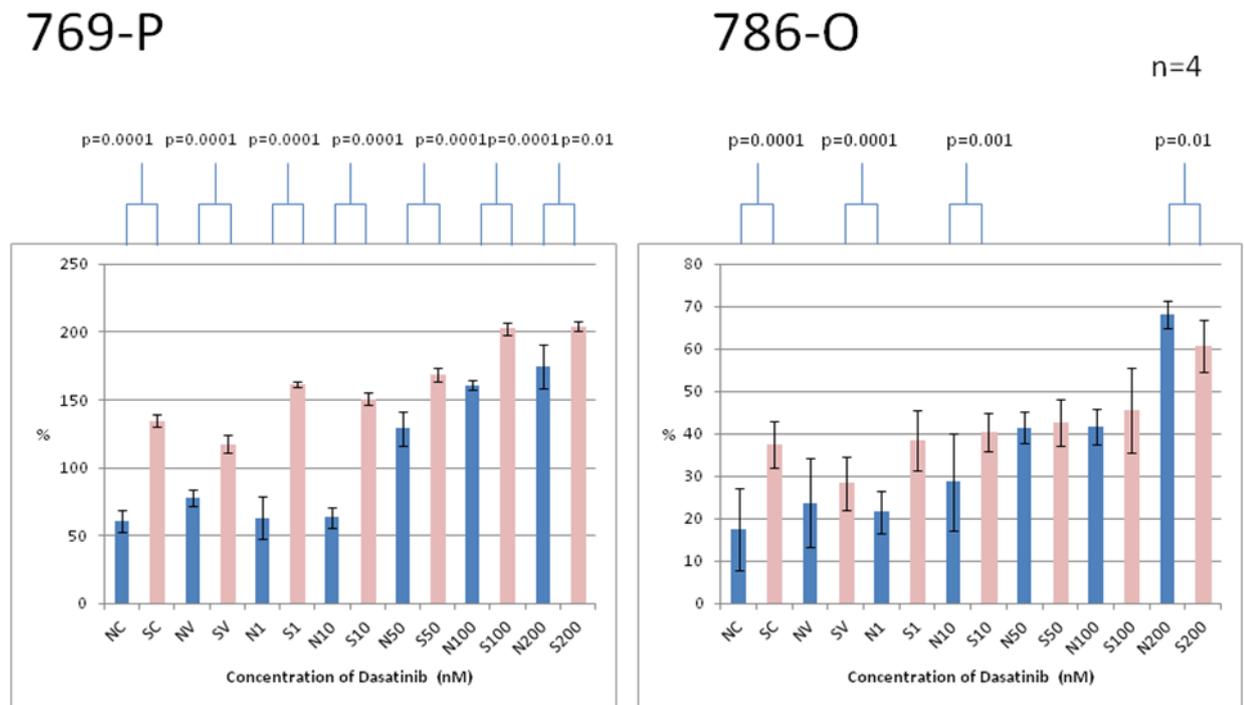


Figure 6.5 demonstrates that cellular apoptosis was induced with increasing doses of Dasatinib in both RCC cell lines and furthermore there is a significant difference in non-silenced and silenced apoptotic rates when c-Src is silenced in both cellular lines.

Figure 6.5 demonstrates that apoptosis was induced in both cell lines with increasing doses of Dasatinib in both cell lines as described in Chapter 5. Furthermore, there is a significant difference in non-silenced and silenced apoptotic rates. When c-Src expression was reduced in both cell lines, apoptotic rates were consistently higher in c-Src silenced cells compared to the non-silenced cells, although Dasatinib still inhibited apoptosis under both conditions. This again suggests that Dasatinib was not functioning via c-Src.

6.4.3 Immunohistochemistry

IHC allows us not only to assess expression of protein but also cellular localisation, we therefore investigated expression and localisation of c-Src, Src Y⁴¹⁶ and FAK Y⁸¹⁶ following silencing for c-Src. Antibody verification for c-Src, Src Y⁴¹⁶ and FAK Y⁸¹⁶ had already been performed as described in Chapter 4.

6-6: Demonstrating the expression of c-Src following silencing for c-Src and treatment with Dasatinib.

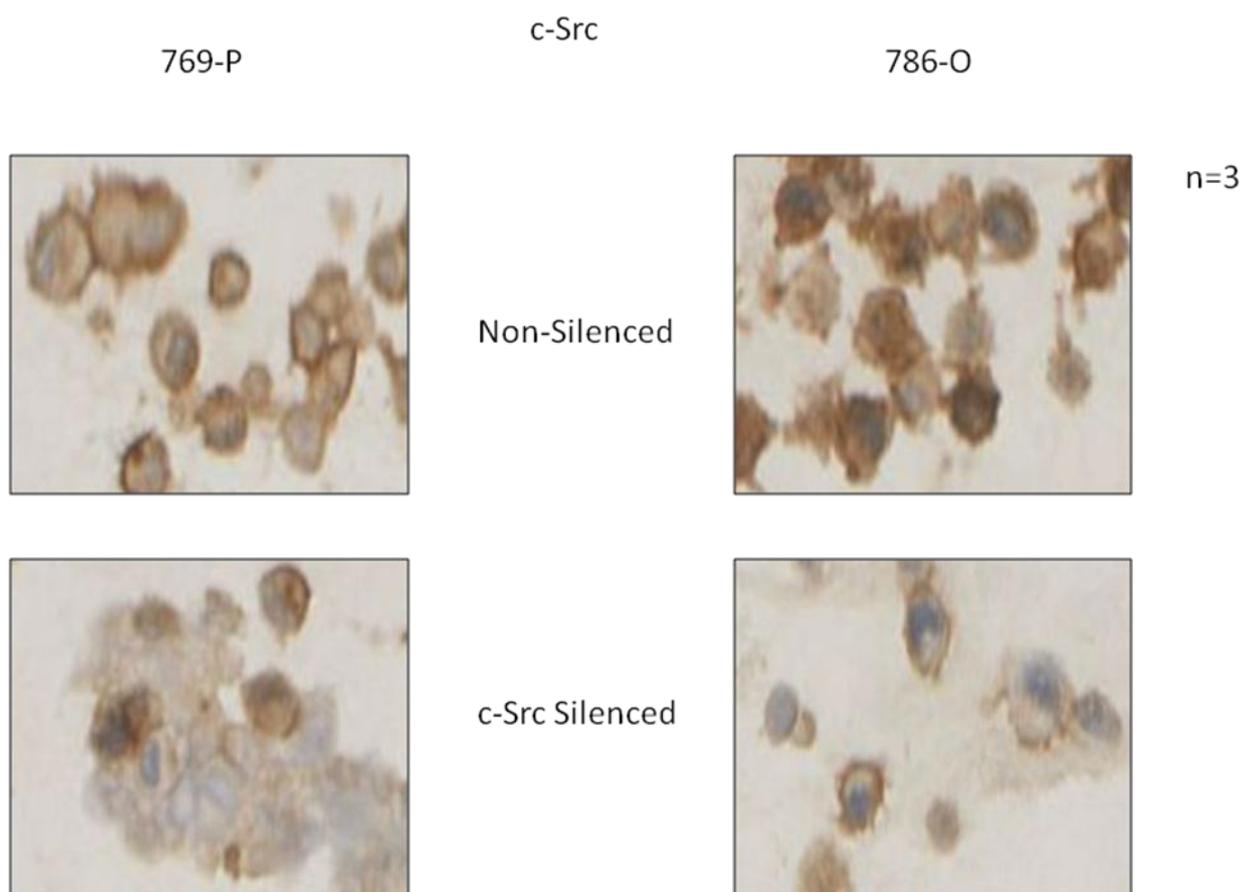


Figure 6.6 demonstrates that there was a decrease in expression of c-Src following silencing of c-Src in both cell lines.

In Figure 6.6, membrane expression of c-Src in the 769-P cell line fell from 100 weighted histoscore units to 20 in the silenced cells and cytoplasmic expression fell from 80 weighted histoscore units to 30 in the silenced cells. In the 786-O cell line, membrane

expression fell from 150 weighted histoscore units to 40 in the silenced cells and cytoplasmic expression fell from 120 weighted histoscore to 10 weighted histoscore units in the silenced cells.

Figure 6-7: Demonstrating the expression of Src Y⁴¹⁶ following silencing for c-Src and treatment with Dasatinib.

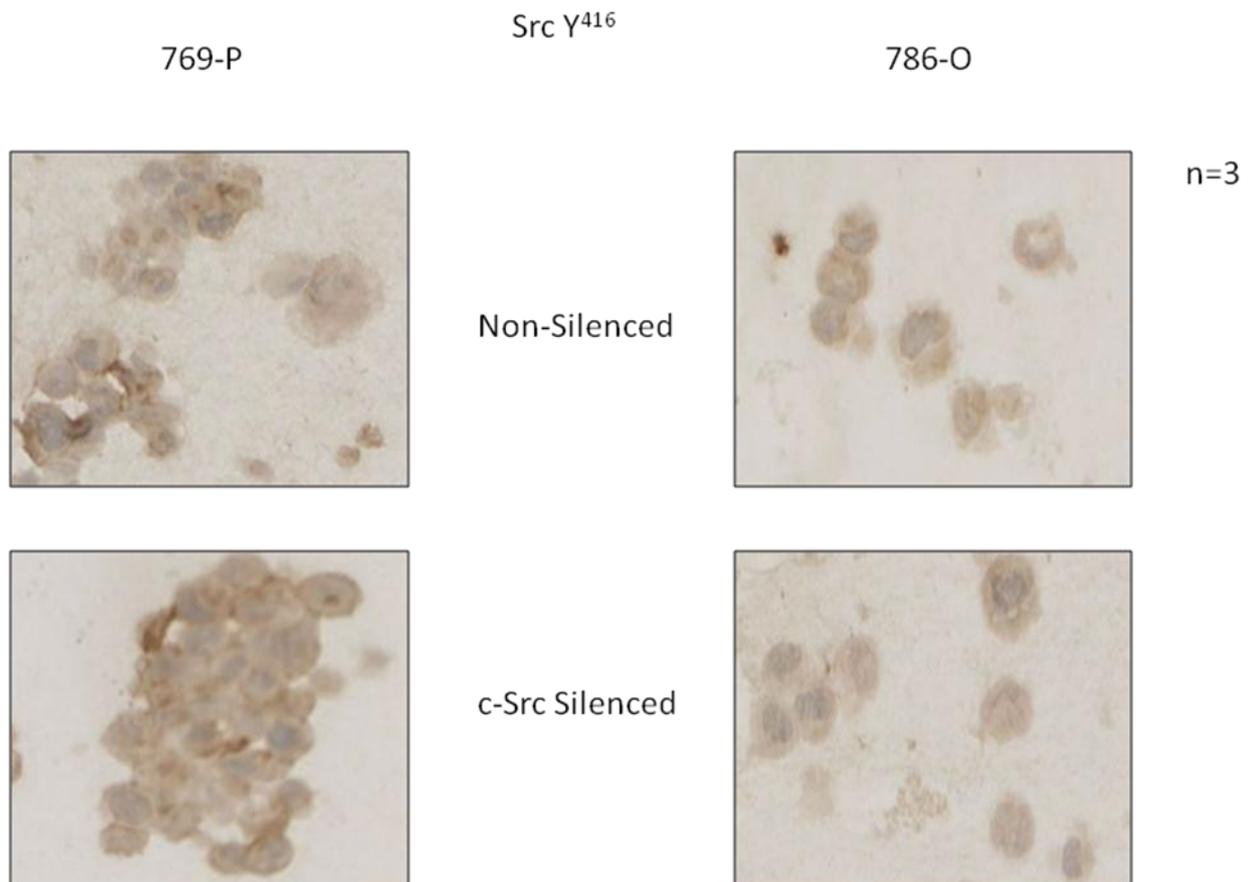


Figure 6.7 demonstrates that there was no obvious difference in expression of Src Y⁴¹⁶ following silencing of c-Src in both cell lines suggesting that an alternative SFK is responsible for Src Y⁴¹⁶.

Figure 6.7 demonstrates that in both cell lines, membrane and cytoplasmic expression was not visually different between the silenced and non-silenced cells.

Figure 6-8: Demonstrating the expression of FAK Y⁸⁶¹ following silencing for c-Src and treatment with Dasatinib.

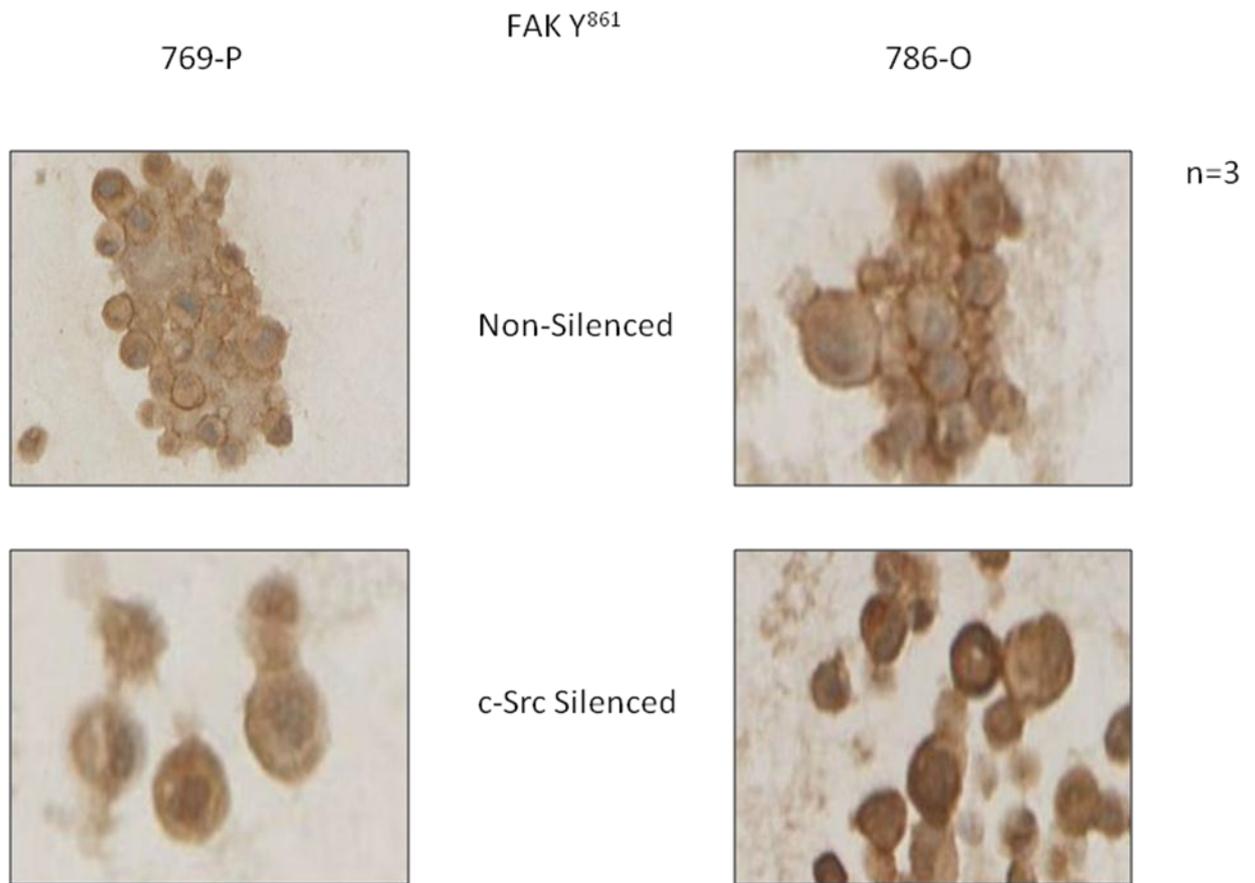


Figure 6.8 demonstrates that expression of FAK Y⁸⁶¹ did not change following silencing of c-Src in both cell lines suggesting that phosphorylation at this site under these conditions is not driven by c-Src but an alternative SFK.

Figure 6.8 demonstrates that in both cell lines, membrane and cytoplasmic expression was not visually different between the silenced and non-silenced cells.

Although c-Src expression decreased following silencing of c-Src as would be expected, there was no corresponding decrease in expression of Src Y⁴¹⁶ and FAK Y⁸⁶¹.

6.5 Discussion

In this chapter we again demonstrated that Dasatinib reduced the rate of cellular metabolic activity and increased the rate of apoptosis in a dose dependent manner. Importantly, it was furthermore demonstrated that cellular metabolic activity rates were significantly higher in the c-Src silenced cells in both cell lines at various concentrations of Dasatinib. In addition, c-Src silenced cells also had significantly higher apoptotic rates. Increased metabolic activity rates following silencing for c-Src would further reinforce the findings from Chapter 4 that the presence of c-Src is associated with increased patient survival; however increased apoptotic rates following c-Src silencing demonstrates that the expression of c-Src would not simply confer improved patient survival by decreasing cellular metabolic activity rates but this relationship is more complex.

We have previously demonstrated in Chapter 5 that a quantitative assessment of c-Src does decrease following treatment with Dasatinib but this does not constitute activity. A reliable marker of SFK activity is Src Y⁴¹⁶ as previously mentioned. Following silencing of c-Src, there was no change in the expressive assessment of SFK activity in both cell lines providing further support that it is not c-Src that constitutes SFK activity. Furthermore, following silencing of c-Src, expressive assessment of FAK Y⁸⁶¹ did not decrease, again providing further support that another SFK member is responsible for the phosphorylation of FAK Y⁸⁶¹.

By performing IHC on cell pellets that have been silenced for c-Src, this allowed analysis of expression of SFK activity and the downstream marker FAK Y⁸⁶¹. Expression of c-Src decreased following silencing, this provided evidence that c-Src had successfully been silenced. Expression of SFK activity did not demonstrate an obvious difference.

Furthermore, it did appear that there was no impact on expression of FAK Y⁸⁶¹ providing further support that c-Src is not responsible for the phosphorylation of FAK Y⁸⁶¹. Although expression was assessed in the cell pellets by the semi quantitative weighted histoscore,

statistics were not applied due to the qualitative nature of the data. However, if time had permitted, the above experiments would have been assessed by western blot to allow a quantitative assessment of expression and therefore perform statistical analysis.

This chapter demonstrates that a level of knockdown was achieved in both cell lines; however this did not impact on downstream expression of Src Y⁴¹⁶ and FAK Y⁸⁶¹.

Chapter 7-Conclusion

Many prognostic markers are utilised in various malignancies, some of these have been demonstrated to have prognostic significance in RCC such as tumour size, nuclear grading and tumour necrosis but there is the suggestion that these can be further refined to improving the prognostic significance. Furthermore, there is evidence that the local and systemic inflammatory response plays a prognostic role in renal cancer and should be further investigated.

We have demonstrated that known prognostic markers such as nuclear grading and tumour necrosis can be further simplified or refined and these do confer increased prognostic significance. It has also been demonstrated that the systemic inflammatory response is an independent prognostic marker associated with poor survival. There were some limitations in this work such as the retrospective nature of the work conducted and limited numbers and despite these findings, these prognostic markers cannot be utilised to assess which patients would benefit from the various therapeutic agents available when recurrence occurs.

It has been more than a hundred years since Rous implicated the role of Src in developing sarcomas [312]. Src has a role in signal transduction of multiple oncogenic cellular processes including migration, adhesion, invasion, angiogenesis, proliferation and differentiation and has significant interactions with other cellular proteins such as growth factor receptors [317]. Due to the role of SFK's in tumourgenesis, they have become important targets for therapeutic intervention. There is a large body of evidence regarding the role of these inhibitors targeting SFK's in various malignancies but their role in RCC is limited. Despite this limited translational evidence, a phase II trial has finished recruiting patients utilising a c-Src inhibitor, Saracitinib, in metastatic RCC, the results of which at the time of writing are not available.

The aim of this project was to fill that gap in knowledge regarding the expression of SFK's, correlation of expression to survival and the role of SFK inhibitor Dasatinib, on RCC.

Assessing mRNA expression of SFK's in RCC, we have demonstrated that all SFK's are expressed in RCC and that they are expressed in various degrees. c-Src was the most highly expressed in RCC. As mRNA expression does not necessarily correlate with protein synthesis and protein expression, further work was required to assess whether c-Src was expressed in RCC, if it correlated to other pathological parameters as well as survival and the expression of the downstream marker FAK Y⁸⁶¹.

In our cohort of patients, it was demonstrated that membrane expression of c-Src was associated with improved disease specific survival as well as a significant association with patients being free of recurrent disease. This evidence alone would suggest that c-Src is associated with improved disease specific survival and utilising treatments such as Saractinib may have a negative impact on survival. In contrast, expression of cytoplasmic FAK Y⁸⁶¹ had a positive relationship with known prognostic markers which confer poor prognosis such as T stage, nuclear grade, recurrence and tumour volume. Expression of cytoplasmic FAK Y⁸⁶¹ was associated with poor prognosis and was an independent predictor of poor survival. Evidence demonstrating that c-Src confers improved survival and the downstream marker of SFK activity, FAK Y⁸⁶¹, confers poor survival would support the theory that another member of the SFK's besides c-Src is responsible for the phosphorylation of FAK Y⁸⁶¹ and therefore is responsible for poor survival.

As described in Chapter 1, there is vast evidence supporting the inhibition of cancer cells with Dasatinib. With SFK inhibitors being utilised in RCC, it was imperative to assess the role of these drugs not only on cellular metabolic activity and apoptosis but also on c-Src which had been shown to be the highest expressed SFK in RCC as well as SFK activity and the downstream marker FAK Y⁸⁶¹.

Treatment with Dasatinib, which is a non-specific SFK inhibitor, on RCC cell lines demonstrated a dose dependant reduction on cellular metabolic activity as well an increase in apoptotic rates. This would support that Dasatinib may be a useful therapeutic drug for RCC. Furthermore, Western Blot analysis demonstrated that assessment of c-Src, Src Y⁴¹⁶ and FAK Y⁸⁶¹ following treatment with Dasatinib reduced in a dose dependant manner. As mentioned earlier, it is important to remember that expression of c-Src does not constitute activity. In combination with earlier results of IHC that in those patients with high cytoplasmic FAK Y⁸⁶¹, exposure with Dasatinib may improve their clinical outcome.

It was further necessary to assess the role of c-Src to support the theory that another SFK is responsible for poor prognosis in RCC. This was undertaken by silencing c-Src and assessing parameters which had previously been analysed and analysing the impact of Dasatinib. Cellular metabolic activity rates increased following silencing further supporting that c-Src confers improved survival, however apoptotic rates also increased showing that the role of c-Src is a complex one. Src Y⁴¹⁶ is a marker of SFK activity and qualitative assessment of this following silencing c-Src did not demonstrate any change in expression and there was also no change in expression of FAK Y⁸⁶¹. This would suggest that c-Src has some form of interaction with another SFK member. This SFK member may be responsible for the phosphorylation of FAK Y⁸⁶¹ and following silencing of c-Src may be up regulated thereby c-Src being associated with a good prognosis. Western Blot analysis allows a quantitative assessment and by performing IHC this allows us to look at the cellular localisation of SFK's in response to treatment and this demonstrated that expression of the downstream marker did not decrease, demonstrating further evidence that another SFK member is responsible for the phosphorylation of FAK Y⁸⁶¹ and therefore associated with poor prognosis.

This research has demonstrated that SFK's are expressed in RCC, the downstream marker of phosphorylation FAK Y⁸⁶¹ is associated with poor prognosis, another SFK member besides c-Src may be responsible for this poor prognosis and SFK inhibitors may have a

role to play in RCC. Further work is required to assess which SFK member is associated with poor prognosis in RCC and the relationship it has with the downstream marker FAK Y⁸⁶¹. It would be imperative to assess the expression of this SFK member in metastatic RCC cell lines as well as the ones that have been utilised. This would allow assessment of therapeutic intervention in either anti-metastatic setting or in latter stages of disease progression.

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9 Appendices

The epidemiology and risk factors for Renal Cancer

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Abstract**Context**

Renal cancer is a frequently occurring malignancy with over 270,000 new cases diagnosed and it being responsible for 110,00 deaths annually on a global basis. Incidence rates have gradually increased whilst mortality rates are starting to plateau.

Objective

To review epidemiology and risk factors for renal cancer.

Methods

The current data is based on a thorough review of available original and review articles on epidemiology and risk factors for renal cancer with a systemic literature search utilising Medline.

Results

The prevalence of associated risk factors such as genetic susceptibility, smoking, hypertension and obesity are changing and could account for the changes in incidence whilst the role of diet and occupational exposure to carcinogens requires further investigation.

Conclusion

Despite the evidence of various associated risk factors, further work is required from well-designed studies to gain a greater understanding of the aetiology of renal cancer.

Incidence and survival rates in Renal Cancer

Worldwide, renal cancer is the 13th most common malignancy with over 270,000 new cases diagnosed in 2008 [1]. In the UK alone, approximately 9000 new cases of renal cancer are diagnosed each year [2]. Incidence rates have increased in the UK with age standardised incidence rates more than doubling between 1975-1977 and 2007-2009 [2]. Age standardised ratios for both incidence and mortality is observed to be 50% lower in women compared to men [2]. Renal cell carcinoma (RCC) accounts for nearly 90% of all renal malignancies. There has been much debate that the increased incidence rate is due to the vast improvement in imaging modalities such as magnetic resonance imaging (MRI) and computed tomography (CT) as well as the increased use of this imaging. It has been reported that there has been an increased rate of detection of incidental tumours which are asymptomatic and localised [3-6] but there has also been an increase detection of more advanced tumours and that the increase in incidence is real and cannot be solely accounted by incidentally detected tumours [3, 7].

Globally, renal cancer was responsible for over 110,000 deaths in 2008 [1]. Nearly 4000 patients died from renal cancer in 2008 accounting for 2% of all cancer deaths in the UK [2]. 20-30% patients present with metastatic disease [8] with another 20% of patients undergoing nephrectomy developing metastases during subsequent follow up [9]. This can account for the increasing mortality rates. Various factors are involved in survival after diagnosis such as tumour involvement as well as overall health but there is only a 50% chance of survival at five years following diagnosis [2].

Risk Factors

Approximately 75% of those diagnosed are over 60 years of age with the disease being rare in those under 50 [2] and reaching a plateau around 70-75 years of age [10]. Incidence rates have increased in all age groups but this increase is predominantly in those over 75

years of age [2]. In addition mortality rates have predominantly increased in those over 75 [2], confirming that renal cancer is predominantly a disease of the elderly.

Age standardised incidences suggest that men are at an increased risk of RCC [1] with it being the 6th most common cancer in men and the 9th most common in women in the UK [2]. The increase in overall incidence is replicated in the increase in incidences in both sexes with there being an obvious higher occurrence in males accounting for the overall increase suggesting that there is a higher predisposition of renal cancer in males than females. It has been reported that between 2007-2030 there will be a 27% and 18% increase in the incidence of renal cancer in males and females respectively [11]. In 2009 the age standardised incidence risk per 100,000 was 15.5% in men compared to 8.2% in women in the UK [2].

Mortality rates are also higher in males than in females as maybe expected given the obvious difference in incidence rates in both sexes. In 2008 the age standardised mortality rate per 100,000 was 6% in men compared to 3.1% in women in the UK [2]. It has long been thought that incidence and mortality rates have been higher in males due to lifestyle factors such as cigarette smoking which has been historically higher in males and also exposure to industrial carcinogens due to differing occupational bias between the sexes.

Smoking is a well-established risk factor for RCC with a meta-analysis reporting not only a difference in a smoker and a non-smoker but also a dose dependant risk with the number of cigarettes smoked [12]. Compared to those whom never smoked, there was a 50% increase in the risk for males and a 20% increase risk for females [12]. This risk can be reduced after smoking cessation for more than 10 years [10, 12, 13]. It is thought that cigarette smoking increases the risk of RCC through chronic tissue hypoxia due to carbon monoxide exposure [14] as well as evidence suggesting higher level of DNA damage in peripheral blood lymphocytes in those with RCC compared to controls [15].

It has been suggested that the different incidence rates observed between males and females maybe due to exposure to potential occupational carcinogens. The most extensively studied is the solvent Trichloroethylene (TCE) which is widely used as a metal degreaser and has been considered a human carcinogen by the International Agency for Research on Cancer (IARC) as well as a common environmental contaminant [16]. A case controlled series in Europe reported an increased risk following exposure to TCE [17] with one review reporting increased risk of various malignancies including renal following exposure [18] and a meta-analysis suggesting a weak association with exposure to TCE [19] whilst others have reported that given the complexities of TCE pharmacokinetics and limitation of studies this prevents a definitive relationship [16, 18, 20]. Various other compounds have been investigated with one study reporting an association with lead which requires further investigation whilst associations have been reported for glass and wool fibres as well as brick dust [21, 22]. Exposure to industrial agents such as cadmium and uranium has shown no relationship to RCC risk [10, 23, 24] and neither have arsenic, nitrate and radon in drinking water [10]. Interestingly an association between agricultural workers and RCC was reported [25] and an inverse relationship between exposure of ultraviolet light in men and RCC risk was observed [22].

Excess body weight has been established as a risk factor for RCC with it accounting for 30% of cases in Europe [26]. Various prospective studies conducted worldwide have reported that overweight and obese individuals were found to have an elevated subsequent risk of RCC [27-30] with a meta-analysis of this work also suggesting that an association between body mass index (BMI) and risk of RCC exists [31]. Some have suggested that body fat distribution is associated with an increased risk of RCC [28, 30] but evidence is limited suggesting that abdominal obesity is independent of BMI with the association with RCC. Two factors closely related to each other and obesity are diet and physical activity.

The majority of studies have demonstrated an inverse relationship between physical activity and RCC risk [32-40] with some authors reporting a dose response with further reduction of risk with increasing levels of activity [35, 37, 38]. Assessing dietary intake has reported mixed results with association with RCC. The role of vitamins that are abundant in fruit and vegetables has produced variable results with the risk of RCC with some reporting an association with RCC [41] whilst others have reported no correlation [42-44] whilst analysis of cohort studies has reported that diets rich in fruits and vegetables are inversely related to RCC [45]. High consumption of fat and protein has not been shown to be associated with an increased risk of RCC [46-48]. The consumption of alcohol has also been demonstrated to have a negative relationship with risk of RCC in a dose response manner [49] whilst in contrast no correlation was demonstrated with total fluid intake from any fluids or from individual types of fluids [50, 51] suggesting that it is not duration of contact with any potential carcinogens which prevents RCC risk with alcohol consumption.

2-3% of all RCC are familial [53, 54] with a two-fold increase in a first degree relative [56]. Of the various subtypes of RCC, each has a corresponding hereditary component caused by a distinct genetic alteration [52]. The most common familial syndrome for RCC is von Hippel Lindau syndrome (VHL) which can also cause patients to develop pheochromocytomas, retinal angiomas and haemangioblastomas of the central nervous system with only 40% suffering from RCC with VHL. The VHL gene is responsible for the degradation of hypoxia inducible factors without which leads to up-regulation of factors which promote angiogenesis and tumour growth such as vascular endothelial growth factor. In those with VHL syndrome, RCC is the most common cause of death.

Various medical conditions have been demonstrated to have an association with an increased risk of RCC. Types of renal tumours have been shown to cause hypertension [64]. Interestingly however, several studies have reported an association with long term

hypertension and risk of RCC [32, 33, 40, 65] as well as some reporting a dose response relationship [32] with the risk of RCC increasing with further elevation of blood pressure and decreasing with a reduction in blood pressure [65]. There have been reports that usage of anti-hypertensive treatment is also associated with an elevated risk of RCC but it is thought that it is hypertension causing this increased risk and not the actual treatment [32, 35, 36, 39, 66]. Obesity as a risk factor has already been mentioned but it has been reported that despite the relationship between obesity and hypertension, both are independent with their association with RCC and risk is higher in amongst those suffering from both conditions than those with only one [32, 36, 39, 65].

Diabetes mellitus is known to be associated with an increased risk of several cancers. Its relationship in RCC has not been demonstrated to be an independent factor but was closely associated with obesity and hypertension [35, 36, 40, 66-71].

It has been demonstrated that acquired renal cystic disease develops in those with end stage renal disease and in those on haemodialysis [72]. The incidence of RCC is higher in those with cystic disease [73, 74] but the evidence suggesting that these cysts undergo malignant change is not conclusive [74, 75]. Those who are undergoing haemodialysis are at a higher risk of RCC [75-77] as well as there being an increased risk of RCC after renal transplantation [77-79].

There has some been controversy surrounding an association between urinary tract infections and RCC, one study suggests that a history of a urinary tract infection increases the risk of RCC and this risk is further exacerbated with a history of smoking [80] whilst another report has demonstrated that no relationship between these parameters is present [81].

Conclusion

The incidence and mortality rates of renal cancer are starting to stabilise. Despite increasing usage of imaging, many tumours present at advanced stages. The rise in hypertension and obesity which are well established factors for RCC are likely to have contributed to the upward trend in recent years. The impact of smoking on RCC will decrease in Western countries but may grow in developing countries. Despite the evidence of various genetic, iatrogenic and lifestyle risk factors associated with RCC further work is required from well-designed epidemiological studies incorporating these various factors to gain further understanding of the aetiology of renal cancer.

Reclassification of the Fuhrman Grading System in Renal Cell Carcinoma-Does it make a difference?

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Purpose

The aim of this study was to determine whether reclassifying the Fuhrman grading system provides further prognostic information.

Materials and Methods

We studied the pathological features and cancer specific survival of 237 patients with clear cell cancer undergoing surgery between 1997-2007 in a single centre. The original Fuhrman grading system was investigated as well as various simplified models utilising the original Fuhrman grade.

Results

The median follow up was 69 months. On univariate analysis, the conventional Fuhrman grading system as well various simplified models were predicative of cancer specific survival. On multivariate analysis, only the three tiered modified model in which grades 1 and 2 were combined whilst grades 3 and 4 were kept separate was an independent predictor of cancer specific survival ($p=0.001$, HR 2.17, 95% CI 1.37-3.43). Furthermore this simplified model demonstrated a stronger relationship to recurrence than the conventional 4 tiered Fuhrman grading system.

Conclusion

A modified, three-tiered Fuhrman grading system has been demonstrated to be an independent predictor of cancer specific survival.

Introduction

In the UK alone, approximately 9000 new cases of renal cancer are diagnosed each year and nearly 4000 die of their disease [2]. Overall survival is poor, even for those patients who undergo resection; the estimated 5 year survival rate is only 50%.

Currently, the TNM stage and tumour grade are the most widely used tools to predict survival. Various grading classifications for clear cell carcinoma based on morphological features have been proposed [83, 136-141] and of these the Fuhrman grading system [138] has achieved widespread usage in pathology practise. The Fuhrman grading system has been demonstrated to be an independent predictor of survival [127] having been acknowledged as optimal for predicting outcome [144] and therefore has been incorporated into the majority of prognostic algorithms including SSIGN [145], UISS [146] and Leibovich [147].

The Fuhrman grading system is based on assessment of the uniformity of nuclear size, nuclear shape and nucleolar prominence [138]. The Fuhrman grading system has been demonstrated to correlate to metastasis with grade 1 tumours having a statistically significant lower metastases rate compared to those with grade 2 to 4 and survival rates being distinguished into 3 categories, those with grade 1, those with grade 4 and those with grades 2 and 3 [138]. Despite the popularity of this grading system, problems have been demonstrated regarding its application [137, 151, 507].

There has been suggestions that the Fuhrman grading system has low-moderate inter-observer agreement [148-151] and that a simplified system improves inter-observer agreement [148, 149] as well as demonstrating as much accuracy as the conventional grading system [152, 153]. Furthermore there are those suggesting that the ideal grading

system is yet to be defined and should consist of three tiers [151] whilst a three tiered system has been shown to be an independent predictor of survival [154, 155]. Given the evidence suggesting that a simplified system improves the prognostic ability of the Fuhrman grading, we aim to evaluate which if any simplified system would further aid in determining prognosis.

Materials and Methods

Patients with clear cell renal cancer were included for this study. These patients had undergone resection based on the surgical findings and the results of CT scans for staging purposes between January 1997 and Dec 2007 in the North Glasgow NHS Trust. The Research Ethics Committee of West of Scotland has approved the study.

Two hundred and thirty seven patients with clear cell renal cancer were identified retrospectively that underwent nephrectomy. The study cohort constituted a representative sample of all surgically treated patients within this period.

Clinicopathological data including T stage, nuclear grade assessment [138] and survival for each patient was collected. Survival was determined from the time of surgical treatment to the time of last follow up. The cause of death was determined by linkage through the Scottish Cancer Registry. In those who were deceased, if the primary cause of death was of renal cancer, these were classed as cancer specific and all other causes were non-cancer specific deaths. Patients notes were accessed for documented evidence of recurrence otherwise they were deemed to have no recurrences

The original Fuhrman grading system was investigated as well as various simplified systems utilising the Fuhrman grade. Table 1 shows the various simplified models that were investigated.

Statistical analysis was undertaken using SPSS (Chicago, IL, USA). Cancer specific survival rates were generated using the Kaplan Meir method. The log rank test was utilised to compare significant differences between subset groups using univariate analysis. Multivariate analysis was carried out based on the results of the univariate analysis. Multivariate Cox regression analysis was performed to identify those factors that were independently associated with cancer specific death. A stepwise backward procedure was

utilised to ascertain which of the variables had a significant independent relationship with survival.

Results

The patient characteristics are shown in Table 2. The median follow up was 69 (2.1-181) months. The median age was 60 (23-86) years. Thirty three patients died of their disease. Within the cohort, the most common tumour stage was pT1 (47%). The most common Fuhrman grades were II (36%) and III (41%).

Univariate analysis of potential predictors of cancer specific survival showed that the majority of the grading models were statistically significant predictors of cancer specific survival (Table 3, Figure 1). On multivariate analysis of those that were significant on univariate, only model 5 which is a modified three tired model combining grades 1 and 2 whilst grades 3 and 4 are kept as separate was found to be an independent prognostic factor in its association with cancer specific survival ($p=0.001$, HR 2.17, 95% CI 1.37-3.43, Table 3).

On χ^2 analysis of the various simplified grading models, whilst majority of the grading models demonstrated a positive correlation with T Stage (Table 4), model 3, which is a two tired model combining grades 1, 2 and 3 whilst grade 4 is kept separate demonstrated the strongest correlation to T Stage ($p<0.001$, Table 4). When analysing the grading models, the majority of these demonstrated a positive correlation to recurrence, whilst models 3 and 5 demonstrated the strongest correlation to this clinicopathological factor ($p<0.001$, Table 4).

Discussion

In this cohort of patients with clear cell carcinoma, a simplified 3-tiered model where grades 1 and 2 are combined whilst grades 3 and 4 were kept separate (model 5) was an independent predictor of cancer specific survival on multivariate analysis. Furthermore, this modified model was also one of only two to correlate to disease recurrence.

Several studies have demonstrated that the Fuhrman grading system is capable of predicting cancer specific survival independent of pathological stage [85, 145]. Studies however have demonstrated that the conventional Fuhrman grading system is complex [142, 154] and that a simplified system improves inter-observer agreement [148, 149]. It has previously been demonstrated that combining grades 1 and 2 improves the prognostic ability of the Fuhrman grading system and a three tiered system combining grades 1 and 2 whilst keeping grades 3 and 4 separate is an independent predictor of survival [154, 155], a finding similar to that reported in this study. There have been reports that this simplified three tiered model has a similar performance in multivariate models predicting outcome to the conventional 4 tiered Fuhrman system [152, 153]. In terms of cancer specific survival, the gap between grades 3 and 4 was more evident than the gap between grades 1 and 2. This result is similar to that demonstrated by several other studies [152-155] where a three tiered model was proposed [154, 155]. This further shows that grades 3 and 4 would be less suitable for combining than grades 1 and 2 and strengthens the argument for a three tiered model.

As with most studies examining the modification of the Fuhrman grading system, the present study is limited due its retrospective nature with no analysis of inter or intra observer variability when assigning the Fuhrman grade and no external review of nuclear grade.

In the present study, a simplified version of the Fuhrman grading system whereby grades 1 and 2 are combined and grades 3 and 4 were kept separate was shown to be an independent predictor of cancer specific survival and demonstrated a positive correlation to disease

recurrence suggesting that this modified model can be considered an option for the purposes of prognosis in those with clear cell renal cancer. Further work is required in terms of a prospective study for validation.

Acknowledgments

This work was supported by Think Pink, Gartnavel Urology Endowment Fund and Renal Cancer Research Fund.

Table 1: Demonstrating the various simplified grading systems investigated.

Fuhrman Grading System	1	2	3	4
Grading System 1	1		2	
Grading System 2	1	2		
Grading System 3	1			2
Grading System 4	1	2	3	
Grading System 5	1		2	3

Table 2: Relationship between clinicopathological characteristics and cancer specific survival.

<i>Variable</i>	<i>Numbers</i>	
		p-value
Age (<60/>60)	108/129	0.918
T Stage (1/2/3/4)	112/35/85/5	<0.001
Nuclear Grade (1/2/3/4)	25/86/97/29	0.005
Recurrence (No/Yes)	178/59	<0.001

Table 3: Relationship between various simplified nuclear grading systems and cancer specific survival.

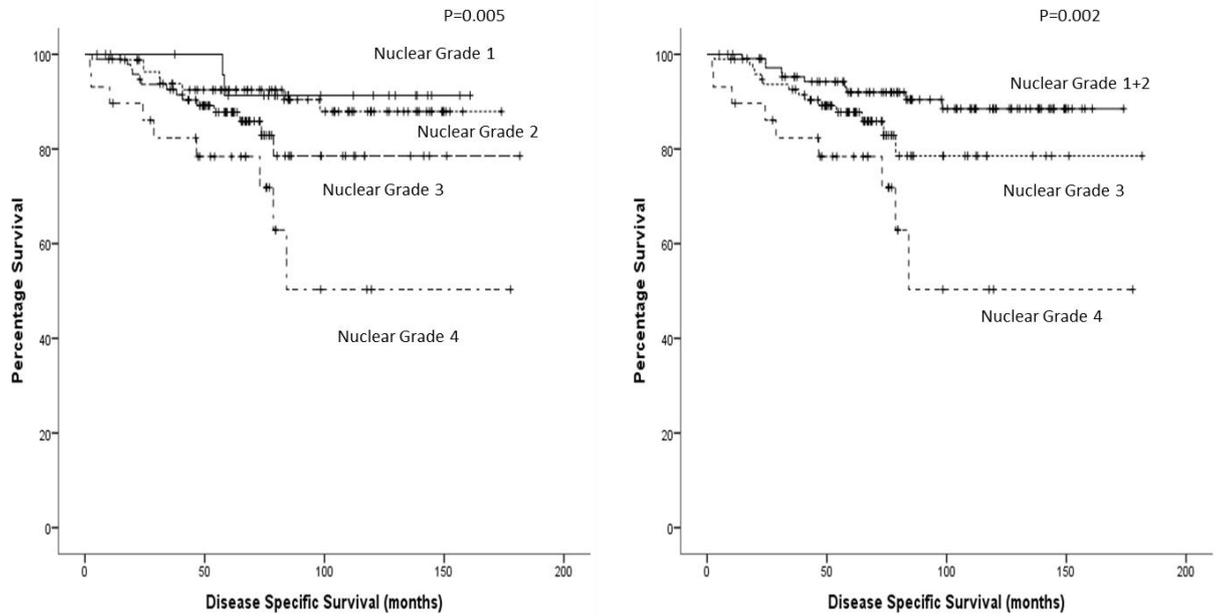
<i>Variable</i>	<i>Numbers</i>	<i>Univariate Analysis</i>	<i>Multivariate Analysis</i>	
		p-value	p-value	HR
Nuclear Grade (1/2/3/4)	25/86/97/29	0.005		
Grading System 1 ((1+2)/(3+4))	111/126	0.008		
Grading System 2 ((1/(2+3+4)))	25/212	0.237		
Grading System 3 ((1+2+3)/4)	208/29	0.002		
Grading System 4 (1/2/(3+4))	25/86/126	0.029		
Grading System 5 ((1+2)/3/4)	111/97/29	0.002	0.001	2.17 (1.37-3.43)

Table 4: Interrelationship between clinicopathological characteristics of patients and various simplified nuclear grading systems.

<i>Variable</i>	<i>Numbers</i>	<i>T Stage</i>	<i>Recurrence</i>
Nuclear Grade (1/2/3/4)	25/86/97/29	0.001	0.001
Grading System 1 ((1+2)/(3+4))	111/126	0.029	0.01
Grading System 2 ((1/(2+3+4))	25/212	0.07	0.278
Grading System 3 ((1+2+3)/4)	208/29	<0.001	<0.001
Grading System 4 (1/2/(3+4))	25/86/126	0.015	0.016
Grading System 5 ((1+2)/3/4)	111/97/29	0.001	<0.001

Figure legends

Figure 1: Kaplan Meier graphs demonstrating the conventional 4 tiered Fuhrman grading system ($p=0.005$) and a simplified model where grades 1 and 2 are combined and grades 3 and 4 are kept separate ($p=0.002$) against disease specific survival.



Is the presence or absence of tumour necrosis a significant predictor of survival in Renal Cell Cancer?

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Short Title: Reclassification of tumour necrosis in renal cancer

Keywords: tumour necrosis, renal cancer, survival

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Introduction

Currently when renal cancer pathology is assessed the presence or absence of necrosis is simply reported. It has been suggested that a presence or absence response ignores its heterogeneity and a classification based on extent of necrosis involvement would aid prognostic value in cancer specific survival. The aim of this study was to determine whether a quantitative assessment of tumour necrosis would provide additional prognostic information.

Materials and Methods

We studied the pathological features and cancer specific survival of 47 patients with renal cancer undergoing surgery with curative intent. A quantitative assessment of tumour necrosis was compared to the presence or absence of necrosis.

Results

Tumour necrosis was present in 27 of 47 cases. A simple assessment of presence or absence was not associated with cancer specific survival ($p=0.052$). When assessed quantitatively, tumour necrosis was associated with decreased cancer specific survival ($p<0.001$). A two tiered assessment, less than 25% and greater than 25% involvement of necrosis, was further utilised and shown to predict cancer specific survival ($p<0.001$). On multivariate analysis, using this two tiered assessment of less than and greater than 25% involvement of necrosis was retained as a significant independent factor for cancer specific survival (HR 11.84, 95% CI 3.81-36.75, $p<0.001$).

Conclusion

A simple assessment of presence/absence of tumour necrosis is reported to be a prognostic factor in renal cell cancer. In this study, the presence/absence was not shown to be a significant prognostic marker of cancer specific survival. However, a more accurate quantitative assessment of tumour necrosis, whereby a two tiered response is still utilised but basing this on less than 25% and greater than 25% involvement of necrosis was statistically significant and independent in predicting cancer specific survival.

Introduction

In the UK alone, approximately 8000 new cases of renal cancer are diagnosed each year [2] and 3800 die of their disease. Of these cases, nearly 80% are clear cell in origin [2].

Various prognostic indicators consisting of anatomical, histological, clinical and immunohistochemical factors are available allowing risk stratification [121, 524]. One of these indicators is the presence or absence of tumour necrosis and this is incorporated into prognostic algorithms such as SSIGN [145] and Leibovich [147] scores. Tumour necrosis occurs when tumours outgrow their blood supply therefore reflecting aggressive tumour biology and rapid proliferation and progression [156]. Several studies have investigated tumour necrosis as a prognostic factor for patients with renal cell cancer and reported conflicting results [137, 156, 166, 525]. Necrosis is evaluated on a presence or absence basis and therefore does not take into account the extent of necrosis. It has been suggested that an extent based classification is superior and retained as an independent prognostic factor [173]. Given the controversy of the presence/ absence of tumour necrosis being of prognostic significance and that an extent based classification being superior, we evaluated the role of necrosis on an extent based classification.

Patients and Methods

Patients with renal cell cancer were included for this study. These patients had undergone resection based on the surgical findings and the results of CT scans for staging purposes between January 1998 and Dec 2000 in the North Glasgow NHS Trust. The Research Ethics Committee of West of Scotland has approved the study.

Forty seven patients with clear cell renal cancer were identified retrospectively that underwent nephrectomy. The study cohort constituted a representative sample of all surgically treated patients within this period. Clinicopathological data including T stage, nuclear grade assessment [138] disease recurrence and survival for each patient was collected. Survival was determined from the time of surgical treatment to the time of last follow up.

The routine haematoxylin and eosin slides were reviewed from pathology archives. Tumour necrosis was evaluated on histological sections and was graded under the supervision of a pathologist (JJG). This was performed according to established histological criteria [156, 515]. Necrosis related to haemorrhage and foci of hyalinization was not considered. The extent of necrosis was graded with 0 when no necrosis was present; a score of 1 given when there was <25% necrosis, a score of 2 when necrosis was 25-50% and 3 for necrosis >50%.

Statistical analysis was undertaken using SPSS. Disease specific survival rates were generated using the Kaplan-Meier method. The log rank test was utilised to compare significant differences between subset groups using univariate analysis. Multivariate analysis was carried out based on the results of the univariate analysis. Multivariate Cox regression analysis was performed to identify those factors that were independently

associated with disease specific death. A stepwise backward procedure was utilised to ascertain which of the variables had a significant independent relationship with survival.

Results

Analysis was based on 47 clear cell renal cancer patients with full clinical follow up. Median age at diagnosis was 59 years (range, 41-80 years). Median follow up was 98 months (range, 0.1-163.3. months). 20 patients died of their disease. 33 patients had T1/2 disease and 14 patients had T3/4 disease. 21 patients had evidence of recurrence at time of follow up.

Necrosis was present in 27 cases (57%). On χ^2 analysis of absence/presence of necrosis, there was no correlation with T stage, nuclear grade, recurrence or tumour volume (Table 1). On univariate analysis, absence/presence of necrosis was associated with poorer survival but failed to reach significance ($p=0.052$) (Table 2).

When analysing extent of involvement of necrosis, 43% of cases had no necrosis, 28% had <25% involvement of necrosis, 21% of cases had between 25-50% involvement and 8% had >50% involvement of necrosis. On χ^2 analysis (Table 3) using an extent based classification, there was no correlation with T stage or nuclear grade. There was a positive correlation with recurrence ($p=0.009$) and tumour volume ($p=0.017$). On univariate analysis, a higher extent of involvement was associated with poor cancer specific survival ($p<0.001$) (Table 2) (Fig 1a).

Further analysis was performed on the quantitative assessment of necrosis to assess if it could be further refined into a simpler two tired system. Analysis of this quantitative assessment into a two tired scoring system, <25% and >25% involvement of necrosis showed 14 cases (30%) had more than 25% involvement of necrosis. On χ^2 analysis there was no correlation with T stage or nuclear grade (Table 4). There was a positive correlation with recurrence ($p=0.003$) and tumour volume ($p=0.007$). On univariate analysis, using the

simpler 2 tiered assessment of <25% and >25% involvement, this was associated with poorer cancer specific survival ($p < 0.001$) (Fig 1b). This significance was maintained on multivariate analysis (HR 11.84, 95% CI 3.81-36.75, $p < 0.001$) (Table 2).

Conclusion

We have shown that the prognostic information provided by an extent based classification is superior to a simple absence/presence response. Furthermore, when utilising this quantitative assessment in a two tiered system, <25% and >25% involvement of tumour necrosis, this was retained as an independent prognostic factor.

Historically, tumour necrosis has been associated with more aggressive tumour activity. Studies have produced conflicting results regarding the prognostic significance of tumour necrosis, with some showing an association with poor survival on univariate analysis but not being retained as an independent prognostic factor whilst others have shown no link with prognosis [137, 145, 156, 166, 525]. Klatte et al have shown that an extent based classification of necrosis is superior to a simple absence/presence response and is retained as an independent prognostic factor [173]. In this study we have also shown that a quantitative assessment of necrosis is superior to an absence/presence response but is not retained as an independent prognostic factor. Klatte et al suggested a cut off of 20% and a 3-tiered system and we used a similar figure of 25% but utilised a 2-tiered system. When using the cut off of 25% and only having two groups, less than 25% and more than 25% involvement of necrosis, we have shown that this was associated as an independent prognostic factor. We felt that using a cut off of 25% and maintaining to use a two-tiered system would allow simpler refinement of the necrosis parameter in prognostic algorithms where a two tiered system already is already utilised and to examine if this were to increase the predictive accuracy of the entire model but this is yet to be tested.

Despite the low number of patients in this study, we were able to show that an extent based classification of necrosis was prognostic of survival and furthermore with a cut off of 25% it was shown to be an independent prognostic factor for cancer specific survival. We feel that further work is required to confirm these findings and to see if this would increase the predictive accuracy of prognostic algorithms and therefore become routine when examining every pathological specimen of clear cell renal carcinoma.

Acknowledgments

This work was supported by Think Pink and the Gartnavel Urology Endowment Fund.

Table 1: Interrelationships between clinicopathological characteristics of patients with renal cancer and presence and absence of tumour necrosis.

Variable	Numbers	T Stage	Nuclear Grade	Tumour Necrosis (negative/positive)	Recurrence	Tumour Volume
Age (<60/>60)	28/19	0.282	0.353	0.96	0.141	0.372
T Stage (1/2/3/4)	22/11/12/2		0.001	0.446	0.003	<0.001
Nuclear Grade (1/2/3/4)	8/20/13/6			0.299	0.003	0.074
Tumour Necrosis (negative/positive)	20/27				0.256	0.226
Recurrence (No/Yes)	26/21					0.011
Tumour Volume (<10cm ³ />10cm ³)	21/26					

Table 2: Relationships between clinicopathological characteristics, various classifications of necrosis and disease specific survival.

Variable	Numbers	Univariate Analysis	Multivariate Analysis	
		p value	p value	HR
Age (<60/>60)	28/19	0.712		
T Stage (1/2/3/4)	22/11/12/2	<0.001	0.006	2.41 (1.28-4.53)
Nuclear Grade (1/2/3/4)	8/20/13/6	0.008	0.012	2.29 (1.20-4.36)
Tumour Necrosis (negative/positive)	20/27	0.052		
Tumour Necrosis (0/1/2/3)	20/13/10/4	<0.001		
Tumour Necrosis (<25%/>25%)	33/14	<0.001	<0.001	11.84 (3.81-36.75)

Table 3: Interrelationships between clinicopathological characteristics of patients with renal cancer and quantifying the extent of necrosis.

Variable	Numbers	T Stage	Nuclear Grade	Tumour Necrosis (0/1/2/3)	Recurrence	Tumour Volume
Age (<60/>60)	28/19	0.282	0.353	0.514	0.141	0.372
T Stage (1/2/3/4)	22/11/12/2		0.001	0.371	0.003	<0.001
Nuclear Grade (1/2/3/4)	8/20/13/6			0.28	0.003	0.074
Tumour Necrosis (0/1/2/3)	20/13/10/4				0.009	0.017
Recurrence (No/Yes)	26/21					0.011
Tumour Volume (<10cm ³ />10cm ³)	21/26					

Table 4: Interrelationships between clinicopathological characteristics of patients with renal cancer and an extent based classification of necrosis with a 25% cut off.

<i>Variable</i>	<i>Numbers</i>	<i>T Stage</i>	<i>Nuclear Grade</i>	<i>Tumour Necrosis (<25%/>25%)</i>	<i>Recurrence</i>	<i>Tumour Volume</i>
Age (<60/>60)	28/19	0.282	0.353	0.96	0.141	0.372
T Stage (1/2/3/4)	22/11/12/2		0.001	0.446	0.003	<0.001
Nuclear Grade (1/2/3/4)	8/20/13/6			0.299	0.003	0.074
Tumour Necrosis (<25%/>25%)	33/14				0.003	0.007
Recurrence (No/Yes)	26/21					0.011
Tumour Volume (<10cm ³ />10cm ³)	21/26					

Figure 1a: Kaplan Meier survival graph for a quantitative based assessment of necrosis against disease specific survival.

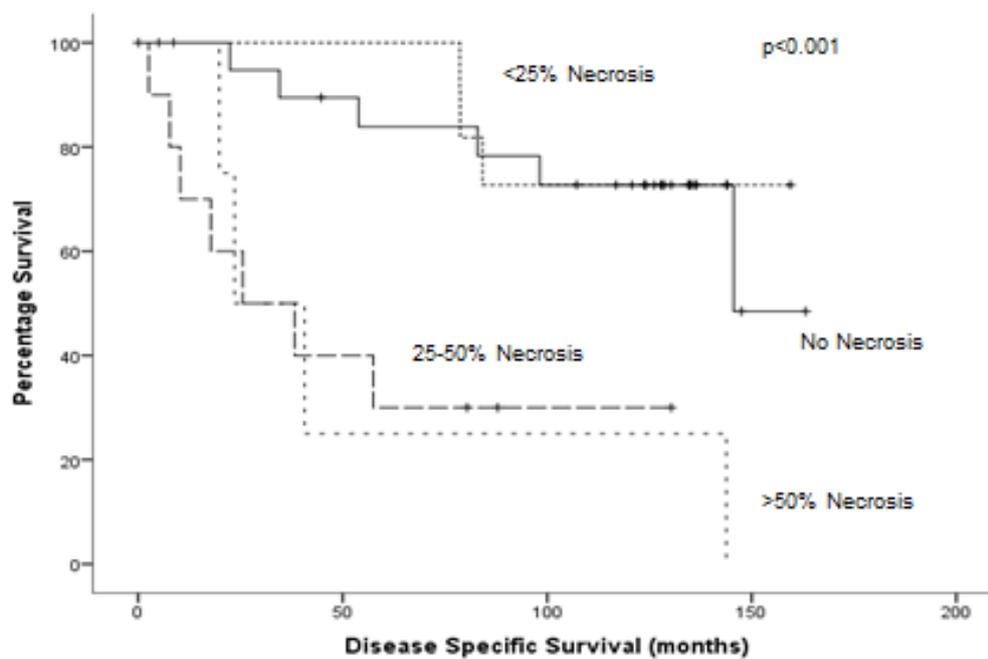
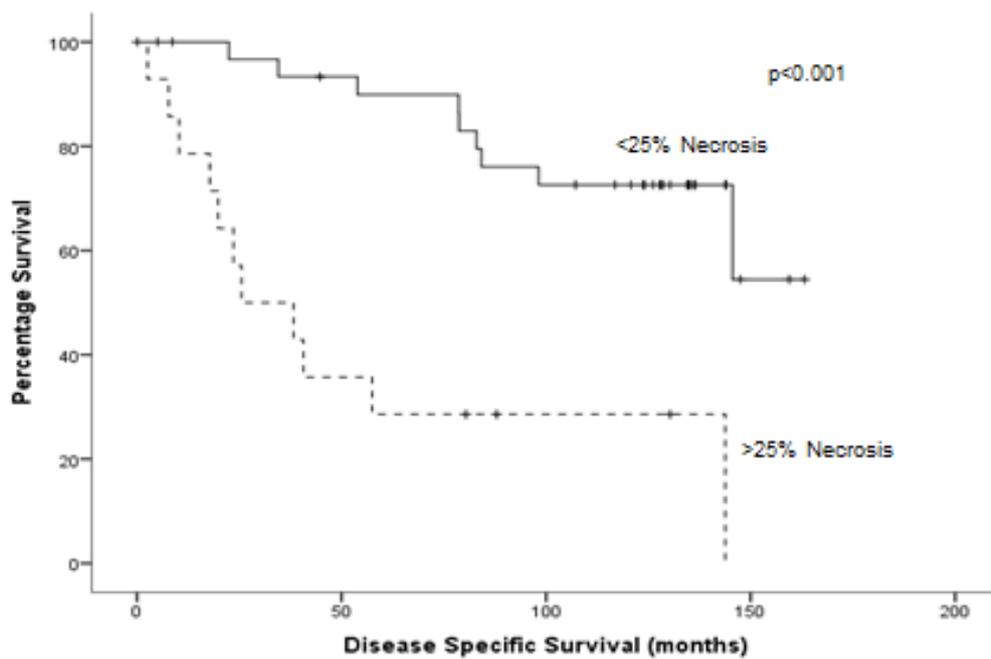


Figure 1b: Kaplan Meier survival graph using a 25% cut off for necrosis against disease specific survival.



A prospective study of the role of inflammation in renal cancer

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Klintrup-Makinen score

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Background: The local and systemic inflammatory responses provide prognostic information in cancer. The modified Glasgow Prognostic Response (mGPS) provides additional prognostic information than C-reactive protein (CRP) alone when assessing the systemic inflammation in cancer. The aim of this study was to determine the role of local and systemic inflammation in renal cancer.

Methods: The cohort consisted of 79 patients who had undergone potential curative resection. Systemic inflammation, mGPS, was constructed by measuring pre-operative CRP and albumin concentrations and the Klintrup-Makinen score was evaluated histologically for the local inflammatory response. Pathological parameters such as T stage, grade and tumour necrosis were also assessed. Preoperative circulating CRP and albumin levels were measured. The local inflammatory response was assessed by examining all inflammatory cells at the tumour edge on diagnostic haematoxylin and eosin slides.

Results: On univariate analysis, T stage ($p < 0.001$), Grade ($p = 0.044$) and mGPS ($p < 0.001$) were significant predictors of cancer specific survival. On multivariate analysis, mGPS (hazard ratio 8.64, 95% confidence interval 3.5-21.29, $p < 0.001$) was the only significant independent predictor of cancer specific survival.

Conclusion: A preoperative systemic inflammatory response as measured by the mGPS is an independent predictor of poor cancer specific survival in renal cancer in patients undergoing potential curative resection.

Introduction

In the UK alone, approximately 8000 new cases of renal cancer are diagnosed each year and 3800 die of their disease [2]. Overall survival is poor, even for those patients who undergo resection; the estimated 5 year survival rate is only 50% [2].

There has been a long standing interest in identifying those patients most at risk of disease progression and ultimately dying from their disease. Ideally, a factor or combination of factors that could clearly stratify patients into those who do not progress and those that progress and are at higher risk of dying from their cancer would be highly beneficial. Currently, the TNM stage and tumour grade are the most widely used tools to predict survival and these are incorporated in the majority of prognostic algorithms including SSIGN [145], UISS [508] and Leibovich[147].

It is now established that disease progression in cancer patients is not solely determined by the tumour characteristics but also by the host response. There is increasing evidence that both local and systemic inflammatory responses play an important role in the progression of various solid tumours [174, 175].

Recent evidence suggests that intensity of local inflammatory infiltrate within the tumour bed predicts prognosis: a pronounced lymphocytic infiltration in colorectal cancer is associated with improved survival [181-183]. Also quantifying the degree of infiltration by lymphocyte subsets such as CD8+ and CD4+ T cells provides prognostic information in various tumour types[184, 185] including renal cancer[186]. The process of assessing lymphocyte infiltration is time consuming and has not been adopted into routine clinical practice. It is therefore of interest that Klintrup and colleagues have reported a simplified method of assessing the inflammatory cell infiltrate at the tumour margin,[187] showing on routine haematoxylin and eosin stained sections, that tumour inflammatory infiltrate,

including all white cell types, can be graded high or low grade, with a high grade infiltrate being associated with improved survival in colorectal cancer[187, 198].

In addition, increasing evidence supports a role of the systemic inflammatory response, indicated by elevated levels of C-reactive protein (CRP) being an independent predictor of survival in patients with a variety of common solid tumours including gastrointestinal, lung, prostate, bladder and renal cancer[194-199]. The modified Glasgow Prognostic Score (mGPS), incorporates CRP and albumin serum levels[200]. The mGPS score has provided additional prognostic information in patients with various solid malignancies including lung, gastroesophageal and colorectal cancers [201-203].

The prognostic role of tumour necrosis is well established in malignancies such as lung and colorectal [163, 514]. Despite using necrosis in prognostic algorithms in renal cancer, there are still some conflicting results regarding its prognostic significance with some showing an association with poor survival on univariate analysis but not being retained as an independent prognostic factor whilst others have shown no link with prognosis[137, 145, 156, 167, 525].

The aim of this study was to assess relationships between a systemic inflammation prognostic score (mGPS), the local tumour inflammatory cell infiltrates, tumour necrosis and cancer specific survival in patients with renal cancer undergoing potential curative resection.

Materials and Methods

Patients with clear cell renal cancer were included for this study. These patients had undergone complete resection of the tumour at time of nephrectomy and pre-operative CT scans showed no evidence of regional or metastatic spread. Patients were staged pathologically and graded according to the TNM classification and Fuhrman grading respectively. The Research Ethics Committee of West of Scotland has approved the study.

Diagnostic haematoxylin and eosin sections from pathology archives were reviewed. A minimum of three slides from the deepest area of tumour invasion were reviewed and were scored according to the Klintrup-Makinen criteria (K/M)[187]. This method is based on scoring inflammation at the deepest point of invasion identified from the three slides. A four point scale was used. A score of 0 was given for no increase of the inflammatory cells at the invasive margin; a score of 1 denoted a mild and patchy increase of inflammatory cells. Score 2 was assigned when inflammatory cells formed a band-like infiltrate at the invasive margin. A score of 3 was given when a prominent inflammatory reaction formed a cup-like zone at the margin. Scores of 0 and 1 were combined (low grade inflammation) and scores of 2 and 3 combined (high grade inflammation). Scoring was supervised by a pathologist (JJG).

Preoperative systemic inflammatory response was assessed using the modified Glasgow Prognostic Score (mGPS) [200]. Patients with both elevated C-reactive protein (>10mg/l) and hypoalbuminaemia (<35g/l) scored 2. Patients in whom both were normal scored 0. Patients with elevated C-reactive protein alone were scored as 1 while those with hypoalbuminaemia alone were scored as 0.

The presence or absence of tumour necrosis was evaluated on histological sections and graded under the supervision of a pathologist (JJG).

Statistical analysis was undertaken using SPSS. Disease specific survival rates were generated using the Kaplan-Meir method. The log rank test was utilised to compare significant differences between subset groups using univariate analysis. Multivariate analysis was carried out based on the results of the univariate analysis. Multivariate Cox regression analysis was performed to identify factors independently associated with disease specific death. A stepwise backward procedure was utilised to ascertain which of the variables had a significant independent relationship with survival.

Results

Seventy nine patients were studied. Median age at diagnosis was 60 years (range 39-82). Median follow up was 93 months (range 0.1-152). 40 patients had recurrence. 19 patients died of their disease.

χ^2 demonstrated that mGPS was positively correlated with tumour stage, grade and necrosis (p=0.001, p=0.044 and p=0.042) (Table 1).

On univariate analysis, T stage (p<0.001), grade (p=0.044) and mGPS (p<0.001, Figure 1) were significant predictors of cancer specific survival whilst local inflammatory response and necrosis did not show significance (p=0.152 and p=0.122) (Table 2). On multivariate analysis of the significant individual covariates mGPS (hazard ratio 8.64, 95% confidence interval 3.5-21.29, p<0.001) was a significant independent predictor of cancer specific survival (Table 2).

Discussion

Results from the present study demonstrate that an elevated mGPS independently correlates to a poor cancer specific survival in those undergoing potentially curative treatment with renal cancer. We have also demonstrated that an elevated mGPS is directly associated with tumour stage, grade and necrosis.

Previous studies have demonstrated that the local inflammatory response [187, 198] and tumour necrosis [163, 514] play a prognostic role in various malignancies. It has been reported that quantifying the degree of infiltration by lymphocyte subsets provides prognostic information in renal cancer[186]. It was therefore of interest that a simplified assessment of the local inflammatory cell infiltrate [187] which is not as time consuming as quantifying lymphocyte subsets has been shown to prognostic in colorectal cancer[187, 198]. The role of necrosis in renal cancer, despite being utilised in prognostic algorithms has produced conflicting results [137, 145, 156, 167, 525]. In this study, neither local inflammatory response nor necrosis were significant in disease specific survival. One of the reasons could be the small cohort study but given previous conflicting results for necrosis with larger study numbers, an increase in numbers may still not show necrosis to be significant in disease specific survival in this study.

mGPS is a well-established marker of systemic inflammation. The variables used are common ones and offer the benefit of being objective and obtainable. We have demonstrated an independent association between preoperative systemic inflammation and cancer specific survival. A limitation of this study is small cohort size, only 79 patients were available for analysis and from these 79 only 19 patients had died of their disease. We therefore would recommend that this study be expanded to a multi-centre study in order to increase cohort size and increase study power.

Conclusion

These results support the evaluation and introduction of the inflammation based (mGPS) scoring system as an independent predictor of poor cancer specific survival in those undergoing potential curative resection for renal cancer.

Conflict of Interest

The authors declare that they have no conflict of interest

Acknowledgments

This work was supported by Think Pink and the Gartnavel Urology Endowment Fund.

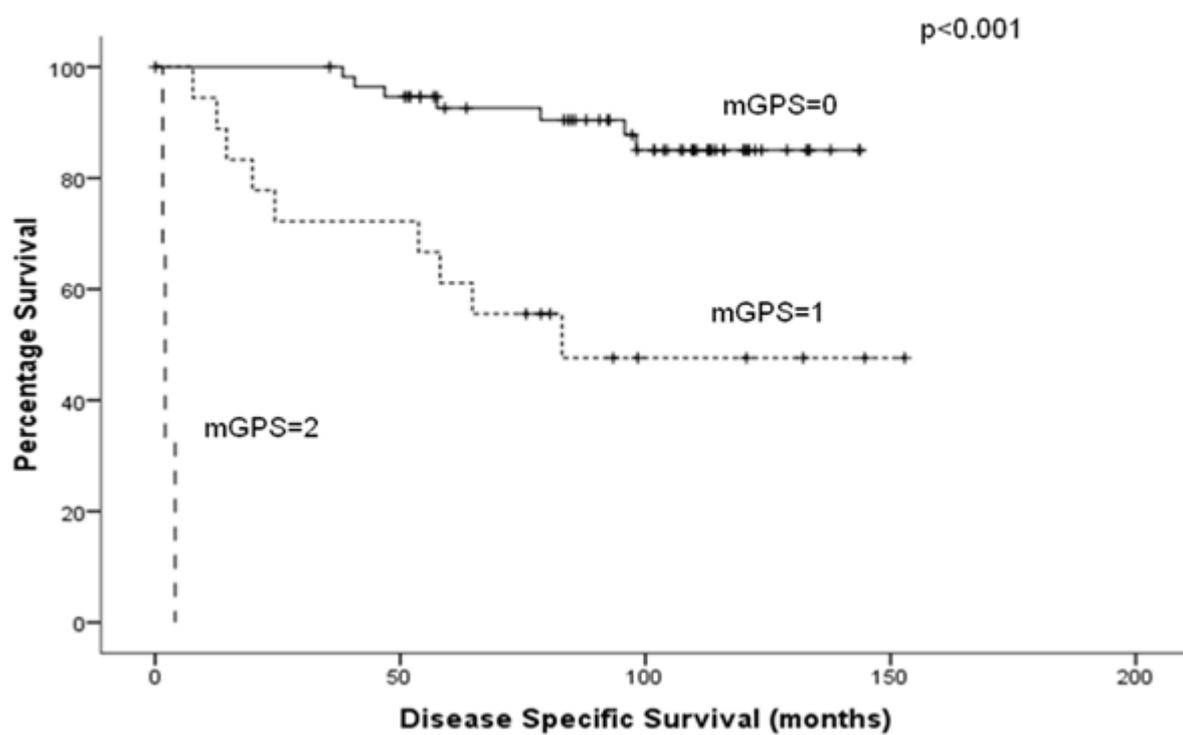
Table 1: Interrelationships between clinicopathological characteristics of patients with renal cancer.

Variable	Numbers	Sex	Grade	T Stage	Tumour Necrosis	Local inflammatory cell infiltrate	mGPS
Age (<65/>65)	40/39	0.316	0.954	0.423	0.291	0.831	0.054
Sex (male/female)	47/32		0.15	0.164	0.233	0.241	0.713
Grade (1/2/3/4)	9/37/21/12			0.005	0.315	0.59	0.044
T Stage (T1/T2/T3/T4)	32/14/29/4				0.166	0.595	0.001
Tumour Necrosis (absence/presence)	39/40					0.194	0.042
Local inflammatory cell infiltrate (low/high)	62/17						0.755
mGPS (0/1/2)	57/19/3						

Table 2: Relationships between clinicopathological characteristics and disease specific survival.

Variable	Numbers	Univariate Analysis	Multivariate Analysis	
		p value	p value	HR
Age (<65/>65)	40/39	0.838		
Sex (male/female)	47/32	0.259		
Grade (1/2/3/4)	9/37/21/12	0.044		
T Stage (T1/T2/T3/T4)	32/14/29/4	<0.001		
Tumour Necrosis (absence/presence)	39/40	0.122		
Local inflammatory cell infiltrate (low/high)	62/17	0.152		
mGPS (0/1/2)	57/19/3	<0.001	<0.001	8.64 (3.5-21.29)

Figure 1: Kaplan Meier survival graph for mGPS against disease specific survival.



Expression and Prognostic significance of Src Family Members in Renal Clear Cell Carcinoma

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- Running Title: The role of Src kinase in Renal Cancer

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Conflict of Interest: None
Word Count 3976

Purpose

The aim of this study was to determine whether Src family kinases (SFK) are expressed in renal cell cancer and to assess their prognostic significance.

Materials and Methods

mRNA expression levels were investigated for the eight SFK members by quantitative real-time PCR in nineteen clear cell cancer tissue samples. Immunohistochemical staining was utilised to assess expression of Src kinase, dephosphorylated Src kinase at Y⁵³⁰ (SrcY⁵³⁰), phosphorylated Src at Y⁴¹⁹ (SrcY⁴¹⁹) and the downstream focal adhesion marker (FAK) at the Y⁸⁶¹ site (FAK Y⁸⁶¹) in a cohort of fifty seven clear cell renal cancer specimens. Expression was assessed using the weighted histoscore method.

Results

Src, Lyn, Hck, Fgr and Fyn were the most highly expressed in renal cancer. All members were more highly expressed in T2 disease and furthermore expression levels between T2 and T3 disease showed a significant decrease for Lck, Lyn, Fyn, Blk and Yes ($p=0.032$).

Assessment of membrane, cytoplasm and nuclear expression of Src kinase, SrcY⁵³⁰ and SrcY⁴¹⁹ were not significantly associated with cancer specific survival.

High expression of cytoplasmic FAK Y⁸⁶¹ was associated with decreased cancer specific survival ($p=0.001$). On multivariate analysis, cytoplasmic FAK Y⁸⁶¹ was independently associated with cancer specific survival (HR 3.35, 95% CI 1.40-7.98, $p=0.006$).

Conclusion

We have reported that all SFK members are expressed in renal cell carcinoma. SFK members had their highest levels of expression prior to the disease no longer being organ confined. We hypothesise that that these SFK members are up-regulated prior to the cancer spreading out-with the organ and given that Src itself is not associated with cancer specific survival but the presence of FAK Y⁸⁶¹, a downstream marker for SFK member activity is associated with decreased cancer specific survival, we hypothesise that another of the SFK members is associated with decreased cancer specific survival in renal cell cancer.

Keywords: Src kinase, Src, FAK, Renal cancer

Introduction

In the UK alone, approximately 8000 new cases of renal cancer are diagnosed each year and 3800 die of their disease [2]. Overall survival is poor, even for those patients who undergo resection; the estimated 5 year survival rate is only 50%.

Treatment options are limited when there is evidence of inoperable metastatic disease. Cytotoxic chemotherapy has a minimal activity and is rarely used [305]. Immunotherapy has been demonstrated to provide a modest survival benefit but is associated with high levels of toxicity [307, 526]. At present, the mainstay of drug therapy for advanced renal cancer involves the sequential use of vascular endothelial growth factor (VEGF) receptor tyrosine kinase inhibitors (such as sunitinib, pazopanib and sorafenib) and inhibitors of mammalian target of rapamycin (mTOR) (such as everolimus or temsirolimus). Despite these recent advances, the outlook for these patients remains poor with little prospect of a cure. Sustained efforts continue to identify activated signalling pathways in renal cancer in order to develop further appropriate targeted therapies.

One potential molecular target is the non-receptor tyrosine kinase Src, the first identified human proto-oncogene. Src kinase has a role in signal transduction of multiple oncogenic cellular processes including migration, adhesion, invasion, angiogenesis, proliferation and differentiation and has significant interactions with other cellular proteins such as growth factor receptors [317]. Src kinase is the prototypical member of the Src kinase family (SFK), with a total of 8 members expressed in mammalian cells (Src kinase, Blk, Fgr, Fyn, Yes, Hck, Lck & Lyn). Src kinase is composed of a C-terminal tail, kinase domain, two protein-protein interaction domains (SH2, SH3) and a unique amino-terminal domain that varies between Src family members. Src kinase is activated by a number of pathways. Src kinase activation involves phosphorylation of a conserved tyrosine residue in the C-

terminal negative regulatory tail region (Y⁵³⁰) and subsequent autophosphorylation of the Y⁴¹⁹ site in the kinase domain [383, 384]. Consequently antibodies to phosphorylated Src kinase at the Y⁴¹⁹ site can be used as a marker for activated Src kinase [519]. In addition, when SFK's are activated, several downstream markers such as focal adhesion kinase (FAK) are phosphorylated and could therefore act as biomarkers for SFK activation [459]. FAK is phosphorylated at several sites by Src such as Y³⁹⁷, Y⁵⁷⁶ and Y⁵⁷⁷ but it has been reported that the Y⁸⁶¹ is the major site of phosphorylation in the carboxyl-terminal domain of FAK [453-455].

There is much evidence showing that levels of SFK are elevated in various malignancies such as prostate, breast, colon and lung [348, 364, 516-518]. Specifically, elevated levels of Src and Lyn (a SFK member) have been observed in breast cancer and expression of Src was associated with decreased survival [516]. Src expression does not always correlate with poor survival, it has been reported that Src expression and activity decreases with bladder tumour stage [357-359, 361, 362] and grade [360]. Expression of Lyn has also been associated with malignancies including breast, colon and prostate [329, 332].

Previous studies have suggested that the Src kinase family member, Src kinase is involved in the progression of urological tumours [348, 527]. Currently Saractinib (AZD05300), an oral Src inhibitor, is being utilised in a Phase II trial in renal cancer (COSAK trial). The aim of the current study is to investigate which if any of the SFK members are expressed in renal cell carcinoma and to assess if Src kinase expression and activation status is associated with poor prognosis.

Materials and Methods

Nineteen clinical specimens were utilised for Real time quantitative PCR (RT-PCR). This consisted of malignant tissue taken from renal cell carcinoma patients at the time of resection. Those specimens were utilised where all tissue was removed at time of surgery and radiological imaging prior to surgery showed no evidence of metastatic spread of disease. The Research Ethics Committee of West of Scotland has approved the study. RT-PCR was utilised to determine mRNA expression of the SFK members. At the time of resection, representative parts of malignant tissue were identified, snap frozen and stored in liquid nitrogen. Total mRNA was extracted from 5-10mg of renal cell cancer tissue using the TRIZOL method according to manufacturer's protocol (Invitrogen, Paisley UK). RNA quality and quantity was examined by UV spectrometry (GeneQuant analyser, GE Healthcare, Little Chalfont, UK).

Once RNA quality and quantity had been assessed, to ensure that no other DNA was present, DNA-free DNase treatment and removal reagent kit was added (Applera, Warrington, UK). Prior to incubation for 30 minutes at 37°C, to ensure that the same volume of cDNA was being utilised for quantification, a starting concentration of 1000ng of RNA was applied for each sample. Random hexamer primers (50ng) were used for First Strand cDNA synthesis using SuperScript II RT according to manufacturer's instructions (Invitrogen). Before using cDNA for PCR amplification, 2 units of RNase H were added to samples and incubated for 20 minutes at 37°C. RTPCR was performed using an ABI Prism 7900 Sequence Detection System (Applied Biosystems, UK) and TaqMan® Gene Expression Assays (table 2.1). For the TaqMan® Gene Expression Assays the manufacturer's protocol with recommended 40 rounds of amplification was applied. Thermal cycler condition were 50°C for 2 min, 95°C for 10 min followed by 40x 95°C for 15 sec and 60°C for 1 min.

Quantitative values were obtained from the threshold cycle (Ct value) at which the increase TaqMan® probe fluorescent signal associated with an exponential increase of each

individual PCR product reaching a fixed threshold value. Each individual primer had a fixed threshold Ct value (Table 1). These fixed threshold values were used for every cDNA sample. Negative controls for each primer were included in each run.

To enable the comparison of different mRNA expression levels, their relation to the average expression level of two housekeeping genes (*GAPDH*, glyceraldehydes-3-phosphate dehydrogenase and *HPRT*, hypoxanthine-guanine phosphoribosyl-transferase) were evaluated. The housekeeping gene with the lowest standard deviation (*GAPDH*) was used for evaluation of the mRNA expression levels. Data was analysed using the Sequence Detection Software, this calculated the threshold cycle (Ct) value. The expression of the target assay was normalised by subtracting the Ct value of the housekeeping gene from the Ct value of the relevant target assay. The fold increase, relative to the control, was obtained by using the formula $2^{-\Delta Ct}$, and then expressed as a percentage ($\times 100$). All samples were measured in triplicates.

Statistical analysis:

Differences in expression levels were analysed using the Mann-Whitney U test or Kruskal-Wallis test, including a Wilcoxon-type test for trends, when appropriate.

Immunohistochemical staining was utilised in a cohort of 57 patients diagnosed with clear cell renal cancer. These patients had undergone complete resection of the tumour at time of nephrectomy and pre-operative CT scans showed no evidence of regional or metastatic spread. Patients were staged pathologically and graded according to the TNM classification and Fuhrman grading respectively. Cancer specific survival rate was the time from diagnosis until time of death or last follow up. The cause of death was determined by linkage through the Scottish Cancer Registry. In those who were deceased, if the primary cause of death was of renal cancer, these were classed as cancer specific and all other causes were non-cancer specific deaths.

Immunohistochemical staining was utilised to assess expression of c-Src kinase, phosphorylated Src at Y⁵³⁰, phosphorylated Src at Y⁴¹⁹ and FAK at the Y⁸⁶¹. Both antibodies for dephosphorylated Src and phosphorylated Src are were not specific for only c-Src kinase but they also detect other family members including Fyn, Yes and Fgr due to high level of conservation between Src family members.

c-Src kinase and activated Src kinase expression (Src Y⁴¹⁹) was investigated using antibodies for c-Src kinase (36D10, Cell Signalling Technology, Beverly MA, USA) and Src Y⁴¹⁶ (Cell Signalling Technology). Dephosphorylated Src and FAK were investigated using antibodies for Src Y⁵²⁷ and FAK Y⁸⁶¹ respectively (Invitrogen, Paisley, UK). In humans the activated phosphorylation sites that were investigated in the current study are amino acids Y⁵³⁰ and Y⁴¹⁹. Antibodies used relate to the rabbit sequence and not the human sequence.

Tissue sections were dewaxed and rehydrated through graded alcohol. Antigen retrieval was performed by heating tissue sections under pressure for five minutes in a pressure cooker using citrate buffer pH 6 for c-Src kinase, Src Y⁵²⁷, FAK Y⁸⁶¹ and EDTA buffer pH 9 for Src Y⁴¹⁶. Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide (H₂O₂). To reduce non-specific binding the tissue sections were then incubated with 5% normal horse serum (Vector Laboratories, Burlingame, CA, USA) in antibody diluent (DAKO Cytomation, Glostrup, Denmark) for twenty minutes at room temperature. Incubation with primary antibody was performed with c-Src kinase (1:200) for 60 minutes at room temperature and overnight at 4°C for antibodies dephosphorylated Src Y⁵²⁷ (1:3000), phosphorylated Src Y⁴¹⁶ (1:25) and FAK Y⁸⁶¹ (1:200). Signal was amplified and visualised using the DAKO Envision Kit (DAKO Cytomation) and the chromagen 3,3'-diaminobenzidine (DAB, Vector Laboratories). Sections were counterstained, dehydrated and mounted. In each run a positive and negative isotype-matched control was included to ensure no false positive staining or intense stromal staining.

Immunohistoscoring: Protein expression for tumour cells was assessed over the full tissue specimen using the weighted histoscore method [520]. The weighted histoscore grades staining intensity as negative (0), weak (1), moderate (2), and strong (3), then multiplication of the percentage of tumour cells within each category. Two observers independently scored all tissue sections. Each cellular location was independently assessed for any evidence of expression (Figure 1). Tumours were then divided into those with high (above median) histoscore or low (below or equal to the median) histoscore expression as has previously been reported [336, 360, 521] for the purposes of analysis. Agreement between observers was excellent (>0.9) for all proteins investigated as measured by interclass correlation coefficient (ICCC).

Statistical analysis was undertaken using SPSS (Chicago, IL, USA). Cancer specific survival rates were generated using the Kaplan-Meier method. The log rank test was utilised to compare significant differences between subset groups using univariate analysis. Multivariate analysis was carried out based on the results of the univariate analysis. Multivariate Cox regression analysis was performed to identify those factors that were independently associated with cancer specific death. A stepwise backward procedure was utilised to ascertain which of the variables had a significant independent relationship with survival. Chi squared analysis was utilised to assess relationships between pathological parameters and the biomarkers at the various locations. Pearson correlation was utilised to assess if relationships could be identified between the various proteins at the various cellular locations. p-values < 0.004 were deemed significant according to Bonferroni's correction.

Results

The cohort for RT-PCR analysis consisted of nineteen renal cell cancers. Median age of diagnosis was 60 years (range 42-72). Table 1 demonstrates clinicopathological parameters of these patients.

The most highly expressed SFK member in renal cell cancer tissue was Src followed by Lyn, Hck, Fgr and Fyn (Table 2, Figure 2). The least expressed SFK member was Blk. When analysing SFK members in correlation to T Stage, expression of Yes and Blk was significant ($p=0.039$, $p=0.045$, Figure 3). Interestingly, higher expression of all SFK members was observed in T2 stage disease (Figure 3). Expression of Lck, Lyn, Fyn, Blk and Yes showed a significant decrease between T2 and T3 disease ($p=0.032$, $p=0.032$, $p=0.032$, $p=0.032$, $p=0.032$).

When analysing SFK member expression in correlation with tumour grade, no significant difference was observed (Figure 4).

Immunohistochemical analysis was based on 57 clear cell renal cancer patients with full clinical follow up. Table 1 demonstrates clinicopathological parameters of these patients. Median age at diagnosis was 60 years (range 41-80). Median follow up was 107 months (range 0.1-163). Twenty two patients died of their disease. Thirty eight patients had T1/2 disease and nineteen patients had T3/4 disease. Tumour volume was recorded for each case; median tumour volume was 9.8cm^3 (range $1\text{-}30\text{ cm}^3$). Twenty five patients subsequently had evidence of recurrence on radiological imaging.

Initial analysis was performed on clinicopathological features which are known prognostic indicators for survival in renal cancer. T stage and nuclear grading were significantly associated with poor prognosis, thus demonstrating that this cohort was associated with classical clinical parameters and validating it for use in a biomarker study (Table 3).

c-Src kinase

As c-Src was the highest expressed of all the family members at the mRNA level, expression at the protein level was further investigated. Of the tumours investigated, 98% showed some degree of nuclear expression, 100% showed some degree of cytoplasmic expression and 97% showed some degree of membrane expression. For the purposes of analysis, the median cut off values of expression at the different cellular locations was utilised, those below and equal to the median score were given a low expression and those above the median were given a score of high as mentioned in the methodology. For nuclear expression this value was 25, cytoplasmic expression was 115 and membrane 100. χ^2 analysis demonstrated that cytoplasmic c-Src kinase expression positively correlated with nuclear grade ($p=0.023$, Table 4) but no correlation was demonstrated with age, T stage, tumour necrosis, recurrence or tumour volume. Membrane c-Src kinase protein expression negatively correlated with recurrence ($p=0.021$, Table 4) but no relationship was demonstrated with age, T stage, nuclear grade, tumour necrosis or tumour volume. These results suggest that membrane c-Src confers good prognosis, however on univariate analysis expression of c-Src at the different cellular locations did not show significance (Table 3). However a trend was observed between high membrane c-Src kinase expression and improved disease specific survival, those patients expressing high membrane c-Src kinase had a median survival of 130 months compared to 97 months for those with low expression, this did not however reach significance ($p=0.097$, Figure 5a). On univariate analysis, expression of Src kinase at any of the cellular locations investigated was not significantly associated with disease specific survival (Table 3). Pearson correlation demonstrated that cytoplasmic c-Src kinase expression demonstrated a positive correlation with nuclear c-Src kinase expression ($p=0.0022$, Table 5).

Y⁵²⁷ Src Kinase

Phosphorylated Src Y⁵²⁷ was investigated. Of the tumours investigated 47% showed some degree of nuclear expression, 96% showed some degree of cytoplasmic expression and 93% showed some degree of membrane expression. There was a difference in the expression of Y⁵²⁷ at the different cellular locations in comparison to c-Src expression with this difference possible as a result of tissue heterogeneity and therefore tissue expression variability. The median cut off values for the purposes of analysis were 0 for nuclear expression, 50 for cytoplasmic and for membrane 85. χ^2 analysis demonstrated that phosphorylated membrane Y⁵²⁷ expression positively correlated with age ($p=0.026$, Table 4) but no correlation was demonstrated with T stage, nuclear grade, tumour necrosis, recurrence or tumour volume. On univariate analysis, expression of Y⁵²⁷ at any of the cellular locations investigated was not significantly associated with disease specific survival (Table 3). Pearson correlation demonstrated that phosphorylated cytoplasmic Y⁵²⁷ expression demonstrated a positive correlation with cytoplasmic c-Src kinase expression ($p=0.0022$, Table 5).

Y⁴¹⁶ Src Kinase

The autophosphorylated status at Y⁴¹⁶ was investigated. Of the tumours investigated 84% showed some degree of nuclear expression, 100% showed some degree of cytoplasmic expression and 81% showed some degree of membrane expression. The median cut off values for the purposes of analysis were 20 for nuclear expression, 100 for cytoplasmic and for membrane 20. χ^2 analysis demonstrated that membrane Y⁴¹⁶ expression positively correlated with age ($p=0.026$, Table 4) but no correlation was demonstrated with T stage, nuclear grade, tumour necrosis, recurrence or tumour volume. On univariate analysis, expression of Y⁴¹⁶ at any of the cellular locations investigated was not associated with disease specific survival (Table 3). Pearson correlation demonstrated that membrane Y⁴¹⁶ expression demonstrated a negative correlation with cytoplasmic c-Src kinase expression

($p=0.0005$, Table 5) and a positive correlation was demonstrated with dephosphorylated membrane Y^{527} expression ($p=0.0001$, Table 5).

Y^{861} FAK

Of the tumours investigated 98% showed some degree of nuclear expression, 39% showed some degree of cytoplasmic expression and 100% showed some degree of membrane expression. The median cut off values for the purposes of analysis were 50 for nuclear expression, 0 for cytoplasmic and for membrane 50. χ^2 analysis demonstrated that expression of cytoplasmic FAK Y^{861} demonstrated a positive relationship with T stage, nuclear grade, recurrence and tumour volume ($p=0.023$, $p=0.001$, $p=0.036$ and $p=0.045$ respectively, Table 4) but no relationship was demonstrated with age or tumour necrosis. On univariate analysis, high expression of cytoplasmic FAK Y^{861} was associated with decreased cancer specific survival, ($p=0.001$, Table 2, Figure 5b). This association was also independent on multivariate analysis (Hazard Ratio 3.34, 95% CI 1.40-7.98, $p=0.006$, Table 3). Pearson correlation demonstrated that membrane FAK Y^{861} expression demonstrated a positive correlation with membrane Y^{527} and membrane Y^{416} expression ($p=0.0002$ and $p=0.0002$, Table 5).

Discussion

We have reported for the first time to our knowledge that SFK members are not only expressed in renal cell carcinoma but are expressed to varying degrees. Of the SFK members, Src is the most highly expressed followed by Lyn, Hck, Fgr and Fyn. Src has a five-fold higher expression than the least SFK member expressed. We have also reported that there is a significant fall in expression of Lck, Lyn, Fyn, Blk and Yes between T2 and T3 disease.

Despite having a limited cohort group, we have shown that there is a significant difference in expression of several SFK members between T2 and T3 disease. In renal cancer, T2 disease is still organ confined with T3 disease invading perinephric fascia and/or the adrenal gland. Src kinase has a role in signal transduction of multiple oncogenic cellular processes including migration and invasion. We hypothesise that SFK members are being up-regulated at a time when the cancer is going beyond being organ confined and starting to metastasise after which up-regulation is no longer required as the cancer has metastasised.

Expressions of SFK members have been demonstrated in a variety of tumours [348, 364, 516-518]. Specifically, expression of Src and Lyn have been demonstrated to be elevated in breast and colon cancer [329, 332, 516]. When assessing activity of SFK members, it has been demonstrated that increased expression of Src is associated with poor survival in breast cancer [336, 516] whereas expression of Src is associated with improved clinical outcome in bladder cancer [358, 360, 361].

Given that the cohort size was limited, significant correlations could not be observed between expression of a particular member and survival. Even if a SFK member is expressed, this does not necessarily constitute activity.

Therefore to gain a better understanding of Src and SFK activation we established a cohort of patients' samples to allow us to assess expression and establish correlations with clinical parameters.

To our knowledge this is the only study investigating the role of Src kinase expression, phosphorylated status (Y⁵²⁷), auto-phosphorylation status (Y⁴¹⁹) and the downstream marker FAK Y⁸⁶¹ in clear cell renal cancer. SFK can be localised at different cellular locations with the inactive form localised at the perinuclear region of cells [399]. Much work has been conducted in breast cancer which has reported high cytoplasmic Src kinase levels being associated with shorter disease specific survival [339] and high expression of activated Src kinase being associated with increased risk of recurrence [336]. In addition, prostate cancer studies have demonstrated that Src kinase expression and activation is associated in quicker time to relapse and shorter disease specific survival [348]. Therefore current literature supports a role for Src as a negative prognostic marker in solid tumours. In contrast in the current study, χ^2 analysis, demonstrates that membrane Src kinase expression was negatively associated with recurrence and a trend towards improved survival was also observed. These results require confirming in a larger patient cohort; however the combination of reduced recurrence and improved survival being associated with Src kinase expression suggests that Src kinase might not be responsible for driving progression in renal cancer. Although Src expression itself might not be involved with renal cancer progression, activation of the Src or other Src family members might be involved.

It has been suggested that a biomarker for prediction of Src kinase activity would be to measure phosphorylation of the protein at a site associated with activity [363, 522].

Currently there are two sites within Src known to be associated with activation.

Phosphorylation of the tyrosine residue 530 on the c-terminal tail by Csk tyrosine kinase acts as a negative regulatory protein-binding site, keeping Src kinase in a closed conformation [523]. Upon dephosphorylation this allows Src kinase to undergo a transformational change allowing the second site of activation, Y⁴¹⁹, to be accessible which needs to be autophosphorylated before full activation of Src kinase. Y⁴¹⁹ is referred to as the classical site and is the most commonly used in cell line studies investigating the

functional relevance of Src kinase activation [475]. In this study, membrane Y⁴¹⁶ expression demonstrated a negative correlation with cytoplasmic Src kinase and a positive correlation with cytoplasmic Y⁵²⁷, confirming validity of results regarding Src kinase needing to undergo dephosphorylation at the Y⁵³⁰ before autophosphorylation at the Y⁴¹⁹ can proceed. Activated phosphorylated Src kinase at the classical site Y⁴¹⁶ when assessed at individual locations was shown to have no correlation to disease specific survival. This antibody is not specific for one particularly Src family member as this part of the sequence is highly conserved between Src family members making it impossible to produce an antibody specific for only one member. Therefore as the antibody cross reacts with all family members that are phosphorylated at this site, this could account for the lack of correlation with disease specific survival.

Autophosphorylation of FAK at the Y³⁹⁷ site occurs as a result of many stimuli thereby creating a high affinity binding site for the Src homology 2 domain of several proteins including Src kinase [453, 454]. Src phosphorylates FAK at several sites but it has been reported that the Y⁸⁶¹ is the major site on the FAK domain associated with activation [455], and therefore maybe used as a surrogate marker not only for SFK activation but also for SFK function [459].

Expression of cytoplasmic Y⁸⁶¹ was associated with reduced disease specific survival and positively correlated with T stage, nuclear grade, recurrence and tumour volume suggesting that the phosphorylation of Y⁸⁶¹ may be associated with more aggressive renal cancers (Table 3). Expression of cytoplasmic Y⁸⁶¹ positively correlated with membrane Y⁴¹⁶ further suggesting that of the Src kinase family members, c-Src kinase may not be responsible for the phosphorylation at the Y⁸⁶¹ site in clear cell renal cancer, therefore implicating a role for an alternative SFK member in renal cancer progression.

The current study has demonstrated that c-Src is the most highly expressed SFK member in renal cell cancer. Activation of c-Src kinase itself is associated with improved survival but the presence of the downstream marker FAK, itself a surrogate marker for SFK member

activation, is associated with decreased cancer specific survival suggesting that another of the SFK members is responsible for poor survival in renal cell cancer.

Acknowledgments

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Table 1: Clinicopathological characteristics of patients utilised for RT-PCR and IHC.

	<i>Numbers</i>	
	RT-PCR Cohort	IHC Cohort
Age (<60/>60)	10/9	29/28
Sex (M/F)	9/10	32/25
T Stage (T1/T2/T3/T4)	5/3/9/2	26/12/17/2
Grade (1/2/3/4)	4/5/6/4	10/26/15/6
Tumour Necrosis (absence/presence)	8/11	32/25
Recurrence (No/Yes)	12/7	32/25
Tumour Volume (<10cm ³ / ^{>} 10cm ³)	7/12	29/28

Table2: Intron-skipping primers used for RTPCR, their fixed threshold Ct values and median expression levels in renal cell cancer tissue.

<i>Gene</i>	<i>Gene Expression Assay ID</i>	<i>Threshold (Ct)Value</i>	<i>Expression Levels</i>
SRC	Hs00178494_m1	0.23	404.9
LCK	Hs00178427_m1	0.17	13.5
LYN	Hs00176719_m1	0.25	233.3
FYN	Hs00176628_m1	0.2	53.2
FGR	Hs00178340_m1	0.2	58.1
HCK	Hs00176654_m1	0.26	75
BLK	Hs00176441_m1	0.19	2.5
YES	Hs00736972_m1	0.26	9.7
GAPDH	N/A	0.2	N/A

Table 3: Interrelationships between clinicopathological characteristics of patients and protein expression/activation with renal cancer (figures in bold denote significant p values).

	Numbers	<i>Univariate</i>	<i>Multivariate</i>	HR
		<i>Analysis</i>	<i>Analysis</i>	
		p value	p value	
Age (<60/>60)	29/28	0.201		
T Stage (T1/T2/T3/T4)	26/12/17/2	0.001	0.001	2.35 (1.41-3.91)
Grade (1/2/3/4)	10/26/15/6	0.012		
Tumour Necrosis (absence/presence)	32/25	0.092		
c-Src nuc (negative/positive)	31/26	0.437		
c-Src cyto (negative/positive)	32/25	0.95		
c-Src mem (negative/positive)	29/28	0.097		
Y527 Src nuc (negative/positive)	34/23	0.72		
Y527 Src cyto (negative/positive)	31/26	0.968		
Y527 Src mem (negative/positive)	29/28	0.329		
Y416 Src nuc (negative/positive)	29/28	0.799		
Y416 Src cyto (negative/positive)	32/25	0.311		
Y416 Src mem (negative/positive)	29/28	0.79		
Y861 Fak nuc (negative/positive)	29/28	0.489		
Y861 Fak cyto (negative/positive)	36/21	0.001	0.006	3.35 (1.40-7.98)
Y861 Fak mem (negative/positive)	30/27	0.678		

Table 4: Interrelationships between clinicopathological characteristics of patients and protein expression/activation with renal cancer using χ^2 (chi squared) analysis (figures in bold denote significant p values).

<i>Variable</i>	<i>Numbers</i>	<i>Age</i>	<i>T Stage</i>	<i>Grade</i>	<i>Tumour Necrosis(absence/presence)</i>	<i>Recurrence (No/Yes)</i>	<i>Tumour Volume (<10cm³/^{>}10cm³)</i>
c-Src nuc (negative/positive)	31/26	0.350	0.187	0.500	0.830	0.196	0.350
c-Src cyto (negative/positive)	32/25	0.703	0.538	0.023	0.985	0.985	0.703
c-Src mem (negative/positive)	29/28	0.148	0.205	0.916	0.363	0.021*	0.692
Y527 Src nuc (negative/positive)	34/23	0.487	0.157	0.793	0.623	0.962	0.487
Y527 Src cyto (negative/positive)	31/26	0.144	0.447	0.708	0.751	0.749	0.684
Y527 Src mem (negative/positive)	29/28	0.026	0.335	0.916	0.882	0.358	0.513
Y416 Src nuc (negative/positive)	29/28	0.692	0.889	0.916	0.227	0.701	0.513
Y416 Src cyto (negative/positive)	32/25	0.227	0.056	0.443	0.985	0.289	0.083
Y416 Src mem (negative/positive)	29/28	0.026	0.667	0.686	0.703	0.222	0.897
Y861 Fak nuc (negative/positive)	29/28	0.897	0.478	0.482	0.703	0.701	0.692
Y861 Fak cyto (negative/positive)	36/21	0.207	0.023	0.001	0.326	0.036	0.045
Y861 Fak mem (negative/positive)	30/27	0.150	0.702	0.753	0.540	0.933	0.361

*-Inverse relationship

Table 5: Interrelationships between protein markers at the various cellular locations using Pearson Correlation. PC-Pearson Correlations, Sig-Significance (2-tailed), ns-non significant p-values (figures in bold denote significant p values according to Bonferronis correction).

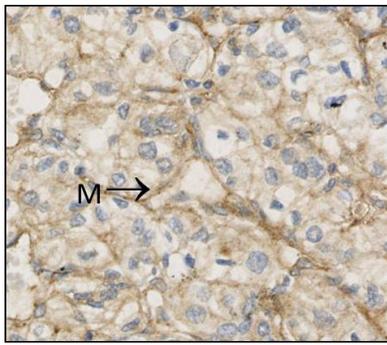
		Correlations										
	c-Src nuc	c-Src cyto	c-Src mem	Y527 Src nuc	Y527 Src cyto	Y527 Src mem	Y416 Src nuc	Y416 Src cyto	Y416 Src mem	Y861 FAK nuc	Y861 FAK cyto	Y861 FAK mem
c-Src nuc	PC	0.3973	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Sig	0.0022	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
c-Src cyto	PC		ns	ns	0.3973	ns	ns	ns	*0.4442	ns	ns	ns
	Sig		ns	ns	0.0022	ns	ns	ns	0.0005	ns	ns	ns
c-Src mem	PC			ns	ns	ns	ns	ns	ns	ns	ns	ns
	Sig			ns	ns	ns	ns	ns	ns	ns	ns	ns
Y527 Src nuc	PC				ns	ns	ns	ns	ns	ns	ns	ns
	Sig				ns	ns	ns	ns	ns	ns	ns	ns
Y527 Src cyto	PC					ns	ns	ns	ns	ns	ns	ns
	Sig					ns	ns	ns	ns	ns	ns	ns
Y527 Src mem	PC						ns	ns	0.5086	ns	ns	0.4735
	Sig						ns	ns	0.0001	ns	ns	0.0002
Y416 Src nuc	PC							ns	ns	ns	ns	ns
	Sig							ns	ns	ns	ns	ns
Y416 Src cyto	PC								ns	ns	ns	ns
	Sig								ns	ns	ns	ns
Y416 Src mem	PC									ns	ns	0.4735
	Sig									ns	ns	0.0002
Y861 Fak nuc	PC										ns	ns
	Sig										ns	ns

Y861 Fak cyto	PC	ns
	Sig	ns
Y861 Fak mem	PC	
	Sig	

*-Inverse relationship

Figure legends

Figure 1: Demonstrates representative images of immunohistochemistry for renal cell carcinoma of c-Src kinase, Src Y⁵²⁷, Src Y⁴¹⁶ and FAK Y⁸⁶¹. Membrane staining denoted by M, cytoplasmic staining by C and nuclear staining N.



•c-Src

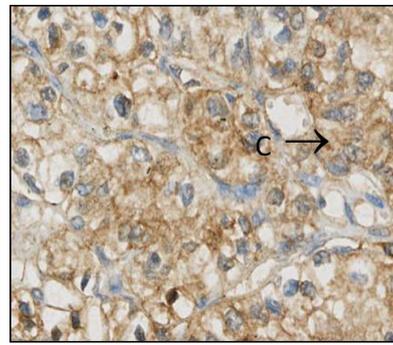
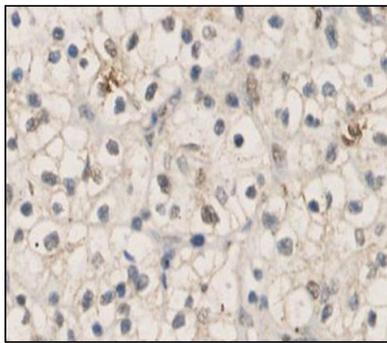
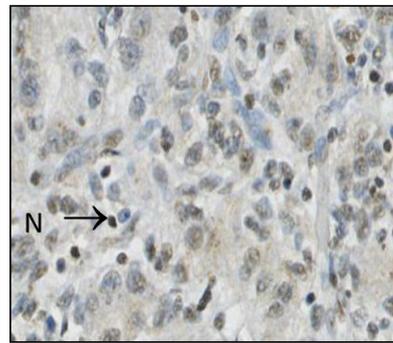
•Y⁵²⁷•Y⁴¹⁶•Y⁸⁶¹

Figure 3: Graphs showing expression of mRNA levels of each SFK member according to T stage.

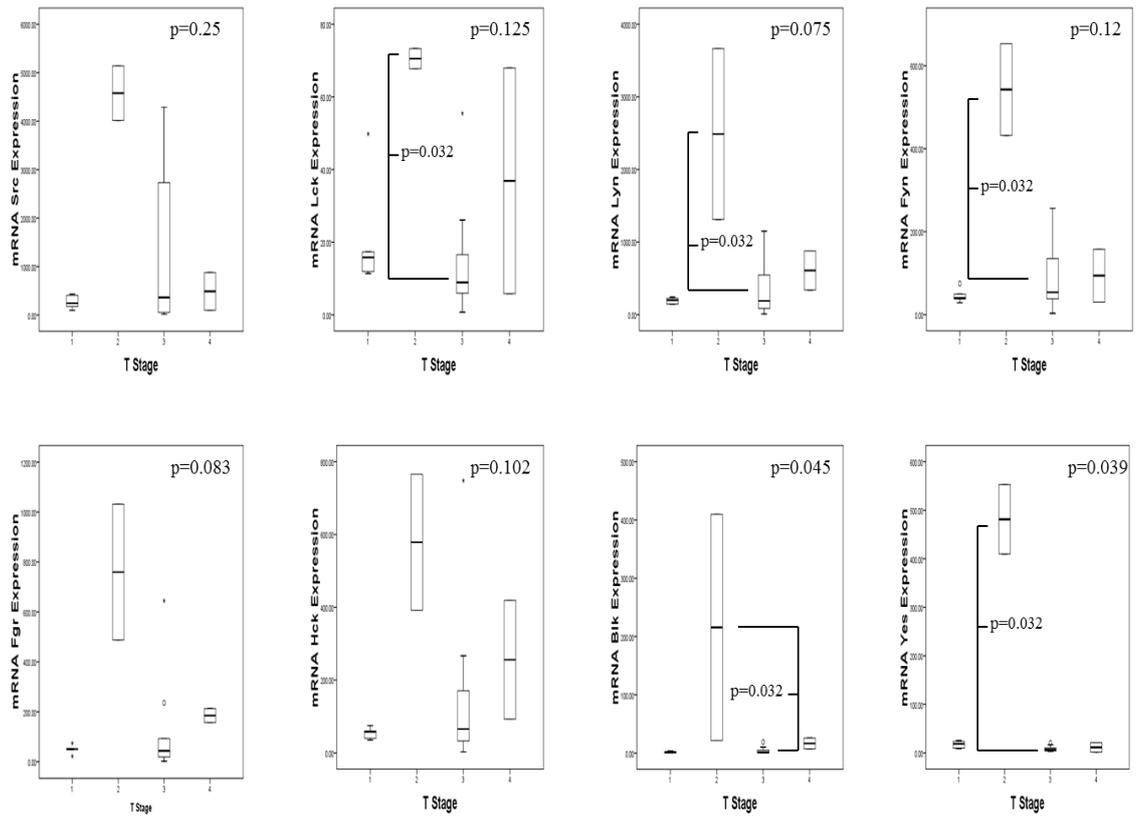


Figure 4: Graphs showing expression of mRNA levels of each SFK member according to tumour grade.

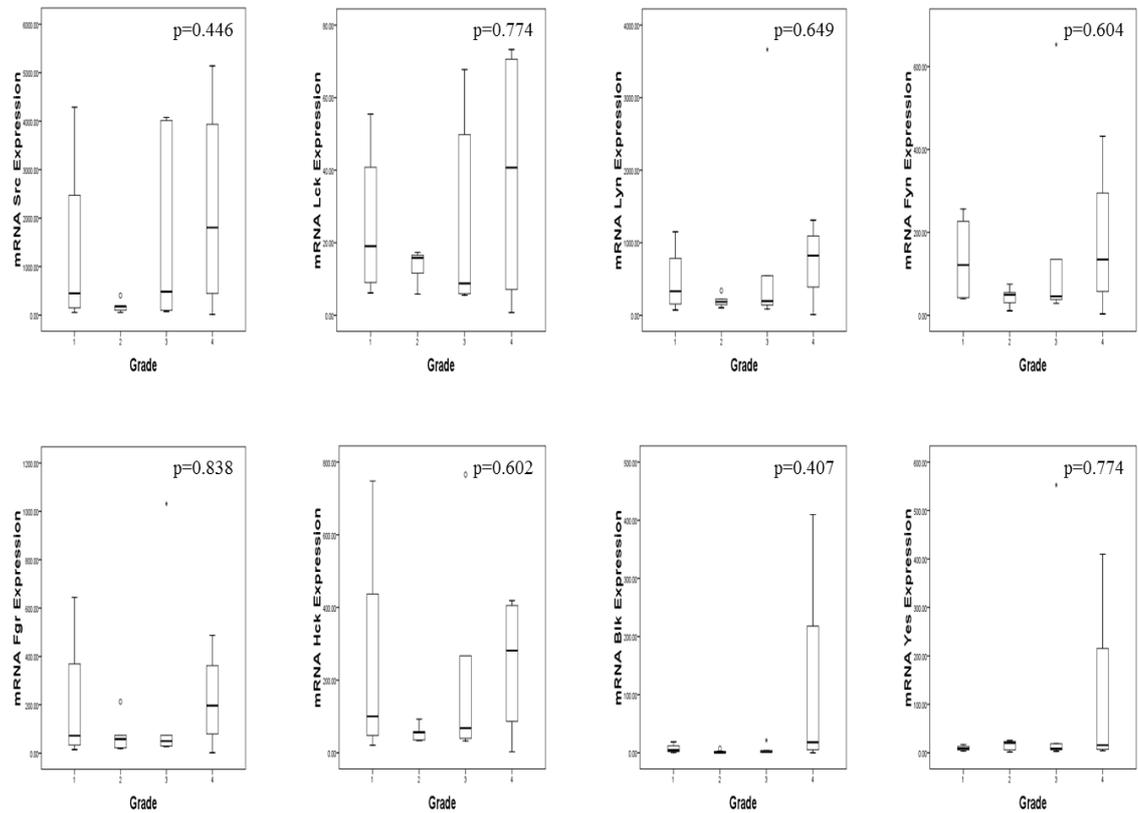


Figure 5

Figure 5a: Kaplan Meier plotted for high and low membrane c-Src kinase expression against disease specific survival, log rank test $p=0.097$.

Figure 5b: Kaplan Meier plotted for high and low cytoplasmic FAK Y⁸⁶¹ expression against disease specific survival, log rank test $p=0.001$.

