MECHANISMS AND IMPLICATIONS OF p53 AND MDM2 EXPRESSION IN RENAL CELL CARCINOMA

Thesis submitted in accordance with the requirements

of the University of Liverpool for the degree of

Doctor of Medicine

by

Aidan Paul Noon

February 2011

ABSTRACT

Despite recent breakthroughs in targeted molecular therapy, renal cancer is still the tenth leading cause of cancer death in British men and the twelfth in women. Cancer is a disease arising from mutations in key growth regulatory genes including loss of function of genes that act as tumour suppressors. The p53 gene is known to be mutated in over 50% of cancers which is not surprising given that its normal function is to be a critical tumour suppressor gene. Unregulated p53 is lethal in mammals and MDM2 (itself a transcriptional target of p53) is an essential negative regulator of p53 function. MDM2 has also been demonstrated to have oncogenic properties independent of p53 and together the p53/MDM2 pathway is one of the most studied in cancer. There have been a number of publications that have suggested that RCCs expressing p53 may have a poor prognosis and in addition one paper has also shown that tumours that express both p53 and MDM2 are associated with poor patient outcome. Initially we wanted to investigate p53 and MDM2 expression (using a recently created tissue microarray), in a different cohort of patients undergoing radical nephrectomy, to determine whether the p53/MDM2 high phenotype was associated with outcome. Secondly we wanted to discover by what means p53 and MDM2 may be up-regulated in RCC. Two possible mechanisms for p53 and MDM2 up-regulation were explored by a series of in vitro studies on a panel of RCC cell lines. One hypothesis tested (using a proteasome inhibitor) was whether failure of normal proteasomal degradation of p53 and MDM2 was responsible for this phenotype. The second mechanism involved inhibiting the HSP90 chaperone complex (which has been shown to stabilise mutant p53) to determine if this led to a decrease in p53 and MDM2 steady state levels. The third part of this study was to develop RCC cell lines that could be made to express high levels of MDM2. With this tool it was hoped to try and understand by what means increased MDM2 expression may promote poorer prognosis. We have found that a subset of RCCs do express both p53 and MDM2 with co-expression of both proteins being significantly linked (P=0.000013). Moreover, increased co-expression of p53 and MDM2 identifies patients with significantly reduced disease specific survival by univariate (P=0.036) and Cox

multiple regression analysis (P=0.027, RR=3.20), despite apparent organ confined disease at the time of their nephrectomy. Testing of the RCC cell line panel revealed no fundamental defect in proteasomal degradation of p53 and MDM2 and therefore proteasomal dysfunction does not appear to be causing a disruption of the p53/MDM2 autoregulatory feedback loop. Inhibition of HSP90 did result in a decrease of both p53 and MDM2 in a cell line specific manner (transient decrease in some cell lines harbouring wild type p53 and more prolonged in one mutant p53 cell line). HSP90 may therefore play some role in the up-regulation of p53 and MDM2 though further studies are required to clarify this. While it was possible to generate renal cell line clones that could inducibly express high levels of a mutated form of MDM2 no clone could be made that would express fully functional MDM2. The reason for this appears to be due to previously documented toxicity resulting from even small increases in MDM2 expression, again highlighting that the ability of renal cancer cells to express high levels of MDM2 is not trivial and worthy of further in vitro investigation especially given the link between high MDM2/p53 expression and poor prognosis.

KEYWORDS: Renal, Carcinoma, p53, MDM2, HSP90, Proteasome, Prognosis

ACKNOWLEDGEMENTS

I would like to thank my supervisors Dr M Boyd and Dr N Vlatkovic for their help and support during my time in the laboratory and with the writing of this thesis. I am also very grateful to Mr K Parsons for his clinical supervision and Mersey Kidney Research who were responsible for providing financial support for the project.

Thanks also to the team in the Division of Surgery for their kindness and my friends in the Boyd Laboratory for their patience, sense of humour and help with keeping my clones alive! Dr M Maguire has been a constant friend and offered invaluable support with this project. I am grateful also to the team in the histopathology department for their assistance with the tissue microarray, in particular Dr H Shawki and Mr A Dodson, and further thanks to Dr H Kalirai and the Liverpool Tissue Bank for all their efforts.

To my wife Vicky for putting up with my mood swings and still encouraging me and to my grandmother who, although She had no understanding of what my "Liverpool degree" involved, persistently asked me if I had finished it – thank you both!

DECLARATION OF ORIGINALITY

This thesis is a result of my own work performed during the course of studies in the Division of Surgery and Oncology, University of Liverpool, between February 2006 and February 2008. The experiments were performed by me with the following exceptions: the MDM2 column affinity presented in Figure 5.2.7, the column experiment was performed by Dr M. Maguire, and the lysates from this experiment were probed for by myself. Experiments presented in figures 4.3.8.1.1 through to 4.3.9.5 were carried out with assistance from Dr M. Maguire as acknowledged in the figure legends. The tissue microarray described in chapter 3 was constructed by Dr Helen Kalirai and the Liverpool Tissue Bank.

The thesis was written wholly by me under guidance of my supervisors

Dr. M. Boyd and Dr. N. Vlatković

A. Noon

February 2011

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ATM	Ataxia telangiectasia mutated
AMP	Adenosine monophosphate
APS	Ammonium persulphate
ATP	Adenosine triphosphate
ccRCC	Clear cell renal cell carcinoma
BALB	Bagg Albino
BHD	Birt-Hogg-Dubé
BSA	Bovine serum albumin
cAMP	Adenosine 3'5' Cyclic Monophosphate
CBP	CREB binding protein
CDK	Cyclin dependent kinase
CHIP	Carboxy terminus of HSP70-interacting protein
CREB	cAMP response element-binding
DMSO	Dimethyl sulfoxide
DNA	Deoxy-ribonucleic acid
DOX	Doxycycline
DSS	Disease specific survival
GA	Geldanamycin
GOI	Gene of interest
ECL	Enhanced chemo-luminescence
ECOG	Eastern Cooperative Oncology Group
E.coli	Escherichia coli
EDTA	Ethylene diamine tetracetic acid
EGFP	Red shifted variant of wild type green fluorescent protein
FASAY	Functional assessment of separated alleles in yeast
FBS	Fetal bovine serum
FFPE	Formalin fixed paraffin embedded
gal	Galactosidase
GFP	Green fluorescent protein
HEPES	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
HDM2	Human double minute 2
HGF	Hepatocyte growth factor
HER	Human epidermal growth factor
HIF	Hypoxia inducible factor
Hip	HSP70 interacting protein
Hop	HSP70/90 organising protein
HRP	Horseradish peroxidase
HSP	Heat shock protein
HSV	Herpes simplex virus
IHC	Immunohistochemistry
IRES	Internal ribosome entry site
LB	Luria-Bertani
LTB	Liverpool Tissue Bank
MET	Mesenchymal epithelial transition factor

MDM2	Murine double minute 2
MDM2RFM	MDM2 RING finger mutant
MOPS	3-(n-Morpholino)Propanesulfonic Acid
MTBP	MDM2 binding protein
NICE	National Institute For Clinical Excellence
NES	Nuclear export signal
NLS	Nuclear localising signal
NOS	Nucleolar localisation signal
OS	Overall survival
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PMSF	Phenylmethylsulphonyl fluoride
PRCC	Papillary renal cell carcinoma
PRO	Proline
RCC	Renal cell carcinoma
RING	Really interesting new gene
r TetR	Reverse Tet repressor protein
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
SLIP	Stuart Linn immuno-precipitation
SNP	Single nucleotide polymorphism
SSCP	Single Strand conformation polymorphism
TAE	Tris – Acetate – EDTA
TEMED	N,N, N',N' – tetramethylethylenediamine
tetO	*tet* operator sequences
TetR	Tet repressor protein
TGF	Transforming growth factor
TMA	Tissue microarray
TPR	Tetratricopeptide repeats
TRE	Tet response elements
VEGF	Vascular endothelial growth factor
VHL	Von Hippel-Lindau
WHO	World Health Organisation

CHAPTER 1 - INTRODUCTION

1.1 Renal cell carcinoma

Renal cell carcinoma (RCC) is the term used to describe malignant tumours arising from the renal parenchyma. As will be described in section (1.1.3) there are many varieties of this disease that can be classified histologically and cytogenetically.

Around 190,000 new cases of kidney cancer (includes RCC and transitional cell carcinoma of the renal pelvis) are diagnosed in the world each year, accounting for just fewer than 2% of all cancers[1]. Cancer of the kidney was the 7th commonest malignancy affecting the UK adult population in 2007 and it has been estimated that the lifetime risk of developing kidney cancer in the UK is 1 in 89 for men and 1 in 162 for women[1]. Data from the UK National Office of Statistics showed 3,848 people died from kidney cancer in 2008[2]. This makes kidney cancer the 12th leading cause of UK cancer death in men and women[1]. In the European Union 63,000 people were diagnosed and 13,000 died of RCC in 2006[3]. Despite advances in diagnosis, new surgical techniques and adjuvant therapy, patients with metastatic RCC still have only a 10% survival rate at 5 years[4].

With improving understanding of cancer molecular biology, the future of prognostication and systemic therapy is dependent on understanding important molecular cancer pathways. The goal of this thesis is to ultimately investigate what role the p53/MDM2 pathway may play in RCC.

1.1.2 Incidence and prevalence of RCC

UK cancer statistics[1] show that 8,228 new cases of kidney cancer were diagnosed in 2007 with an incidence of 13.5 / 100,000 population. There is a higher incidence in men (17.3 / 100,000) than women (9.9 / 100,000) and the incidence increases with age, see Figure 1.1.2 from the same data source.

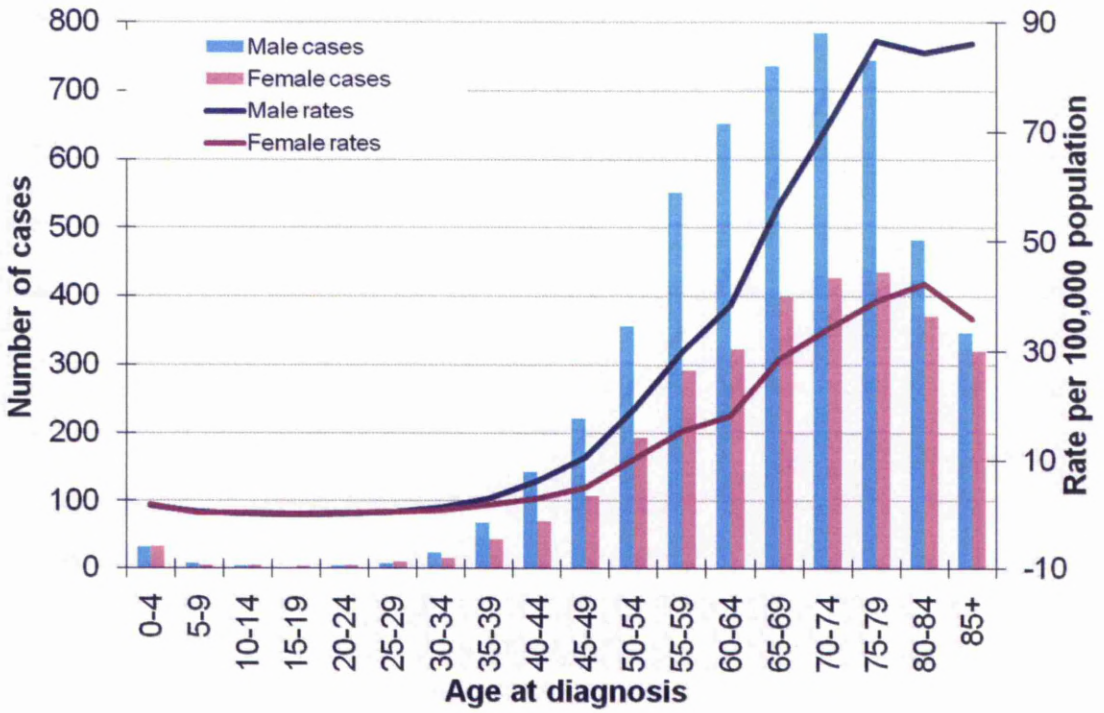


Figure 1.1.2 Numbers of new cases of renal cancer and age specific incident rates, by sex, in the UK 2007[1].

There is a 10-fold difference in the incidence of RCC between Eastern Europe (the highest rates) and some Asian and African countries (the lowest rates)[5]. The American

Cancer Society estimates that 58,240 people (35,370 men and 22,870 women) will have been diagnosed with Kidney cancer in 2010[6]. Some of the observed geographical differences may be in part due to differences in the frequency of the use of diagnostic imaging techniques and autopsy rates[5].

The incidence of RCC in Great Britain has risen since the mid 1970s for both men and women[7]. Rates have increased in men by 79% from 7.1 per 100,000 in 1975 to 12.7 per 100,000 in 2002. In women rates have increased over the same period from 3.2 to 6.1 per 100,000, a rise of 90%. Most of the increase in males has occurred in men aged over 65. The widespread availability of new imaging methods such as ultrasound and computed tomography has to led to an increase in detection of incidental (pre symptomatic) kidney cancer[5]. However, there have also been increases in more advanced tumours, suggesting that increase in detection of pre-symptomatic tumours by imaging does not fully explain the increases seen for RCC overall[8].

1.1.3 Pathology and histology of RCC

There are various histological and genetically distinct types of RCC. The most recent (2004) edition of the World Healthcare Organisation lists 10 types of malignant RCC[9]. Table 1.1.3 outlines the more common types which account for 90% of all RCC and gives some information on clinical characteristics.

RCC Subtype	Incidence (%)	Clinical prognosis
Clear Cell (ccRCC)	75	Aggressiveness according to grade, stage and sarcomatoid change. Better response to systemic therapy than PRCC and Chromophobe RCC.
Papillary (PRCC)	10	Type 1 and 2 are recognised. Type 1 is less aggressive than ccRCC, type 2 more aggressive[10]. Aggressiveness according to grade, stage and sarcomatoid change.
Chromophobe	5	Least aggressive RCC type. Reported Mortality 10%

Table 1.1.3 Three most frequent RCC subtypes according to WHO classification 2004[11].

The remaining types are collecting duct, multilocular cystic, medullary, Xp11 translocation, after neuroblastoma and mucinous tubular and spindle cell (MTSC - see Noon et al for further information[12]). This latest classification no longer recognises sarcomatoid RCC as a separate type. All types of RCC can progress to sarcomatoid change[11] and RCC with sarcomatoid change is highly malignant[13]. The different expression of p53 in these various subtypes is reviewed later (see Section 1.4).

1.1.3.1 Clear cell RCC

Clear cell RCC is the most common form of RCC accounting for 75% of adult malignant renal tumours and arising from cells of the proximal tubule[4]. The name "clear cell" derives from its typical histological appearance of round cells with abundant

cytoplasm. Approximately 4% of these tumours are multi-centric and 0.5 – 3% are bilateral at presentation[11].

1.1.3.2 Papillary RCC

PRCC is the second commonest form of adult RCC. This tumour is divided into two separate histological types. Type 1 is characterised by papillae covered by small cells with scanty cytoplasm arranged in a single layer and Type 2 by tumour cells of higher nuclear grade, eosinophilic cytoplasm and pseudostratified nuclei[11]. Type 1 has the propensity to be multicentric and there is evidence from univariate and multivariate analyses to show that Type 1 PRCC has a better prognosis than Type 2[14].

1.1.3.3. Chromophobe RCC

These tumours represent 5% of RCC and are thought to arise from the intercalated cells of the collecting duct. Although thought to be less aggressive than ccRCC it is associated with a 10% mortality rate[11]. Chromophobe RCC should be differentiated from oncocytoma which is a benign tumour again arising from the intercalated cells of the collecting duct.

A recent large meta-analysis of 3,564 RCC tumours showed no prognostic difference between the various histological types of RCC when stratified for grade, stage and patient performance status[15]. Previous studies have shown poorer prognosis with ccRCC

compared to PRCC and chromophobe tumours[16]. There is evidence that chromophobe RCC and PRCC have extremely poor responses to interleukin-2 therapy[17]. As mentioned earlier sarcomatoid is no longer considered a separate subtype of RCC, nevertheless it is associated with a poor prognosis. For example, De - Peruna [18] reviewed 101 cases and found the 5 and 10 year survival rates for sarcomatoid tumours to be 22% and 13% respectively which compares with 79% and 76% for non sarcomatoid tumours

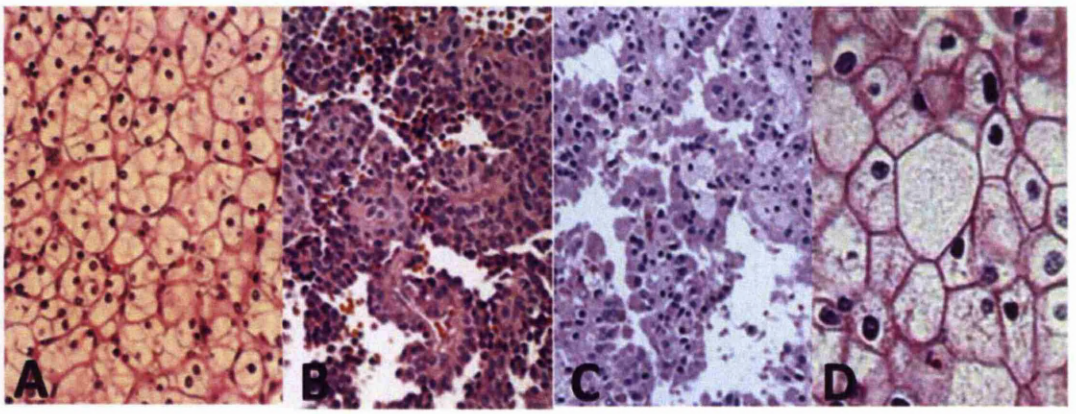


Figure 1.1.3 Microscopic appearances of RCC subtypes. A = ccRCC, B = PRCC Type 1, C = PRCC Type 2 and D = Chromophobe RCC. Adapted from Lopez-Beltran[11].

1.1.3.4 Staging in RCC

RCC is currently staged according to the 7th edition (2009) TNM/UICC classification[19], analyses performed in chapter 3 of this thesis were performed using the 6th edition (2002) TNM/UICC classification. The two major changes in the 7th edition are; stage T2 is now split into T2a size 7 – 10cm and T2b size >10cm and spread into the adrenal gland (considered T3a in 6th edition) is now classified as T4.

Primary tumour (T):

TX: Primary tumour cannot be assessed (information not available)
T0: No evidence of a primary tumour.
T1a: Tumour is 4 cm in diameter or smaller and is limited to the kidney.
T1b: Tumour is larger than 4 cm but smaller than 7 cm limited to the kidney.
T2: Tumour is larger than 7 cm but is still limited to the kidney.
T3a: Tumour has spread into the adrenal gland or into peri-renal fat.
T3b: Tumour has spread into the renal vein or sub-diaphragmatic vena cava
T3c: Tumour has reached the vena cava above the diaphragm
T4: Tumour has spread beyond Gerota's fascia

Regional lymph nodes (N):

NX: Regional lymph nodes cannot be assessed.
N0: No regional lymph node metastasis.
N1: Metastasis to one regional lymph node.
N2: Metastasis to more than one regional lymph node.

Distant metastasis (M):

MX: Presence of distant metastasis cannot be assessed .
M0: No distant metastasis.
M1: Distant metastasis present; includes metastasis to nonregional lymph nodes and/or to other organs.

Using the 2002 TNM system the five year cancer specific survival has been described as 97%, 87%, 71%, 53%, 44%, 37% and 20% in patients with pT1a, pT1b, pT2, pT3a, pT3b, pT3c and pT4 RCC[20]

1.1.3.5 Tumour grade

Most urologists in Europe and USA use the Fuhrman nuclear grading system[21]. The system evaluates nuclear diameter (in microns), nuclear outline: regular or irregular and nucleoli (visibility): present or not and at what power (low or high power). Fuhrman's grade (I-IV) is the sum of the points for all 3 parameters (see Table 1.1.3.51). Prognosis is worse as the grade increases. There is a strong correlation between Fuhrman grade and 5 year survival[22]. Tsui and colleagues [22] showed the 5 year cancer specific survival to be 89% for grade I, 65% for grade II and 46% for grades III and IV. This appears to be independent of the tumour stage as the 5 year survival for stage T1 tumours was 91%, 83%, 60% and 0% for Fuhrman grade I – IV. Tsui showed that Grade, TNM stage (Pathological stage - a combination of Tumour, Nodal and Metastases) and patient performance status were independent prognostic factors in multivariate analyses. Tumour stage alone (i.e only the T part of TNM staging) was not found to be an independent prognostic variable (see Table 1.1.3.5.2).

Grade	Nuclear size	Nuclear Shape	Nucleoli	Other features
1	10μm	Round and uniform	Inconspicuous or absent	
2	15μm	Irregular	Present	Examine at x400 magnification
3	20μm	Obviously irregular	Large	Examine at x100 magnification
4	20+μm	Bizzare or multilobulated	Large	Clumped chromatin spindle cells

Table 1.1.3.5.1 The Fuhrman grading system[21].

Variables	Category	Overall Survival Hazard Ratio (95% CI)	SE	*p* Value
Tumour stage	T1, T2, T3, T4	0.133 (0.310-0.428)	0.089906	0.138
ECOG status	0, 1-2	0.386 (0.034-0.737)	0.179206	0.031
Disease grade	1, 2, 3 + 4	0.321 (0.134-0.508)	0.095546	0.000
Pathological stage	I, II, III, IV	0.643 (0.454-0.832)	0.096642	0.000

Table 1.1.3.5.2. Multivariate analysis of prognostic variables for RCC. From Tsui et al[22].

1.1.4 Important aetiological factors in RCC

This next section reviews important aetiological factors that have been associated with renal cell carcinoma.

1.1.4.1 Lifestyle factors

There is no convincing evidence that social class variables, alcohol consumption or diet influence the incidence of RCC[23]. Cigarette smoking has been repeatedly associated with RCC, and estimated to account for 30% of RCC in men and 10% - 20% of RCC in women[24]. Obesity has been shown to be associated with RCC[25]. In this meta-analysis the authors reported a relative risk of RCC of 1.07 (95% CI 1.05–1.09) per unit of increase in BMI (corresponding to 3 kg body weight increase for a subject of average height). The mechanism by which obesity causes RCC has been investigated by Gago-Dominguez [26] who found lipid peroxidation of proximal renal tubules to be carcinogenic in animal models. Other mechanisms by which obesity may contribute to cancer has been reviewed by Calle et al [27]. The increasing rate of adult obesity may be in part responsible for the increased incidence of RCC. There is some epidemiological evidence that hypertension, independent of associated obesity or antihypertensive medication, leads to an increased risk of RCC[23].

1.1.4.2 Inheritable RCC syndromes

The incidence of a familial predisposition to RCC is reported to be c. 4% [9]. Just as there are a number of different histological types of RCC (see Section 1.1.3), a number of different hereditary conditions have also been reported. The identification of the genes involved in these conditions has helped us to gain an understanding of the key molecular events involved in RCC. To date no specific familial RCC syndrome has been associated

with the p53/MDM2 pathway. Patients with Li Fraumeni syndrome (an inheritable germline mutation of *p53*) do not have a higher incidence of RCC[28]. However patients with Li Fraumeni and a single nucleotide polymorphism of the intronic promoter of MDM2 (See section 1.3.5.1), develop malignancies on average 8 years earlier than Li Fraumeni patients lacking this polymorphism[29].

1.1.4.2.1 Von Hippel-Lindau disease and the VHL gene

Patients with VHL disease have an inherited (germline) mutated copy of the VHL gene[30]. This predisposes them to bilateral multifocal ccRCC, bilateral multifocal phaeochromocytoma, pancreatic tumours and cysts, cerebellar and spinal haemangiomas, retinal angiomas and endolymphatic sac tumours of the inner ear[30]. VHL mutation is important in sporadic ccRCC where VHL has been shown to be mutated in 57% of sporadic tumours[31].

The VHL gene is a tumour suppressor gene, located on the short arm of chromosome 3. VHL protein comprises an α domain which binds a protein called Elongin C and recruits an E3 ubiquitin ligase complex. The β domain of VHL can bind hydroxylated HIF -1α (hypoxia inducible transcription factor). HIF-1α is hydroxylated by enzymes utilising oxygen as a substrate. Therefore under normoxic conditions, VHL can ubiquitylate HIF-1α, leading to its degradation by proteasomes[32]. Under hypoxic conditions HIF-1α can accumulate and heterodimerises with HIF-1β, together they bind hypoxia-responsive elements in target genes such as *VEGF* (vascular endothelial growth factor), which is a

key regulator of angiogenesis, *PDGF* (platelet derived growth factor), *TGF-α* (transforming growth factor) and genes encoding glucose transporters and glycolytic enzymes [10, 32]. This response acts to enable the tissue to adapt to hypoxic conditions. In ccRCC mutation of VHL can result in highly vascular tumours as a result of VEGF expression[32].

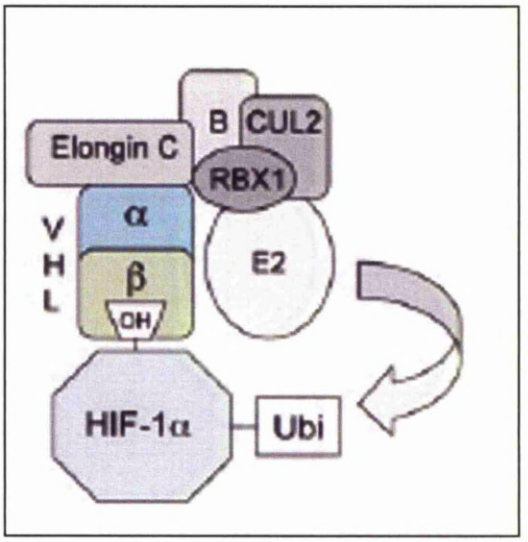


Figure 1.1.4.2.1 VHL binding HIF-1α (from Semenza 2006[32]). Ubi = Ubiquitin, CUL2 = Cullin 2, RBX2 = Ring box protein 2

1.1.4.2.2 Hereditary papillary renal carcinoma (HPRRC)

Germline mutations of the mesenchymal-epithelial transition factor (Met) proto-oncogene, located on the long arm of chromosome 7 are responsible for this condition. Patients develop Type 1 papillary renal cell carcinoma (PRCC) most commonly in the 4th, 5th and 6th decades of life. Tumours tend to be bilateral and multicentric. There is an autosomal dominant inheritance pattern with high penetrance[10 30].

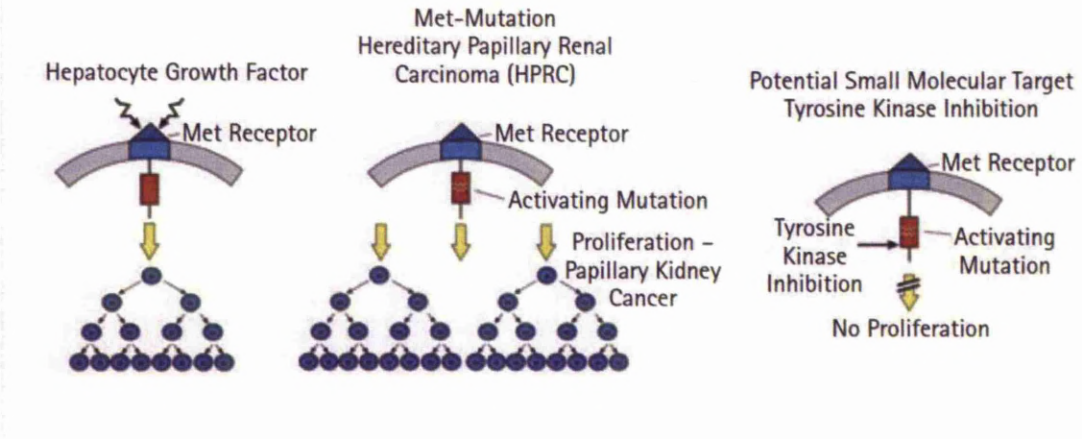


Figure 1.1.4.2.2 Hereditary papillary renal carcinoma is associated with germline mutation of the proto-oncogene Met from Vira et al[10]. Met encodes the cell surface receptor for the hepatocyte growth factor (HGF). Met = mesenchymal epithelial transition factor).

1.1.4.2.3 Birt-Hogg-Dubé syndrome

The Birt-Hogg-Dubé (BHD) gene is located at 17p11.2 and encodes a tumour suppressor protein, called folliculin, important in cellular energy sensing[33]. 15% – 30% of patients with this disease develop kidney tumours. There are a variety of kidney tumours seen in BHD patients. The various incidence of the tumours are as follows: the chromophobe type (see 1.3.3.3) in 34%, hybrid oncocytic neoplasms 50%, ccRCC 9% and oncocytomas 7% & 2% PRCC[34]. Other clinical manifestations include cutaneous lesions and pulmonary cysts with spontaneous pneumothorax[30].

1.1.5 Presentation and Diagnosis of RCC

It has been estimated that 48% – 66% of RCCs are now incidentally detected following radiological imaging [35]. Other symptoms include haematuria, loin pain, mass or symptoms of metastatic disease. 70% of RCC present as tumours 4cm or less (stage T1a)[36] and 1 to 3% of tumours are bilateral. 25 to 30% of patients present with metastases. Frequent sites of metastases include the lung parenchyma (50 to 60% patients with metastases), bone (in 30 to 40%), liver (in 30 to 40%), and brain (5%). Paraneoplastic syndromes are seen in less than 5% of patients. These include erythrocytosis, hypercalcaemia, hepatic dysfunction (Stauffer's syndrome), and amyloidosis. Their causes include tumour-produced hormone-like substances (erythropoietin and parathyroid hormone–related protein) and the formation of immune complexes (as in amyloidosis)[4].

1.1.6 Summary of Treatment options for RCC

The gold standard treatment for all histological types of organ confined RCC (Stages 1 - 3) is radical nephrectomy. Modern surgical practice allows tumours to be removed laparoscopically, robotically and via an open procedure depending on anatomical, tumour, patient factors and availability of robotic or laparoscopic facilities. There is a current vogue to try to spare nephrons and where appropriate patients may be offered a partial or nephron sparing nephrectomy[37]. Although not yet established nationwide, some patients are undergoing minimally invasive procedures to treat (ablate) smaller

tumours by such techniques as cryotherapy[38], radiofrequency ablation[38] and high intensity focused ultrasound (HIFU)[39].

For patients with inoperable disease, metastases and recurrent disease post nephrectomy - effective treatment options are limited. All histological forms of renal cell carcinoma are relatively chemo- and radio- resistant. Established therapeutic options include cytokine therapy, with agents including interferon α and interleukin 2.

The last 8 years have heralded a new era in systemic treatment of RCC. Advances in the understanding of the VHL pathway and the synthesis of small molecular inhibitors have seen new therapeutic options for patients. A recent Cochrane review[40] highlights the improved efficacy of these drugs over the cytokine based treatments. It also points out the paucity of information regarding the efficacy of these drugs in non clear cell carcinomas.

At the time of writing this thesis, courses of these drugs can cost in the region of £30,000 (per patient per annum) and as a result the National Institute for Clinical Excellence (NICE) has restricted their use[41]. A phase III trial of first-line monotherapy for metastatic disease, compared treatment with sunitinib (an oxindol tyrosine kinase inhibitor) to interferon-alpha and found median survival times were 26.4 for sunitinib versus 20.0 months for interferon-alpha[42]. Although these new treatments are producing encouraging results there is still need for further work in particular the role of kinase inhibitors in the adjuvant setting is uncertain (currently being investigated in the ASSURE, STAR & SORCE trial[43]) and in patients with metastatic disease undergoing cytoreductive nephrectomy (currently being investigated in the CARMINA trial[44]).

1.2 p53 overview

Over the last 30 years, p53 has become one of the most studied molecules in nature. The huge interest originated since the discovery in 1979 that p53 bound, and was inactivated by, oncogenic viral proteins [45-47]. Later it was shown that p53 function is compromised in more than 50% of human cancers [48]. The following section reviews p53's important role in cancer, its regulation and its role in RCC.

1.2.1 The p53 gene

The TP53 (tumour protein 53) gene is situated on chromosome 17 at position 17p13.1. The gene encodes a protein 53 kilodaltons in mass, hence its name, comprising 393 amino acids[49]. The domain structure of the p53 protein can be seen in Figure 1.2.1 p53 has three main domains, the N –terminal transactivation domain and site of MDM2 binding (see Section 1.3.2), the central core DNA binding domain and the C – terminal oligomerisation and regulatory domain (p53's ability to bind DNA is optimum as a tetramer and this has significance later when we consider p53 mutation) [49].

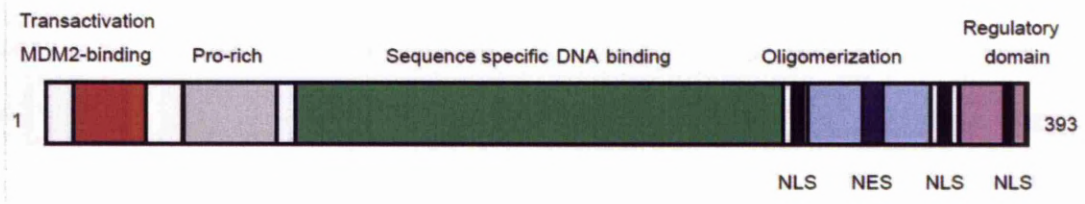


Figure 1.2.1 The p53 protein domains (from Balint & Vousden[49]) NLS – nuclear localising signal, NES nuclear export signal, Pro - proline.

1.2.3 Tumour suppressor functions of p53

There are two major main downstream responses to p53 activation; cell cycle arrest and programmed cell death. p53 also regulates genes involved in DNA repair, senescence, inhibition of angiogenesis and metastasis, and oxidative stress (see Figure 1.2.3)[50]. The exact response depends on the particular cellular stress and cell type, but appears to be primarily due to p53 transcription or repression of target genes.

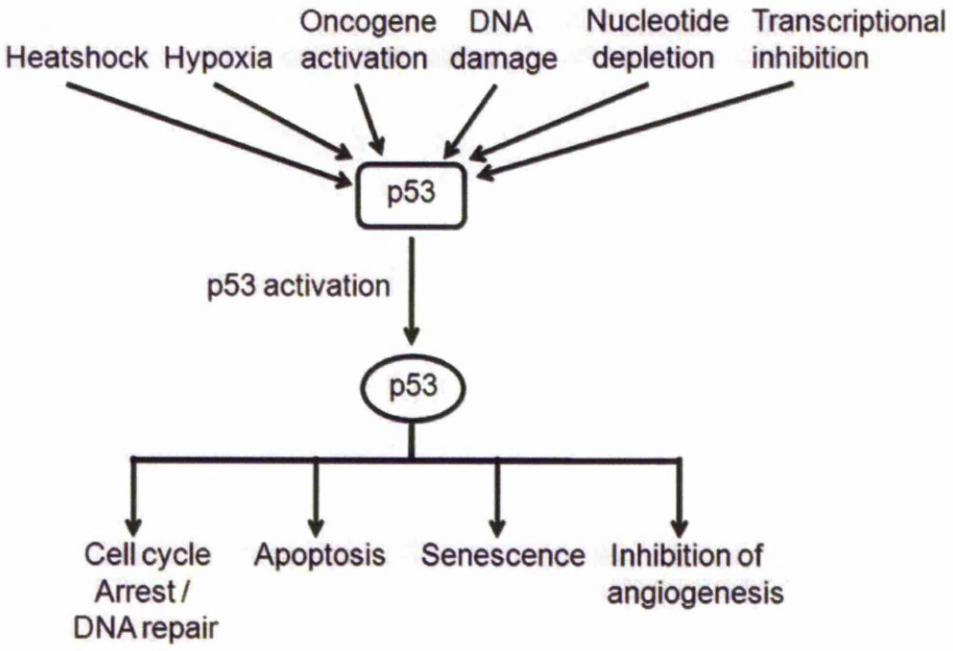


Figure 1.2.3 Activation and response of p53.

1.2.4 Activation of the p53 Response

A variety of cellular stresses can lead to activation of p53 e.g. genotoxicity, heat shock, hypoxia, hyperoxia, cytokines, growth factors, metabolic changes and activated

oncogenes amongst others[51]. Some of these factors can bring about conditions which may lead to the development of cancer if left unchecked. The dramatic effects of p53 activation have to be tightly regulated to prevent p53 mediated apoptosis or cell cycle arrest. The half life of p53 in normal cells is very short (minutes) and this is brought about by negative regulators such as MDM2 (see Section 1.3.3). In response to cellular stress the p53 molecule is stabilised and prevented from undergoing normal degradation. This mechanism has yet to be completely defined, but appears to involve ataxia telangiectasia mutated (ATM) and other damage activated kinases, phosphorylating p53 and possibly also phosphorylating MDM2[52]. The consequences of p53 phosphorylation are to reduce the affinity of p53 for MDM2 and its homologue MDM4[52, 53]. A simplified model exists by which p53 is stabilised and accumulates under conditions of cellular stress. This in turn leads to increased transcription of MDM2 and other p53 responsive genes (reviewed by Robins[54]). If the cell survives and the cellular stress signals decrease, p53 is gradually destabilised by the accumulated MDM2. A decrease in p53, leads to a decrease in MDM2 transcription and MDM2 levels fall. Thus p53 positively regulates MDM2 while MDM2 negatively regulates p53 creating an autoregulatory feedback loop (see Figure 1.2.4)[55].

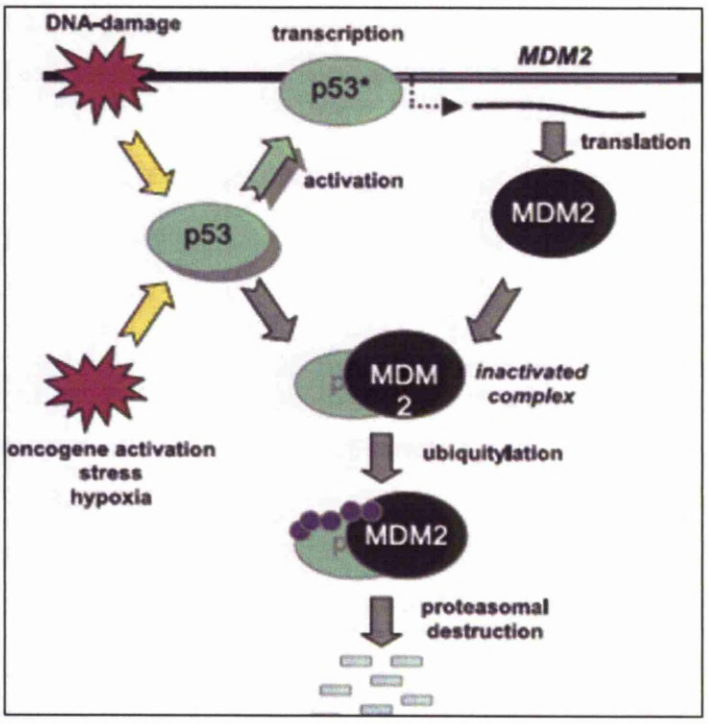


Figure 1.2.4. The p53 / MDM2 autoregulatory loop[56]

1.3 Overview of MDM2

1.3.1 MDM2 Introduction

MDM2 is a 90 kilodalton protein first identified in a transformed cell line from a BALB (Bagg Albino) /c strain. The Mdm2 gene was amplified on **m**urine **d**ouble **m**inute chromosomes (extrachromosomal nuclear bodies). Mdm2 descibes the murine protein whereas MDM2 or sometimes HDM2 describes the human protein. Mdm2 was shown to be able to oncogenically transform cells when over expressed (reviewed in Iwakuma and Lorenzo 2003[57]). Momand et al showed that MDM2 bound and inactivated p53[58]. Later it was shown that MDM2 is an E3 ligase for both p53 and itself, capable of promoting

its own and p53's degradation, by the ubiquitin/ proteasome pathway (see section 1.3.3.1)[59-61].

1.3.2 MDM2 gene and protein structure

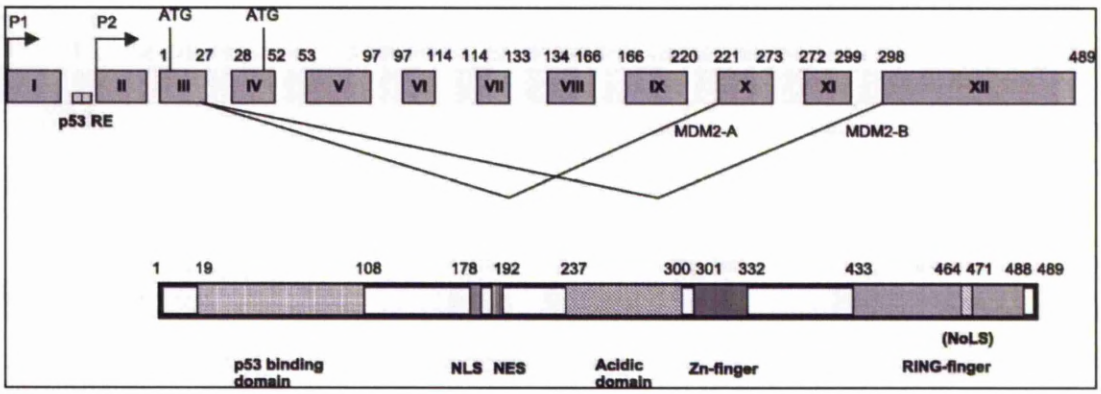


Figure 1.3.2 MDM2 gene and protein structure, from Iwakuma et al[57]. The two major alternative splice variants MDM2-A and MDM2-B are shown. NLS = nuclear localisation signal, NES = nuclear export signal & NoLS = nucleolar localisation signal, Zn = zinc.

The MDM2 gene is situated at 12q14.3-q15 and the gene consists of 12 exons. Transcription from the first promoter, labelled P1 in Figure 1.3.2, is not p53 responsive and results in relatively low constitutive levels of MDM2. The P2 promoter is p53 responsive and results in MDM2 up-regulation in response to p53. Both promoters give rise to a full length P90 protein and a shorter P76 isoform that lacks the p53 binding domain. Alternative splicing can occur generating many shorter forms[57]. The two major

splice variants are shown in Figure 1.3.2. Splice variants may have a role in cancer and are discussed later (see Section 1.3.5.2).

The N-terminal p53 interaction domain of MDM2 binds the N-terminus transactivation domain of p53, reducing its ability to function as a transcription activator. The central acidic domain of MDM2 is necessary for interaction with p300/CBP (CREB-binding protein), this is important in p53 degradation[62]. The zinc finger domain may be important for binding ribosomal protein 11 and for the degradation of p53[63] (see later). The RING (really interesting new gene) finger domain contains the E3 ligase activity responsible for ubiquitylation[57].

1.3.3 MDM2 Function

The principal function of MDM2 is to negatively regulate p53 function. Transgenic mice that are Mdm2 null display early embryonic lethality and die at around day 5 or 6 of embryogenesis. This lethality can be rescued by concomitant deletion of the p53 gene[64]. MDM2 is clearly needed to obviate the lethal effects of p53. This has traditionally been thought to occur through two mechanisms. Firstly by binding to the transactivation domain of p53, Mdm2 and Mdm4 (see later) prevent p53 transcription. Binding at this site may also prevent p53 acetylation by p300 and CREB-binding-protein (CBP) [58, 65]. Recent work reviewed in Clegg et al [66] describes a re-evaluation of this situation. These authors have shown that Knock-in mice homozygous for an Mdm2 RING finger mutation (ie. they are unable to ubiquitylate p53), are not able to rescue p53 embryonic

lethality despite the fact that this form of Mdm2 still binds to p53. This suggests that although MDM2 may be able to negatively regulate some of p53's functions by binding to it, this is not sufficient. The second method (just alluded to) is based on the E3 ligase activity of MDM2 through which MDM2 is able to target p53 for degradation by the proteasome, thus decreasing the abundance and therefore activity of p53.

1.3.3.1 Ubiquitin-proteasome pathway

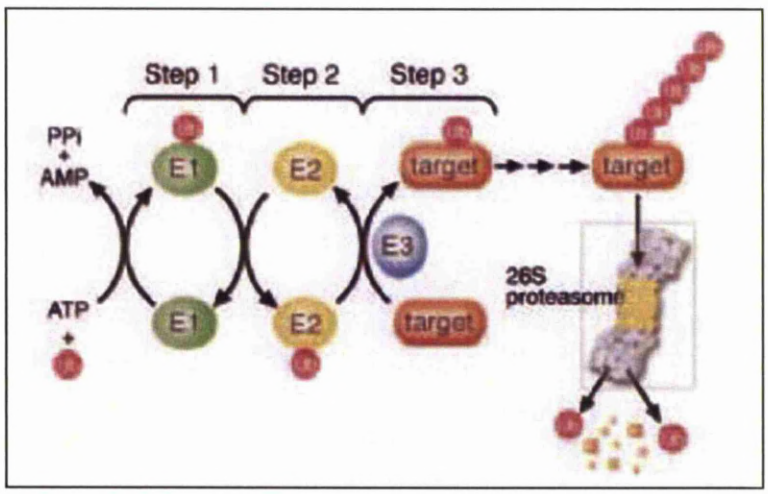


Figure 1.3.3.1 The ubiquitin – proteasome pathway, from Mani et al [67] PPI = pyrophosphate, AMP = adenosine monophosphate, ATP = adenosine triphosphate, Red circle = Ubiquitin, E1 – E3 represents the enzymatic cascade.

The ubiquitin system can selectively degrade a number of short lived proteins within the cell including p53 and MDM2. Figure 1.3.3.1 illustrates the ubiquitin cascade resulting in proteasomal degradation. The first stage of this process starts when an ubiquitin-

activating enzyme binds ubiquitin in an ATP dependent step. The ubiquitin is then transferred to an E2 ubiquitin conjugating enzyme. Ubiquitin is then transferred to the target protein via an E3 ubiquitin ligase. An attached chain of at least four ubiquitin molecules, is needed to target the protein to the proteasome for degradation[68].

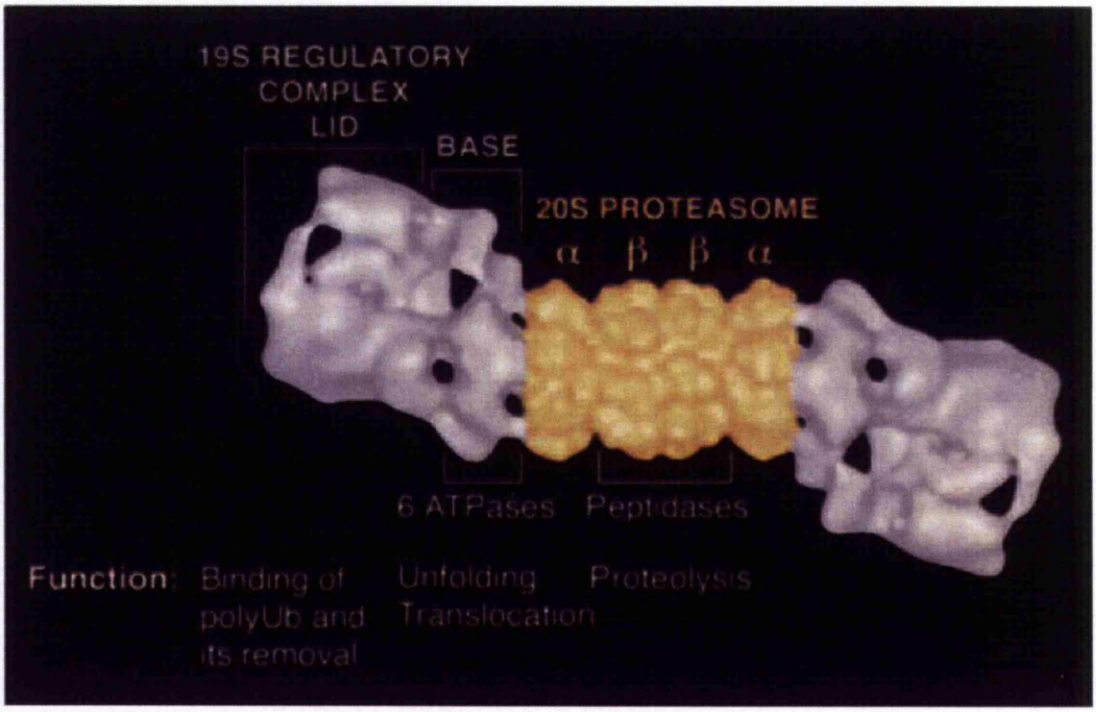


Figure 1.3.3.2. The 26S proteasome structure, from Mani et al[67].

The 26S proteasome is a multisubunit protein complex (see Figure 1.3.3.2). The 19S components assemble at each end of the 20S subunit. The 19S subunit is responsible for recognising proteins targeted by ubiquitinylation for degradation. Together with the 20S core subunit, proteins are unfolded and hydrolysed in an ATP dependent process. Both the 19S and the 20S subunits are needed for protein degradation[67].

The situation with regard to p53 ubiquitinylation is complex. MDM2 collaborates, as a homo or heterodimer, with a related protein, MDM4 (also called MDMX) to control p53 levels[69]. MDM4 is not an E3 ubiquitin ligase but is ubiquitinylated and degraded by MDM2. Gu et al [69] showed that MDM4 stabilises MDM2 and at an appropriate ratio increases MDM2's ability to degrade p53. If MDM4 is absent, MDM2 is not stable enough to degrade p53. Likewise if MDM2 is absent MDM4 does not distribute into the nucleus to be able to bind and cause p53 degradation by the proteasome. Deletion of either MDM2 or MDM4 leads to embryonic lethality.

1.3.4 p53 independent oncogenic properties of MDM2

The observation from histochemical studies of RCC, bladder cancer and sarcoma that MDM2 and p53 co-expression are associated with a poorer prognosis than either protein alone (see section 1.4.5) may suggest oncogenic properties of MDM2 independent of p53. In an animal model experiment in which p53 null mice were generated that "over-expressed" Mdm2; mice were found to have increased tumourigenesis[70]. This suggests a p53 independent oncogenic effect of Mdm2. MDM2 has been shown to interact with a number of other cellular proteins independent of p53 (see Figure 1.3.4). Some of these such as NF-kβ (nuclear factor kappa-light-chain-enhancer of activated B cells) , MTBP (MDM2 binding protein) and TGF-β (Transforming Growth Factor-β) may mediate the tumourigenic properties of MDM2 see review by Ganguli et al[71].

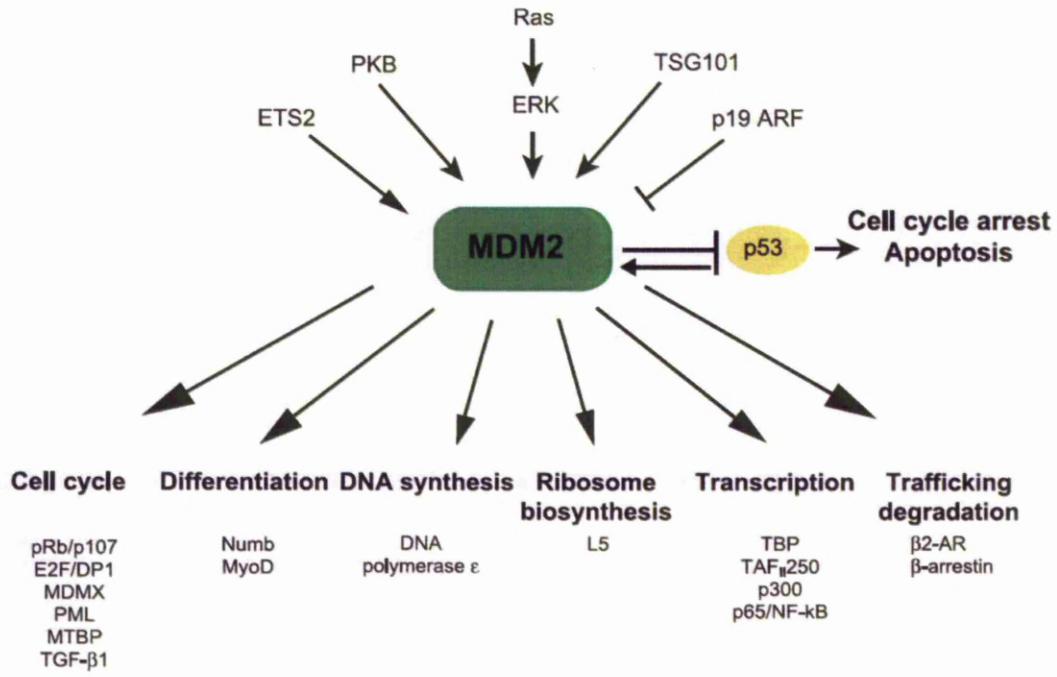


Figure 1.3.4 Putative oncogenic interactions of MDM2, from Ganguli et al[71].

1.3.5 Abnormalities of MDM2 in human cancers

MDM2 protein over expression was first found associated with gene amplification leading to the appearance of double minute chromosomes (hence the name)[72]. However MDM2 is not just expressed but rather is up-regulated often described as over-expressed in the absence of gene amplification[73].

1.3.5.1 Single nucleotide polymorphisms

A single nucleotide polymorphism (SNP) at nucleotide 309 (a T to G change) in the first intron of the MDM2 promoter, has been shown to increase the affinity of the promoter for the transcriptional activator Sp1[29]. This has been shown in the same study to lead to a >3 fold increase in MDM2 levels in subjects homozygous for the polymorphism (SNP309 G/G) compared to subjects with wild type for SNP309 (T/T). A fourfold increase in MDM2 protein levels were seen in cancer cell lines with SNP309 (G/G) compared to SNP309 (T/T) cell lines. The heightened levels of MDM2 lead to a decrease in functional p53 levels. This leads to an increased risk of tumour development in subjects carrying the SNP309. It has been shown that in RCC, differences in the SNP309 genotype lead to different levels of MDM2 expression as detected by IHC (immunohistochemistry) and moreover the GG SNP309 genotype is an independent predictor of poor prognosis (HR = 1.87, $p = 0.03$)[74]. This phenotype was seen in 62/200 (31%) of patients with RCC versus 40/200 (20%) of normal age matched controls. Clearly further analysis of this polymorphism is warranted.

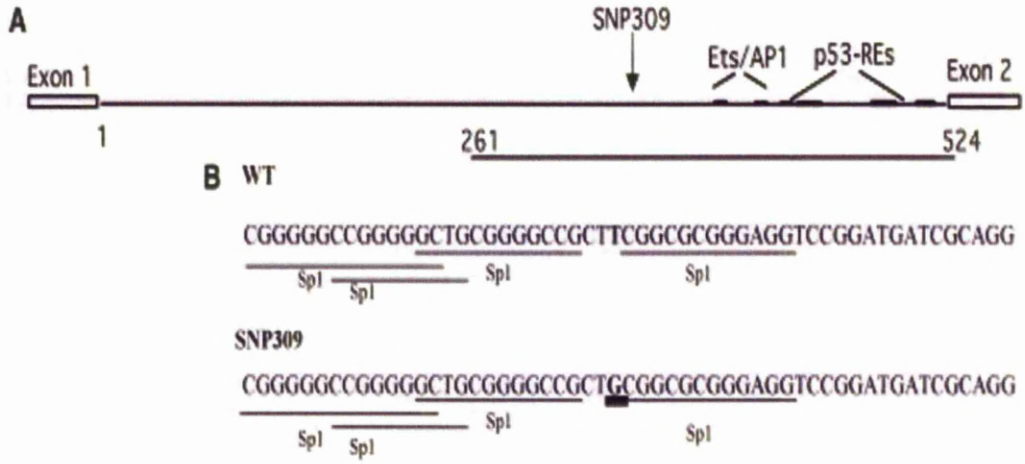


Figure 1.3.5.1 SNP309 in the human MDM2 gene from Bond et al[29]. **A** represents the intronic promoter of the MDM2 gene. The position of SNP309 is indicated in relation to the exon/intron boundaries and the transcription factor binding sites for p53 and Ets/AP-1. The region analysed for sequence variation (shown in B) is marked by the bar drawn below the diagram. **B** - The analysis of transcription factor binding sites in the region containing SNP309 is depicted. Potential Sp1 sites are underlined.

1.3.5.2 MDM2 splice variants

To date at least 40 different transcripts of alternatively spliced MDM2 mRNA, have been documented in human cancers[75]. The incidence of splice variants in RCC is not known. The significance of these in malignancy is also uncertain. Fridman et al [76] have shown that some variants are as oncogenic as full length MDM2. The authors also make the point that many splice variants lack the epitopes used by common antibodies aimed at detecting MDM2. This could mean that the oncogenic properties of MDM2 in cancer have been underestimated by IHC analyses. The exact mechanisms of how splice

variants are oncogenic are also not known. It may be that they interact and negate full length MDM2 (dominant negative effect) or their over-expression is oncogenic[75].

1.3.5.3 MDM2 mutants

Point mutations of *MDM2*, affecting the central zinc finger domain, have been reported in some human cancers[77]. Lindsrom et al[63] have shown that mutation of MDM2's central zinc finger disrupts the interaction of MDM2 with ribosomal proteins L5 and L11. Zinc finger mutants are impaired in undergoing nuclear export and proteasomal degradation as well as in promoting p53 degradation. They retain however the function of suppressing p53 transcriptional activity. Unlike wild type MDM2, where p53-suppressive activity can be inhibited by L11, the MDM2 zinc finger mutant escapes L11 inhibition. The incidence of MDM2 mutation in RCC has not been reported.

1.4 p53 and MDM2 in RCC

1.4.1 p53 expression in RCC

To date over 30 studies have investigated the expression of p53 in RCC. These studies have been critically reviewed, as part of my project, and a paper published in Cancer Journal[78] (see appendix 2). These studies have used IHC staining of paraffin embedded tumour samples. More recent studies have employed a modification of this approach in which a number of samples are arrayed onto single slides as tissue microarray (TMA) [79]

that allows for more consistent direct IHC comparisons to be made between multiple tumour samples. The published studies to date of p53 expression in RCC, are presented below in Table 1.4.1. Where the data is available, the percentage of the sample size that is ccRCC is indicated, as is the stage range, and number in the sample with metastases. The samples all contained a mixture of high and low grade tumours. The majority of papers used the p53 DO-7 antibody that targets the amino-terminal region of the p53 molecule, where available the criteria for dichotomising p53 expression/staining are given. The percentage of RCC specimens reported as staining positive for p53, ranges from 0 to 60%. From the published data (excluding papers by Klatte et al[80] and Kim et al[81] – where no details of p53 positivity are quoted) 2,519 tumours were stained for p53 and of these 618 were deemed positive for p53 by the authors. This gives a p53 positive staining percentage of 24.5% however this does not reflect the heterogeneity of the samples (see below). Such variability in percentage positive staining may be explained by the fact that there is no universal criterion for designating a tumour positive for p53 expression. There are also differences between studies in antibody and processing techniques. Interpretation of these results is also hindered by the variation in the histological subtypes of RCC tumours, tumour stage and grade and the presence of metastases, in the sample populations.

One paper [82] used two different p53 antibodies which resulted in a higher overall positive p53 expression in 60% of specimens analysed (positivity of DO-7; 51% and pAb240; 30%). This highlights the fact that using different antibodies can result in apparent differences in p53 expression As for the rarer histological subtypes of RCC,

three studies reported the incidence of p53 expression in PRCC to be 42% (5/12)[83], 70% (14/20) [84] and 24% (12/50)[14]. Perret[14] showed that p53 expression was significantly higher in type II PRCC compared to type I, however there was a higher proportion of patients with metastatic disease in this group (0 vs 32%) . For chromophobe tumours the reported rates are as follows; 20% (1/5)[83], 27.3% (6/22)[84] and 100% (2/2)[85]. Kanamaru et al[86] studied 11 RCC with sarcomatoid features and found no difference in the immunohistochemical expression of p53 in sarcomatoid compared to non-sarcomatous areas of the tumour. However, the small numbers employed in these series make interpretation of the incidence of mutations in p53 in these rarer histological classifications difficult.

There does appear to be an increased expression of p53 in studies including high stage and patients with metastatic disease. The study by Zigeuner and colleagues [84] analysed p53 staining in primary and in metastatic samples. These authors found a higher incidence of p53 positive staining in metastatic compared to primary samples (51.8% versus 22%). This together with the finding that many tumours have distinct areas of p53 staining rather than a widespread field change, suggest p53 expression is a later event in the evolution of RCC. If this is the case one might well expect p53 expression to be associated with a poorer prognosis.

1.4.2 p53 and prognosis in RCC

The prognostic implications of IHC expression and mutation of p53 have been evaluated and the results are summarised in table 1.4.1. Of the 28 studies that evaluated p53 expression as a clinical outcome predictor, 18 studies found it predicted poor outcome and 10 did not. The ten papers that did not, had a smaller study size (mean of 62 patients per study compared with 119 in the positive studies). The four largest studies to date, all using TMAs, showed p53 to be a prognostic predictor. Kim et al[81] evaluated 318 ccRCC-only patients with local and metastatic disease (49% i.e.155/318 had metastatic disease). These authors found that p53 was an independent predictor of decreased disease specific survival on univariate (p<0.001) and on multivariate Cox regression analysis (p=0.014) with the presence of metastasis as a covariate [81]. Klatte [80] in a similar study of 170 ccRCC patients again found p53 was retained in multivariate Cox regression analysis for predicting disease free survival. In the study by Klatte[80] the percentage of tumour staining for p53 was evaluated obviating simplification necessitated by describing a tumour as positive or negative for p53 staining. Zigeuner[84] and colleagues showed that p53 was a predictor of disease progression (metastasis free survival) on multivariate Cox regression analysis in their study of 130 ccRCC patients. Patients were followed for a median of 26 months and 9 of 16 patients with p53 positive staining tumours progressed versus 20/114 negative tumours (p=0.0005). In another substantial study Shvarts et al[87] evaluated p53 staining as a predictor of 5 year recurrence in 193 patients operated on for localized disease. They again showed a p53

positive cut-off of 20% expression detected by IHC to be a predictor of recurrence (HR 3.28, P=0.0108) on both univariate and multivariate Cox regression analysis

Study	Specimens (% ccRCC)	Technique	Antibody	Positive criteria (%)	Number positive (%)	p53 prognostic	Comments
Klattel[80]	170 (100) All stages M=0	TMA	?	Any	?	YES	Decreased disease free survival
Peret [14]	50 (0) All PRCC 25 Type I 25 Type II, M=8	IHC	DO-7	>20	24 (48) Type I = 12 Type 2 = 36	YES	Decreased overall survival in Type II PRCC
Phuoc [66]	119 (100) All stages, M=23	IHC	DO-7	>10	64(54)	YES	Decreased disease specific survival in all cases
Kankuri [69]	117 / (86) All stages, M=29	IHC	DO-7	>10	15(12.8)	YES	Decreased overall survival in patients with metastases
Kramer[90]	117 (89) All stages, M=21	IHC	?	>5	16(13.6)	NO	
Langer[91]	95 (75) Stage pT1 only, M=0	TMA	DO-7	?	23(24)	Not evaluated	
Cho[92]	92 / (100) All stages, M=7	IHC	?	>10	11(12)	YES	Decreased cancer specific survival
Shvarts[87]	193 (85) All stages, M=0	TMA	DO-7	Any >20	111(57.5) 14(7.3)	YES	20% cutoff predicted recurrence
Uzunlar[93]	57 (77.1) All stages & grades, M=?[1]	IHC	?	>1	20(35)	YES	Decreased Disease specific survival
Zigeuner [84]	184 (70.7) 56 mets (94.8)	TMA	DO-7	>1	42(22.8) 29(51.8)	YES	Decreased metastasis free survival in CCRCC only
Kim[81]	318 All stages M = 155	TMA	D0 – 7	>15	?[1]	YES	Decreased disease specific survival
Uchida[94]	112 / (78) All stages, M=?[1]	IHC	DO-7	>1	15(13.4)	YES	Decreased overall survival
Olumi[82]	43 / (100) All stages, M=14	IHC	DO-7 & PAB240	>10 either	22(51) 13(30) 26(60)	NO	Combined antibody positivity was 60%
Ljungberg[83]	99 / (74) All stages, M=?[1]	IHC	DO-7	>5	17(19)	YES	Decreased survival in non CCRCC
Girgin[95]	50 (62) All stages, M=0	IHC	DO-1	>20	16(20)	YES	Decreased disease specific death
Haitel[96]	97 (100) All stages, M=15	IHC	DO-1	>5	35(36)	YES	Decreased disease specific survival
Rioux-Leclerq[97]	66 (?[1]) All stages, M=10	IHC	DO-7	>20	11(17)	YES	Decreased disease specific survival
Sejima[98]	53 (?[1]) All stages, M=25	IHC	RSP53	?	1(2)	NO	
Vasavada[99]	39 (71) T1 & T2 only, M=0	IHC	DO-7	>1	0	NO	
Sinik[100]	39 (100) All stages, M=?[1]	IHC	DO-7	>10	7(17.9)	NO	
Papadoul[101]	90 / (?[1]) T1 & T2, M=14	IHC	DO-1	Any positive nuclei	30(33)	NO	
Zhang[102]	70 (?[1]) All stages, M=?[1]	IHC	Ab – 6	>10	16(23)	Not evaluated	
Gelb[103]	52 (100) T1 & T2 only, M=0	IHC	DO-7	>5	2(2)	NO	
Shina[104]	72 (?[1]) All stages, M=6	IHC	DO-7	>10	29(40.3)	YES	Decreased overall survival

Moch[105]	50 (100) T3 only, M=1	IHC	DO-7	NG	8(16)	YES	Decreased overall survival
Hofmokel[106]	31 (?[1]) T1 – T3, M=0	IHC	DO-7	>1%	5(16)	NO	
Chemeris[85]	82 (40), M=?[1]	IHC	DO-1	?	43(52)	Not evaluated	
Lipponen[107]	123 (?[1]), M=29	IHC	CM1	Any positive	41(33)	YES	Increased recurrence free survival
Kamel[108]	56 (?[1]) All stages, M=13	IHC	CM1	>1	6(11)	NO	
Bot[109]	100 (74) T1 to T3, M=0	IHC	DO-7	>50	32(32)	NO	
Uhlman[110]	175 (?[1]) All stages, M=45	IHC	?	>1	49(28)	YES	Decreased disease specific survival

Table 1.4.1 p53 expression and prognosis in renal cell carcinoma. M = Metastases, ? = Not given

When considering such studies of biomarkers of disease outcome the possibility of publication bias leading to fewer papers being published that demonstrate no disease outcome correlation should be considered. Nevertheless, the trend appears to be that more recent studies with higher numbers of cases find that p53 protein levels are prognostically significant in renal cell carcinoma.Both Kim et al[81] and Klatte et al[80] devised prognostic nomograms which included p53. Neither of these two nomograms have been validated by other groups and at present p53 is not routinely evaluated in RCC by pathologists. Another important factor that must be taken into consideration is that an immunohistochemical study simply tries to detect the expression of a protein and compares it to normal tissue (or to different types/grades of tumours). It does not tell us whether the protein is functional or mutated.

1.4.3 p53 mutation in RCC

To date, at least fifteen papers have evaluated whether p53 is mutated in RCC (see table 1.4.3). Most studies have used a technique called Single-Strand Conformation Polymorphism or SSCP as a first step in screening for mutations. This principle, devised by Orita et al [111], is based on the observation that DNA single strand mobility in a polyacrylamide gel under non-denaturing conditions, is markedly altered by single nucleotide sequence changes because of the alternative conformations adopted by single stranded DNAs with altered sequences.

Having detected altered migration of conformational polymorphisms the PCR (polymerase chain reaction) products are usually then directly sequenced to determine the identity of the alteration. In the majority of these 15 studies, analysis was restricted to the central or core domain of the gene (exons 4-8 or 5–8), as this is the most common site of p53 mutation[112]. Approximately 15% of p53 mutations occur outside exons 5-8 in exons 4, 9 & 10[113] and so it is likely that there will be some under-reporting of p53 mutations in these studies. The frequency of p53 mutations reported is between 0 and 44% (excluding the study by Oda[13], which evaluated sarcomatoid tumours). For comparison, in other tumours the incidence of p53 mutations has typically been reported as; 60 - 65% for lung & colon, 40 - 45% for stomach, oesophageal and bladder cancer, 25 – 30% for breast, liver, prostate and lymphomas and 10 – 15% for leukaemia's data from the I.A.R.C p53 database [112].

Study reference	Sample type	Technique	p53 Mutation frequency (%)[1]
Gad et al[114]	46 chromophobe 19 ccRCC 9 papillary	Dir. Seq. Exons 2-11	11 (23.9) 1 (5.3) 1 (11.1)
Kawasaki et al[115]	5	SSCP and Dir. Seq. Exons 4 – 8	1 (20)
Zhang et al[102]	16 (all p53 positive by IHC)[1]	SSCP only Exons 5 – 8	7 (44)[1]
Contractor et al[116]	30 ccRCC 20 chromophobe	SSCP and Dir. Seq. or sub clone seq. Exons 5 – 8	1 (3) 6 (30)
Dahiva et al[117]	40	SSCP and Dir. Seq. Exons 5 – 9	14 (35)
Dijkhuizen et al[118]	14 papillary	SSCP only Exons 2 – 11	0
Oda et al[13]	14 sarcomatoid	Sub clone seq. Exons 1 – 8	11 (79)
Chemeris et al[85]	29 (all p53 positive by IHC)[1]	SSCP and Dir. Seq. Exons 4 – 8	0[1]
Kuczyk et al[119]	33	SSCP and Dir. Seq. Exons 5 – 8	2 (6)
Uchida et al[120]	36	SSCP and Dir. Seq. Exons 5 – 8	2 (5.6)
Kikuchi et al[121]	118	SSCP and sub clone seq. Exons 4 – 9	2 (1.7)
Imai et al[122]	53	SSCP and Dir. Seq. Exons 4 – 8	5 (9)
Reiter et al[123]	33 RCC cell lines	SSCP and sub clone seq. Exons 5 – 9	12 (36)
Suzuki et al[124]	23	SSCP and Dir. Seq. Exons 5 – 8	1 (4.3)
Torigoe et al[125]	21	SSCP Exons 2-11	2 (10)

Table 1.4.3 p53 mutation in RCC. Dir = direct, seq = sequencing. [1] Where IHC positivity has been used to pre-select samples for genetic analysis, p53 mutation frequency only refers to the percentage of mutations found in the samples thus analysed

One intriguing observation derives from the study by Chemeris et al[85] who found that 0/29 RCC samples, all positive for p53 by IHC, had a p53 point mutation [85]. However, in another study by Zhang and colleagues it was found that 44% of tumours with p53 staining (n=16) had a p53 point mutation [102]. It is possible that contamination of samples with normal tissue might lead to reduced detection of p53 mutations by SSCP and thus variations in the extent of this might explain the differences between these two studies. This conclusion is indirectly supported by the observation that 33% of RCC-derived tumour cell lines were found to harbour p53 mutations [123]. However this assertion should be tempered by the possibility that selection of cells to adapt to growth in vitro may have resulted in an increased p53 mutational frequency. Moreover, this data from ccRCC cell lines contrasts with a study by Dijkhuizen et al [118] that identified no p53 mutations in 29 PRCC-derived tumour cell lines and thus it seems possible that different mechanisms other than direct p53 mutation may inactivate p53 more frequently in PRCC than in RCC and that these also might result in higher expression [83,84]. One other finding of particular note is the high p53 mutation rate reported in sarcomatoid tumours[118]. Sarcomatoid change is a histological finding associated with renal carcinoma with a poor prognosis and also a high (79%) p53 mutation rate (n=14) [13]. Notwithstanding these differences between individual studies and RCC sub-types, it seems clear that in contrast to both other cancers and p53 protein detection in RCC, p53 mutational analysis has yet to demonstrate prognostic utility. Until much larger studies are performed, the question of whether the lack of significance of p53 mutation as a biomarker in RCC is due to differences in p53 inactivation between RCC and other

cancers, or is simply due to a lack of power in the studies to date is a critical question that requires resolution.

1.4.5 MDM2 expression and prognosis in RCC

Four studies have evaluated MDM2 and its prognostic value in RCC. Imai et al [122] screened 53 RCC tumour samples for MDM2 gene amplifications, using dot-blot and southern blot analysis, and found none. In another study, IHC was used and MDM2 expression was detected in 2% (2/112) of tumour samples [94]. In another study by Moch et al[105], 50 consecutive tumours of stage T3 and T4, were screened by IHC, the expression of MDM2 was identified in 30% of cases, but it was found to be of no prognostic significance in terms of overall survival. However, Moch et al's[105] study also found that 7 of the 8 patients that expressed p53 also expressed MDM2 (p=0.0006), raising the possibility that MDM2 expression may be linked to up-regulation or activation of p53. Another study which analyzed this question was performed by Haitel et al [96]. In this IHC study of 97 ccRCC of all stages, MDM2 expression was detected in 19% of tumours and this was significantly more frequent in high grade tumours (p=0.01490). In addition, it was also shown that MDM2 staining was strongly associated with tumour progression (p=0.00113). p53 expression was detected in 36% of the samples, and was correlated with decreased progression free survival (p=0.00291). When different p53 and MDM2 phenotypes were compared (figure 1.4.5), it was found that tumours expressing both MDM2 and p53 have the shortest progression free survival time (p=0.00179). Perhaps most interesting from a mechanistic perspective and in

accordance with the study by Moch and colleagues, these authors found a highly significant correlation between MDM2 detection and p53 positivity ($p<0.00004$). Three of these studies analysed relatively small numbers of patients and they have employed different cut-off values for MDM2 expression. Nevertheless, the association of p53 and MDM2 detected by Moch et al[105] and Haitel et al[96] suggests that tumour progression in RCC may present a tissue specific pattern not seen in other (often) better documented cancers. For example, this link between p53 and MDM2 has not been observed in soft tissue sarcomas, [73, 126] nor in bladder cancer [127], even though patients expressing mutant p53 and increased MDM2 in these tumours had a poorer prognosis similar to the situation reported in RCC.

As will be explained later, to get cells to stably express MDM2 in culture is very difficult. Could the adaptive changes needed to express high p53 and MDM2 lead to possible targets for clinical drugs in poor prognosis renal cell carcinoma?

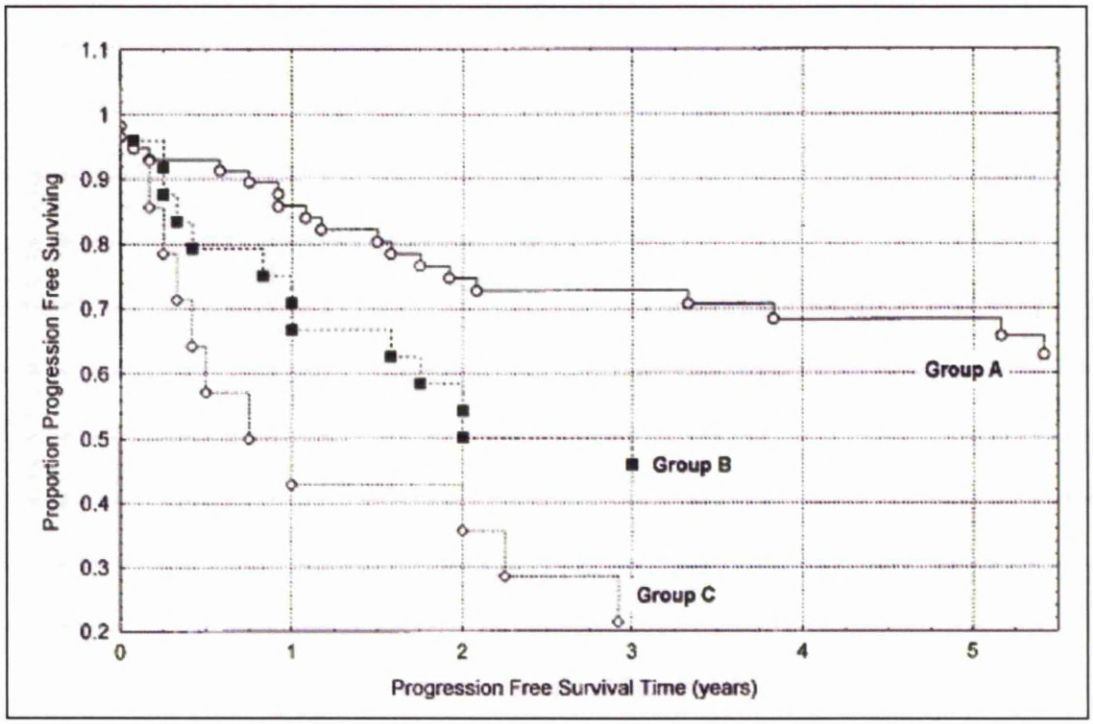


Figure 1.4.5 Kaplan-Meier disease free survival curves for patients with different p53 and MDM2 expressions in RCC[96]. Group A = MDM2 - / p53 -, Group B = MDM2 - / p53 + & MDM2 + / p53 -, Group C = MDM2 + / p53 +

1.4.6 In vitro studies of p53 and MDM2 in RCC

Several groups including our own have used in vitro analyses to investigate the functional relationship between p53 and MDM2 in RCC cells. My MD project predecessor in the laboratory (Hazel Warburton – HW) has shown that p53 is regulated by MDM2 in a panel of RCC cell lines, several of which retain relatively high levels of both wild type p53 and MDM2 [128]. As mentioned already, p53 mutations have been detected in around 30% of RCC-derived cell lines [123]. This, together with other studies

showing that p53 expression is an independent prognostic indicator; suggest that p53 function, or rather its loss or aberration contribute to tumour evolution in the kidney. It has been suggested, however, that an alternative novel dominant mechanism leads to inactivation of p53 in RCC [129]. Again HW and others have provided evidence that appears not to support this conclusion[128, 130]. Nevertheless, there remain good reasons to continue to investigate this possibility and two arguments for this are immediately apparent. Firstly there is evidence from several studies that p53 is not as frequently mutated in patient samples from RCC as it is in many other cancers. Secondly, the strong association between p53 and MDM2 expression may suggest a functional link between these, with one obvious possibility being that MDM2 expression may be driven by wild-type p53. One further point that merits particular attention is the recent discovery in vitro of a novel and potentially significant and complex interplay between MDM2 and several angiogenic factors in RCC[130]. MDM2 has also recently been shown to play a role in metastasis[131] and given its association with poor outcome in RCC, either or both of these phenotypic connections (metastasis and/or angiogenesis) may have important consequences.

1.5 Renal cell cancer cell lines

A panel of 14 RCC cell lines has been assembled by my predecessor HW. This panel forms the basis of a number of experiments designed to examine the mechanisms of MDM2 and p53 steady state levels of expression in RCC. The following section and Figure (1.5) summarises important information about each of the cell lines. Further work undertaken by HW has shown that the increased expression of MDM2 is not due to increased transcription or gene amplification.

Cell Line	Histological subtype of RCC	P53 Status	VHL status
111	?	mt 173 T-G[123]	?
115	ccCRC?	mt 280 R-T[132]	?
117	?	wt	3p LOH
121	"Granular"* RCC	mt 275 G-A[123]	?
122	ccRCC	mt 294 G – A[123]	?
154	Lymph Node RCC metastasis	mt 248 G - A[123]	?
A498	Unclassified RCC	wt	null
ACHN	Pleural RCC metastasis	wt	wt
786-0	?	Mt 278 C-G	wt
769-P	ccRCC?	wt	?
Caki-1	ccRCC? Metastasis to skin	wt	?
Caki-2	ccRCC	wt	?
A704	ccRCC	?	null
SW156	ccRCC	wt	?

Table 1.5 The RCC cell lines and p53 mutational status. The information about the origin of the cell line, p53 status, p53 mutation and VHL This information comes from Anglard et al[133] and Reiter et al [123] that established the cell lines. *The term "granular" is no longer used to describe RCC. It has previously been used to describe chromophobe RCC, PRCC & ccRCC[134]. Mt = mutant p53, wt = wild type p53, ?= not known

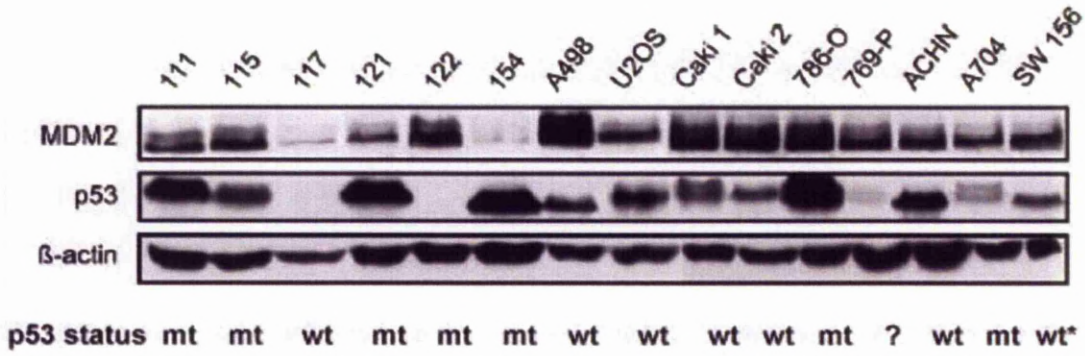


Figure 1.5 Western blot analysis of MDM2 and p53 expression in the RCC cell lines (produced by HW). mt = mutant p53 wt = wild type p53

1.6 Generating stable "high" MDM2 expressing 117 clones

As previously stated high co-expression of MDM2 and p53 detected by IHC, has been shown to be associated with poor prognosis in renal cell carcinoma. Previous work in our group by HW tried to generate high MDM2 expressing clones from the RCC line 117 (low wild type p53 and low MDM2), see Figure 1.6.1. To generate the clones, 117 cells were transfected with an MDM2 expression marker that also contained a gene for antibiotic resistance. Culturing the cells in media containing the appropriate selection antibiotic increased the chances of colonies forming that stably expressed higher levels of MDM2. Clones were then screened using western blot analysis to look to see if high MDM2 expression occurred. No MDM2 expressing clones could be generated after multiple attempts.

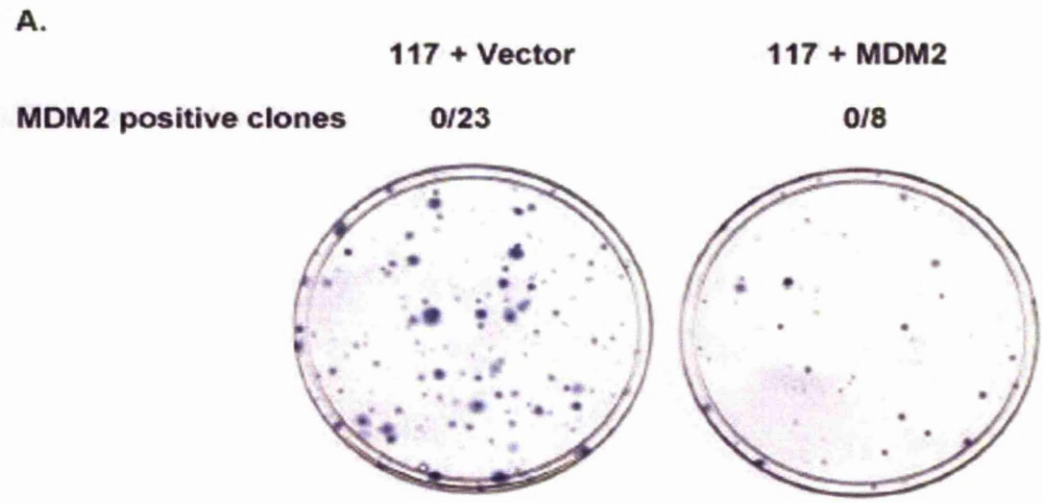


Figure 1.6.1 A representative colony formation assay of the RCC 117 cell line following transfection with either pcmv-Neo-Bam (empty vector) labelled 117 + Vector or pcmv-Neo-Bam-MDM2 labelled 117 + MDM2. No MDM2 expressing clones were created.

Clearly some other adaptive change must take place in RCC cells to allow toleration of high MDM2 levels. Or put another way, some other event is needed that leads RCC to express high MDM2 levels. In the next stage of experimentation (see Figure 1.6.2) HW investigated if the presence of non-functional/mutated p53, rendered the cells permissive for stable expression of high MDM2. Again 117 cell lines were transfected with a dominant negative p53 mutant (R175H) construct. On screening clones were found that expressed higher levels of MDM2. Surprisingly on screening of the clones that had only been transfected with the empty vector control (i.e. no MDM2 has been transfected into

these cells, just the vector containing antibiotic resistance) some were also found to express MDM2. As a result of this cloning experiment (without a MDM2 expression construct), 117 cells were identified that could express high MDM2 levels.

HW next selected a clone from the previous R175H experiment with very low MDM2 expression levels, similar to normal 117 cells. This clone was designated ΔNp53 (please note that the "Δ" is not meant to signify that p53 is truncated, the "ΔN" was used as an abbreviation for "dominant negative". A clone from the empty vector control with p53 and MDM2 expression levels similar to normal 117 cells was selected. This clone was designated pCEP3. These two clones (ΔNp53 and pCEP3) were now transfected with MDM2 and subjected to antibiotic selection, in a repeat of the first experiment (as in Figure 1.6.1). This time, on screening, some clones were generated that stably expressed MDM2 from both ΔNp53 and pCEP3 clones (see Figure 1.6.3).

As a result of these cloning experiments, the 117 RCC cell line that normally expresses low p53 and low MDM2 and cannot be manipulated to stably express high MDM2 levels, has been altered (maybe spontaneously) into progeny clones that can be made to stably express high MDM2 levels.

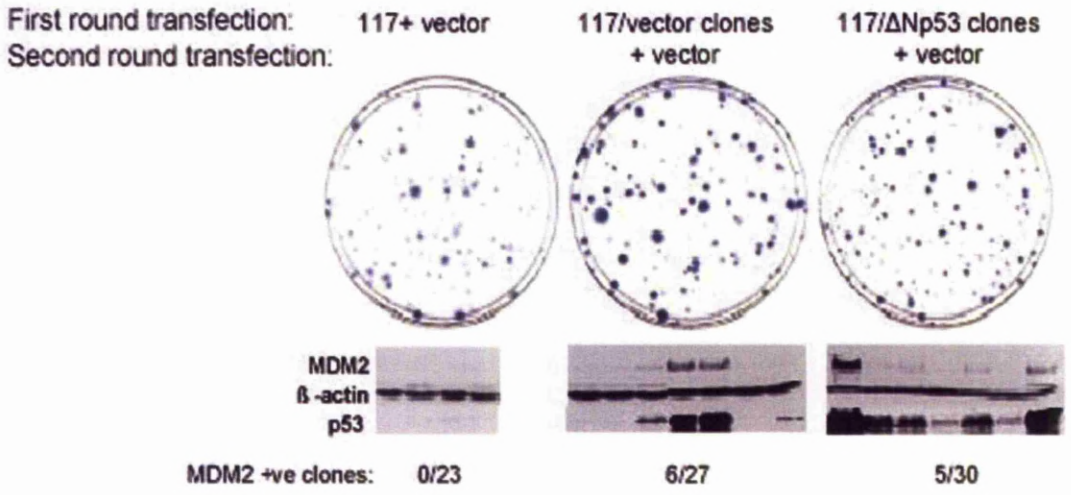


Figure 1.6.2 Spontaneous development of MDM2 expressing clones. Clones expressing increased endogenous MDM2 (no MDM2 expression construct transfected) can arise spontaneously in 117 RCC cells previously cloned after transfection with either an empty vector (pCEP4) or with a p53 dominant negative mutant R175H expressed from pCEP4. Numbers relate to all clones analysed, colony formation and western blots are from a representative sample.

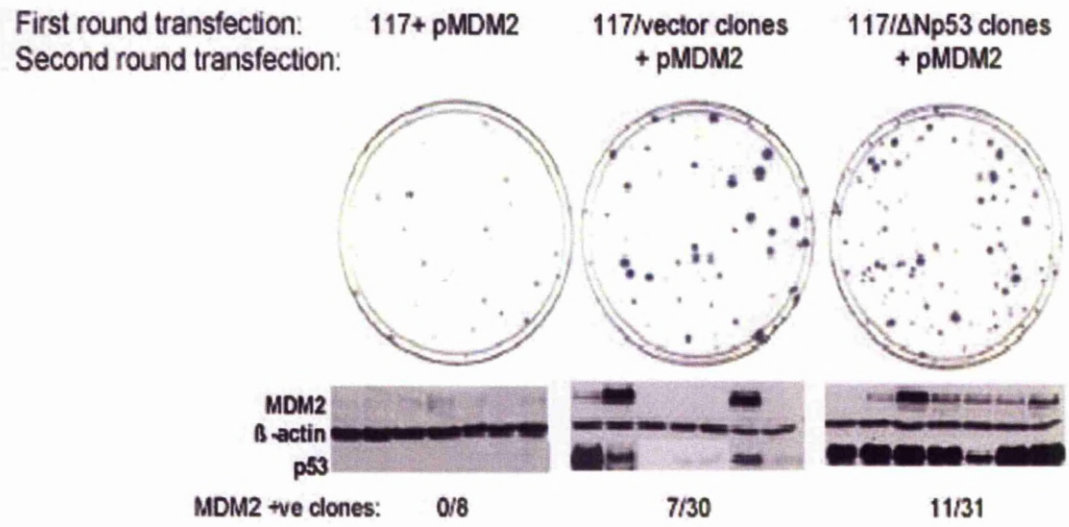


Figure 1.6.3 Transfected cells can stably express high levels of MDM2. Clones expressing MDM2 from an expression vector (pcmv-Neo-Bam-MDM2) can be generated in 117 RCC cells previously cloned after transfection with either an empty vector (pCEP4) or with a p53 dominant negative mutant R175H expressed from pCEP4. Numbers relate to all clones analysed, colony formation and western blots are from a representative sample. Analysis of clones transfected with an empty vector (pCEP4) in the first round and with an MDM2 expression construct in the second round shows that clones expressing MDM2 also express p53 (note that no p53 has been transfected into these cells)

As a result of these experiments three cell lines are able to be used as a model (see figure 1.6.4) to try and understand the adaption of RCC cells to allow high MDM2 and p53

expression and also possibly the consequences of this phenotype. The cell lines are the 117 RCC cell line that cannot be manipulated to generate high MDM2 expressing progeny and ΔNp53 and pCEP3 both of which can be manipulated to generate high MDM2 expressing progeny.

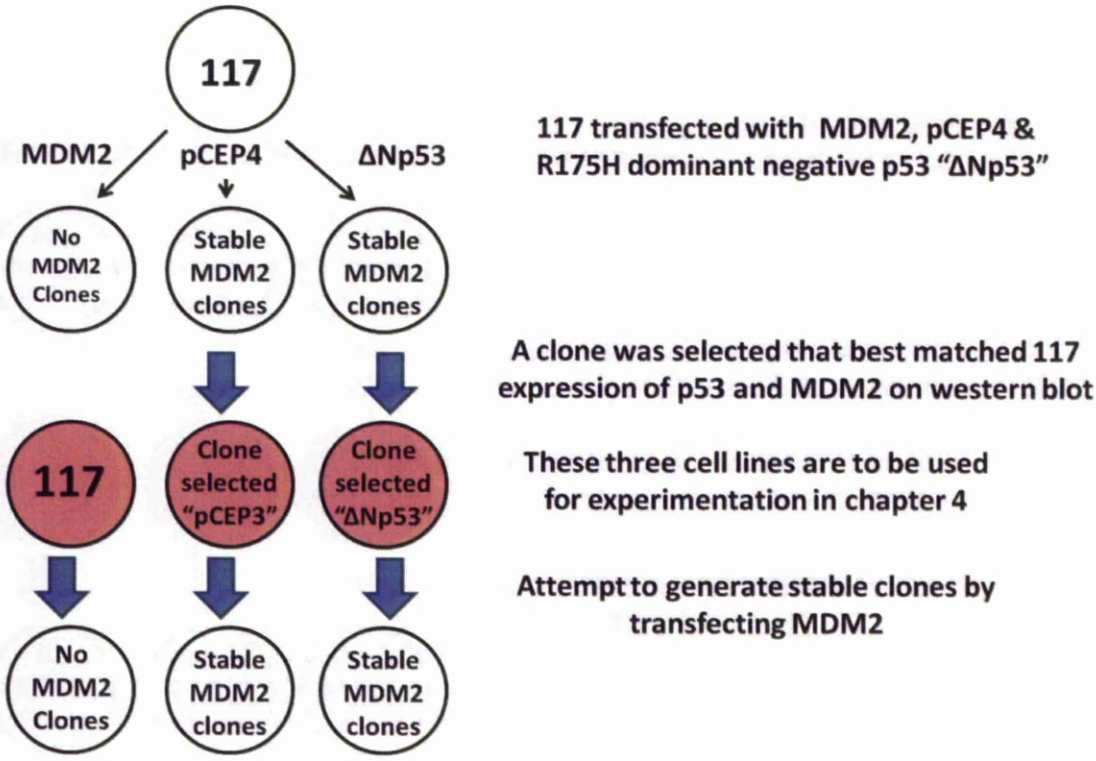


Figure 1.6.4 Overview of the cloning experiments performed by HW

1.7 Heat Shock Protein 90 (HSP90) and p53 / MDM2 in RCC

In order to explain the high levels of p53 and MDM2 expression in RCC cell lines the heat shock protein 90 (HSP90) complex will be investigated. As will be described later in this section there is evidence to suggest that the HSP90 complex may interact with p53 and MDM2 in certain conditions. This interaction of HSP90 with p53 and MDM2 may explain the higher levels of p53 and MDM2 seen in RCC.

1.7.1 Heat Shock Protein 90 (HSP90) overview

HSP90 is a molecular chaperone. There are at least five major chaperone families grouped and classified by their molecular weights. These include HSP27, HSP60, HSP70, HSP90 and HSP110[135]. HSP90 can bind unstable forms of other proteins (client proteins) allowing correct folding, oligomeric assembly, intracellular transport or disposal/degradation of the client protein[136]. During heat shock and other cellular stresses, HSP90 functions to refold damaged proteins and prevent aggregation of mis-folded proteins. There is also evidence that HSP90 is involved in the maintenance of the cytoarchitecture and also in antigen presentation[135]. HSP90 has amongst its client proteins a number of proteins important in oncogenesis e.g HER2, RAF – 1 and HIF-1α [137] see Figure 1.7.1.

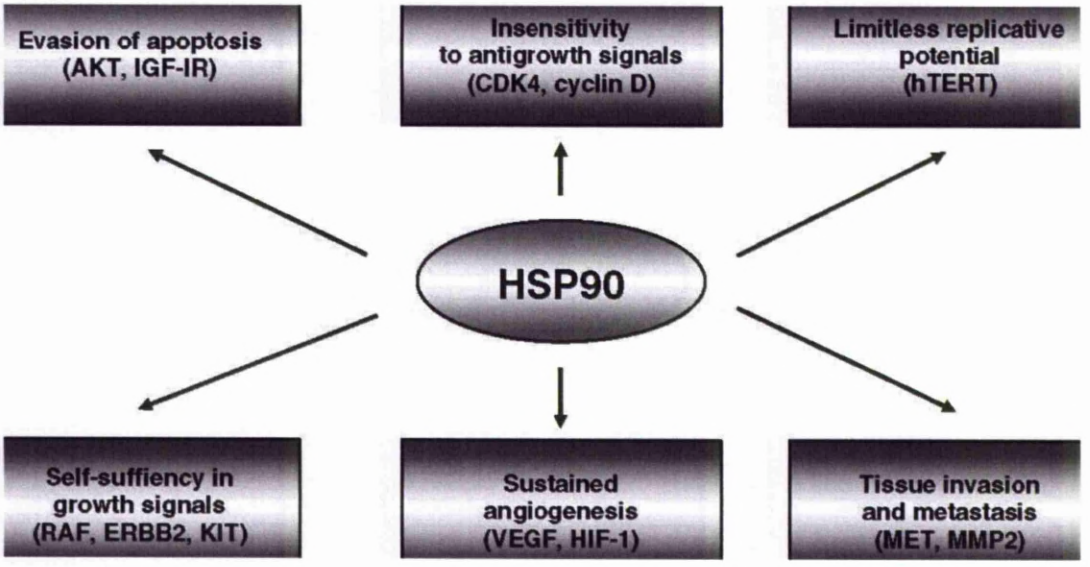


Figure 1.7.1 HSP90 client proteins implicated in cancer, from Sharp 2006[138]

It has also been shown that mutant p53 complexes with HSP90[139] and is stabilised by it[140]. Therefore it is possible that HSP90, may have a role in the high expression of p53 seen in RCC.

1.7.2 Structure, function and regulation of the HSP90 chaperone complex

HSP90 has two isoforms, HSP90-α and HSP90-β. HSP90-β is less inducible than HSP90-α and is also termed HSC90, the C standing for constitutively expressed[135].

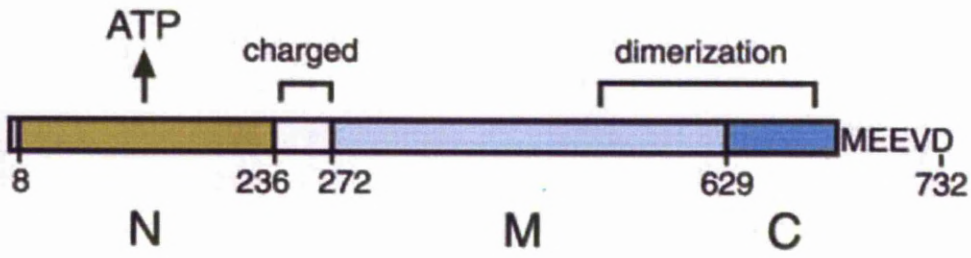


Figure 1.7.2 Schematic of HSP90 domain structures from Young[141]. N = N terminal domain, M = middle domain and C = carboxyl terminal domain.

The N terminal domain of HSP90 (labelled N in Figure 1.7.2) is the binding site for both ATP and for client proteins. The charged region has also been shown to be important for client protein binding. The carboxyl terminal domain contains a dimerisation motif and HSP90 must homodimerise in order to function. There is also evidence that the Carboxy– terminus houses a second ATP binding domain[137]. At the very end of the C-Terminus is the MEEVD pentapide. This is responsible for interaction with the tetratricopeptide repeats (TPRs) of co – chaperones[137] (see Figure 1.7.3).

1.7.3 The HSP90 chaperone complex in action

A model for the normal function of HSP90 is illustrated below in Figure 1.7.3. A client protein first binds to an HSP70 molecule co-chaperoned with Hip (HSP70 interacting protein) aided by HSP40 (also called dnaJ protein) [142]. These molecules are then bound to the HSP90 dimer by Hop (HSP70/90 organising protein – previously

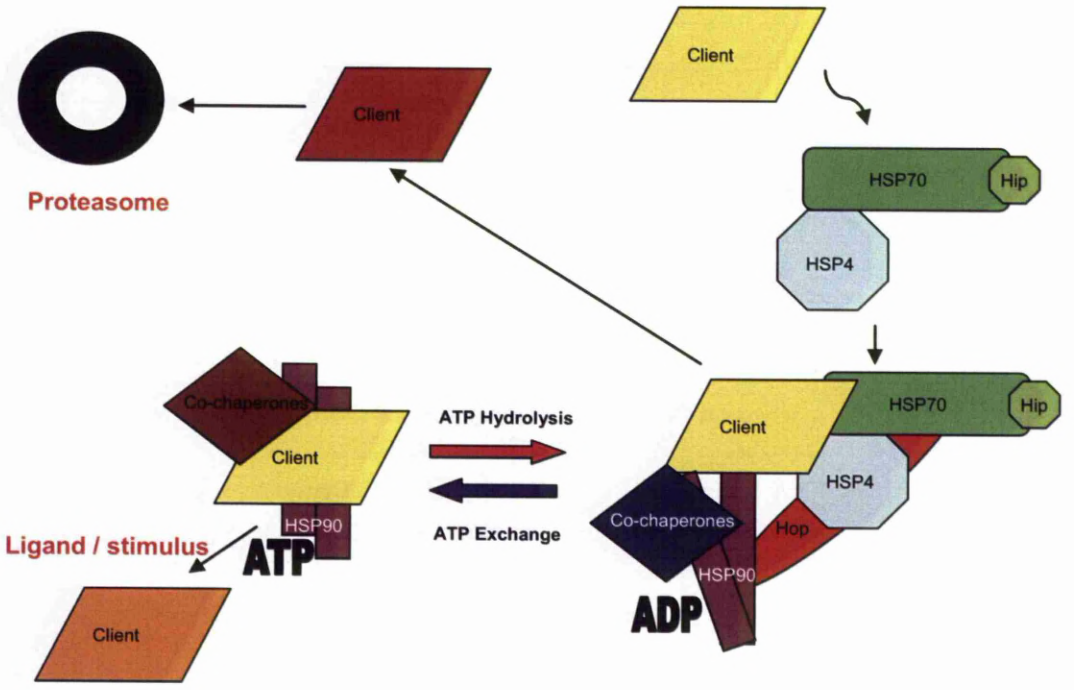


Figure 1.7.3 Schematic of HSP90 Chaperone function adapted from Isaacs 2003[143].

called HSP60). Hop acts as an inhibitor of the HSP90 ATPase, by preventing access to the nucleotide binding site of HSP90. It is thought this allows the client protein to be transferred to the nucleotide free state of HSP90[141]. Binding of the substrate allows replacement of ADP with ATP altering the conformation of the HSP90 dimer, releasing Hop/HSP70/40 and recruiting other co-chaperones such as p23, immunophilins or p50[143]. This new state allows the client protein to interact with ligands (for example steroid hormones) or other cellular stimuli (such as cystosolic kinases)[143]. If the client protein fails to interact it may be degraded, as upon ATP hydrolysis the HSP90 reverts back to the previous ADP bound conformation. While associated with the ADP bound

confirmation of HSP90, the client protein is no longer folded correctly and under the influence of co-chaperones such as CHIP (carboxyl terminus of HSP70-interacting protein) may be ubiquitinylated and targeted for proteasome destruction (see Figure 1.7.3).

Not all substrates are degraded, for example HSF1 (heat shock factor 1) is normally bound to and sequestered by HSP90. At times of cellular stress mis-folded proteins compete with HSF1 for HSP90 binding, leading to the liberation of HSF1. The inactive monomeric form of HSF1 that is bound to by HSP90 can then oligomerise into the active trimer whereupon it can bind the heat shock responsive elements in the promoter regions of the heat shock genes leading to transcription of heat shock proteins[135, 144]. Inhibitors of HSP90 (see Figure 1.7.4) also lead to liberation of HSF1 and transcription of Heat shock proteins.

1.7.4 Drug inhibition of HSP90

HSP90 has been identified for possible cancer therapy as it has been shown to chaperone a number of proteins important for oncogenesis. Interestingly HSP90 in cancer cells seems to be 100 times more susceptible to inhibition than in normal cells. This is thought to be because HSP90 exists in a high affinity conformation in cancer cells enabling it to chaperone oncogenic proteins more readily[145]. For a full review of HSP90 inhibitors see Sharp 2006[138]. For the purposes of this introduction, the bensoquinone ansamycin antibiotics will be further discussed. Geldanamycin (GA) was the first

member of this group to be discovered. It has been shown to bind to the amino – terminal ATP binding domain with greater affinity than ATP or ADP. The binding of GA results in arrest of the HSP90 cycle in the conformation that promotes client protein degradation (see Figure 1.7.4.1)[146].

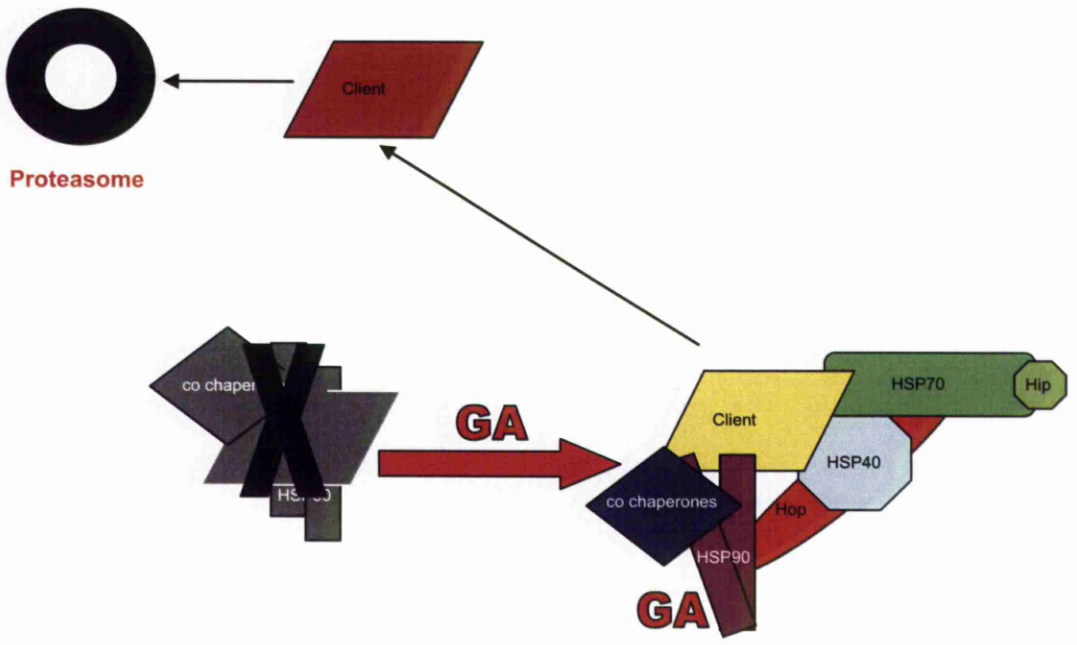


Figure 1.7.4 Inhibition of the HSP90 chaperone by geldanamicin, from Isaacs[143]. GA = geldanamicin

GA has been shown in clinical trials to be hepatotoxic. This has led to the development of two less toxic derivatives with better bioavailaility – 17 - AAG and 17 - DMAG. Another inhibitor of interest is the coumarin antibiotic novobiocin. This drug is unique as it seems to target the ATP binding site in the Carboxyl – terminus resulting in disruption of the binding of HSP90 with both p23 and HSP70[141].

1.7.5 HSP90 interactions with p53

In 1995 Blagosklonny et al[147] reported that GA destabilised mutant p53 in breast, prostate and leukaemic cell lines. Further work by this group revealed that it was HSP90 inhibition by GA that was responsible for mutated p53 destabilisation[148] and that the destabilisation was mediated by the proteasome[149]. Whitesell et al [150] found that mutant and not wild type 53 is chaperoned by HSP90. Marcu et al[151] in a different study showed that mutant p53 is also destabilised following HSP90 inhibition with novobiocin, which has a different mode of HSP90 inhibition (see section 1.7.4). Marcu et al[151] also concluded that MDM2 was unlikely to mediate the degradation of mutant p53 upon GA treatment, as MDM2 levels did not increase. This fails to exclude the possibility that MDM2 is also chaperoned and degraded following HSP90 inhibition. Nagata et al[140] again showed that HSP90 prevented mutant p53 and not wild type p53 degradation via the ubiquitin-proteasome system. Nagata et al[140] postulated that the reason mutant and not wild type p53 is chaperoned is because the mutant conformation, and not wild type p53, reveals hydrophobic residues selectively recognised by HSP90 as incompletely or misfolded. It is worth noting that these experiments, just described used transfected mutant and wild type p53. The observed effects may be a consequence of higher amounts of p53 and MDM2 present as a result of transfection.

These papers provide clear evidence that mutant p53 is stably bound to HSP90. After HSP90 inhibition with GA or novobiocin, mutant p53 is ubiquitylated and degraded by the proteasome. These results have been derived from in vitro systems or transfected cell

lines. It is not known if the effects are tumour and or cell line specific. The question still remains what role HSP90 has with wild type p53 in RCC.

Using nuclear magnetic resonance spectroscopy combined with cross-correlated relaxation enhanced polarization transfer (CRIPNET-TROSY), Rudiger et al[152] evaluated the conditions at which p53 binded to HSP90. Rudiger found that HSP90 binding was not restricted to certain mutational conformations but solely on their stability and liability to unfold. The truncated unmutated core domain of p53 was found to denature and bind at temperatures higher than that for four other mutant conformations. This may mean in vivo that HSP90 is binding mutated p53 because a higher proportion of this protein is unfolded at physiological temperatures. Evidence for in vivo interactions between HSP90 and wild type p53 come from Muller et al[153] and Walerych et al[154] who used a transformed human fibroblast cell line containing functionally active p53 as an experimental model. Treatment of this cell line with GA and radicol reduced the degree of upregulation of p53 seen after DNA damage was induced with camptothecin. Wang & Wang[155] in a similar set of experiments demonstrated less p53 stabilisation after HSP90 inhibition when the cells were heat shocked.

1.7.6 HSP90 and MDM2

As mentioned earlier in this introduction it has been observed that some cell lines with mutated p53 sometimes have high levels of MDM2. Given that mutated p53 does not

promote MDM2 transcription other mechanisms must be responsible for these high MDM2 levels. The HSP90 molecule may in some instances be responsible for maintaining high levels of MDM2. Peng et al[156] showed MDM2 co-precipitated with HSP90, but only in the presence of mutated p53. In this study destabilisation of MDM2 after HSP90 inhibition was blocked by proteasome inhibition.

1.8 Project aims

Our working hypothesis is that RCC specimens that express high levels of p53 and MDM2, on histochemical staining, have a worse prognosis. The mechanisms responsible for this phenotype are currently unknown but HSP90 may be in part responsible.

1.8.1 Investigate if there is a correlation between p53 and MDM2 expression in RCC. I will also investigate what if any, is the prognostic significance of p53 and MDM2 expressing phenotypes in RCC.

1.8.2 Develop a model for investigating the mechanisms and consequences of high expression of MDM2 in 117 cell lines. I intend to follow on from my predecessors (HW) work on the 117 cell line. I will investigate the effects of high MDM2 expression in the parental 117 cell line and its generated clones that can stably express high MDM2 levels. To achieve this I will first need to find a reproducible method of making these cells express MDM2 in high levels.

1.8.3 Investigate the effects of proteasome inhibition on the levels of p53 and MDM2 expression in RCC cell lines.

Previous work in the Boyd laboratory has suggested that high levels of MDM2 expression are not due to MDM2 gene amplification or increased transcription. Therefore, some other mechanism must exist to explain high expression. One mechanism of up-regulation may involve loss of ubiquitinylation and degradation of MDM2 by the proteasome. I aim to inhibit the proteasome in the RCC cell lines to see if MDM2 is still stabilised.

1.8.4 Investigate the effects of HSP90 inhibition in the RCC cell lines

The activities of the HSP90 chaperone complex may offer a mechanism (chaperoning may prevent their degradation) to explain the co-upregulation MDM2 and p53 in RCC cell lines. I will inhibit the function of HSP90 using a HSP90 inhibitor and look for changes in MDM2 and p53 protein expression.

CHAPTER 2 - METHODS

2.1 Reagents, buffers and solutions

All prepared in water with a resistance >15 MΩ

2.1.1 SLIP (Stuart Linn immuno-precipitation) buffer

Made up fresh each time.

50mM HEPES (pH 7.5)

10% (v/v) glycerol

0.1% (v/v) Triton X-100

150mM NaCl

2.1.2 Pepstatin (Roche Applied Science)

Stored as 1000x stock at -80°C

1mg/ml in 100% methanol

2.1.3 Leupeptin (Roche Applied Science)

Stored as 1000x stock at -80°C

0.5mg/ml in H_20

2.1.4 Aprotinin (Roche Applied Science)

Stored as 1000x stock at -80°C

2mg/ml in PBS

2.1.5 Soybean trypsin inhibitor (Roche Applied Science)

Stored as 1000x stock at -80°C

100μg/ml in H_20

2.1.6 Phenyl methane sulfonyl fluoride (Sigma)

Made Fresh

100x stock

0.0174g/ml in 100% ethanol 17M

2.1.7 4x Sample buffer

0.25M Tris (pH 6.8)

8% (v/v) SDS

40% (v/v) glycerol

4mg/ml bromophenol blue

1% (v/v) β – mercaptoethanol

2.1.8 SDS Polyacrylamide stacking gel

40% acrylamide mix

1M Tris (pH 6.8)

10% (v/v) SDS

10% APS (v/v) (Made freshly)

1% (v/v) TEMED

2.1.9 SDS Polyacrylamide separating gel (10ml)

	10%	12%
H_20	4.8ml	4.3ml
40%(v/v) Acrylamide mix	2.5ml	3ml
1.5M Tris (pH 8.8)	2.5ml	2.5ml
SDS	0.1ml	0.1ml
APS	0.1ml	0.1ml
TEMED	0.08ml	0.008ml

Table 2.1.9 SDS Polyacrylamide separating Gel (10ml)

2.1.10 Tris-glycine electrophoresis buffer

25mM Tris base

250mM glycine

0.1% (v/v) SDS

2.1.11 Electrophoresis transfer buffer

25mM Tris

192mM glycine

20% (v/v) methanol

2.1.12 Ponceau S (Sigma) 10X stock

2% (v/v) Ponceau S (3-hydroxy-4-[2-sulpho-phenylazo)phenylazo]-2,7-napthalene disulphonic acid

30% (v/v) trichloroacetic acid

30% (v/v) sulphosalicylic acid

2.1.13 PBS / Tween

0.065 M Na_2HPO_4

0.015 M $NaH_2PO_4.2H_2O$

0.075 M NaCl

0.1% (v/v)Tween 20

2.1.14 β-gal fixing buffer

0.5% (v/v) glutaraldehyde in PBS

2.1.15 β-gal substrate buffer

Diluted in PBS

3mM potassium ferrocyanide

3mM potassium ferricyanide

1mM magnesium chloride

0.5mg/ml X-gal

2.1.16 Sodium Phosphate buffer 1M (pH 7.4) 100ml

77.4ml Na_2HPO_4

22.6ml NaH_2PO_4

2.1.17 B-galactosidase reporter lysis buffer

Made up in H_20

200mM sodium phosphate buffer

2mM $MgCl_2$

100mM β-mercaptoethanol

1.33mg/ml o-nitrophenyl-β-D-galactopyranoside

2.1.18 MACS™ separation buffer

Made up in H_20

1mg of PBS

0.5% (w/v) BSA

2mM EDTA

2.1.19 1x TAE

0.04M Tris-acetate

0.001M EDTA (pH 8.0)

2.2 Tissue culturing and experimental treatment of cells

2.2.1 Cell lines

The following cell lines were used for experimentation:

H1299 (non small cell lung carcinoma) from the Division of Surgery and Oncology, Liverpool University

U2OS (osteosarcoma) from the Division of Surgery and Oncology, Liverpool University

The following RCC lines were obtained from the American Type Culture Collection, Manassas, VA 20110 – 2209 (ATCC):

A498, 786-O, 769-P, ACHN, A704, SW156, Caki – 1, Caki – 2.

The following RCC lines were kindly provided by Prof W. Marston Linehan MD, NIH.

UOK 111, 115, 117, 121, 122, 154.

2.2.2 Cell culture requirements

Cell culture was performed using a sterile technique in a class II laminar flow cabinet. Cells were cultured as a monolayer at 37°C with 5% CO_2 in tissue culture flasks. Cells were passaged when they reached a confluence of 80 – 100% depending upon the cell line. The media requirements for 500ml are detailed in Table 2.2.2. All reagents were obtained from Sigma-Aldrich.

Cell Lines	Media	Additives (Final Concentrations)
H1299 & U2OS	RPMI 1640	10% FBS
786-O, 769-P	RPMI 1640	10% FBS 1mM Sodium Pyruvate 0.5% D-Glucose 1% HEPES
A498 ACHN A704 SW156 UOK 111,115,117,121,122,154 *117 pCEP4 & 117 ΔNp53	Eagle's minimum essential medium	10% FBS (Sigma) 2mM L-glutamine 1mM Sodium pyruvate 1% non essential amino acids *250μg/ml of hygromycin B
Caki-1 Caki-2	McCoy's 5a Medium	10% FBS 2mM L-glutamine

Table 2.2.2 Culture media for experimental cell lines.

2.2.3 Cell harvesting

All cell lines were harvested in the following manner. Volumes used depended on the culture vessel, please see Table 2.2.3. Media was removed and the cells were briefly washed with trypsin (Sigma-Aldrich) which was then removed. Cells were then covered in trypsin and left in the incubator for approximately two minutes until they were no longer adherent. This was confirmed by light microscopy. The trypsin was then

neutralised with an equal volume of culture media containing FBS (complete media). The cell suspension was then repeatedly aspirated with a pipette to produce a single cell suspension. To serially passage the cell line, the cell suspension was then reseeded into the culture vessel with the appropriate volume of media. For routine passage of cells a split ratio of 1:4 was used.

Vessel (surface area cm^2)	Trypsin (ml)	Media (ml)
Flask (175)	6	25
10cm Dish (56)	3	10
6 well plate (9.5)	1	2

Table 2.2.3 Cell harvesting media and trypsin requirements

2.2.4 Cryogenic storage of viable cells

Routinely a 90% confluent $175cm^2$ flask was used. The cells were harvested as described in section 2.2.3. The cell suspension was centrifuged at 300rcf for 5 minutes. The media was removed and the cell pellet resuspended in 1ml of freeze media (FBS with 10% DMSO (dimethyl sulfoxide). The cell suspension was then transferred to a 1ml polypropylene cryo-vial and placed in a controlled rate freezing apparatus at -80°C for 24 hours, before storing in liquid nitrogen. Recovery of cells from the liquid nitrogen involved rapid defrosting of the cells at 37°C before transferring into a pre-warmed flask containing the appropriate volume of complete media.

2.3 Protein analysis

2.3.1 Cellular lysate preparation

Cell pellets were harvested as described above and stored at -80°C for at least 2 hours. Routinely 10µl of the protease inhibitors aprotinin (2.1.3), leupeptin (2.1.4), pepstatin (2.1.2), Soybean trypsin inhibitor (2.1.5) and 100µl of PMSF (2.1.6) was added to 10ml of SLIP buffer (2.1.1). SLIP buffer was added to each pellet and repeatedly pipetted until the pellet was just translucent (20µl - 55µl for pellets obtained from a confluent 10cm dish). Samples were left on ice for 10 minutes before being centrifuged at 16000rcf for 10minutes at 4°c. The resulting supernatant was aspirated to a clean tube for further analysis.

2.3.2 Protein concentration determination (Bradford assay)

A set of standard protein concentrations was made as follows, using the same SLIP used for protein lysis. 20mg of BSA (Sigma – Aldrich) was dissolved in 1ml of SLIP containing protease inhibitors. This was used to generate concentrations of 20, 10, 5, 2.5, 0.625, 0.3125 mg/ml (A micro Bradford calibration is calculated in the same way but two further dilutions are used. The concentrations range from 20 down to 0.07 mg/ml). Bradford protein assay reagent (Biorad, Hemel Hampstead UK) was diluted 1:5 with water. 1ml of the diluted Bradford reagent was added to appropriately labelled tubes. 2µl of each standard was added to the 1ml Bradford reagent in each labelled tube. After a

minimum of 30 seconds each tube was then vortexed for 8 seconds. The optical density for each sample, at 595nm, was recorded using a spectrophotometer. This enabled a standard curve of protein concentrations to be produced and stored by the spectrophotometer. Using the stored calibration curve, protein concentrations for each sample, were calculated. Samples for western blotting were then adjusted to the desired concentration (typically 50µg/20µl) using sample buffer (2.1.7). Samples were stored at -80°C until needed.

2.3.3 Western blotting

This technique allows proteins to be separated by their apparent mass by gel electrophoresis. Separated proteins can then be detected by immunoblotting [157]. Protean III (Biorad, Hemel Hampstead UK) equipment was used for the running of gels. 75mm glass plates were cleaned with ethanol and air dried. The majority of experiments used a 10% SDS polyacrylamide separating gel (2.1.9) but for lower molecular weight proteins such as cyclin – D1, a 12% gel was used (see table 2.1.9). The gel was poured to within 1.5cm of the top, overlaid with water and left for 20 minutes to set. The water was removed and the stacking gel (2.1.8) was poured onto the separating gel. A 10 well comb was inserted into the stacking gel and left for 20 minutes to set. The comb was removed and the plates containing the gel were transferred to an electrophoresis chamber. The chamber was filled with Tris-Glycine electrophoresis buffer (2.1.10) to ensure the tops of the wells were covered. Protein samples were boiled for 5 minutes to denature proteins and then centrifuged at 16000rcf for 1 minute. 20µl of sample was

loaded alongside 20µl of broad range pre-stained molecular weight marker (New England Biolabs, Hitchin UK). The samples were then electrophoresed at 200V for 1 hour. A piece of Hybond nitrocellulose membrane (Amersham Biosciences) was cut to the size of the gel (7cm x 9cm). The membrane, 3mm Whatman chromatography paper (VWR) and transfer sponges were pre-soaked in transfer buffer (2.1.11). The stacking gel was removed and the separating gel was "sandwiched" as indicated in Figure 2.3.3, submerged in transfer buffer. This "sandwich" was placed between two sponges and returned to the chamber along with an ice block. The chamber was filled with transfer buffer and transferred for 1 hour at 100V. The membrane was then removed and stained in Ponceau S (2.1.12) for 1 minute. The membrane was then washed in water to remove excess stain. The membrane was then cut into strips, to contain the area of the desired protein, as indicated by the marker ladder. The membrane was then washed in PBS / Tween (2.1.13). The membrane was incubated in 5% blotting grade non-fat dry milk powder (Biorad) in PBS / Tween for 16 hours.

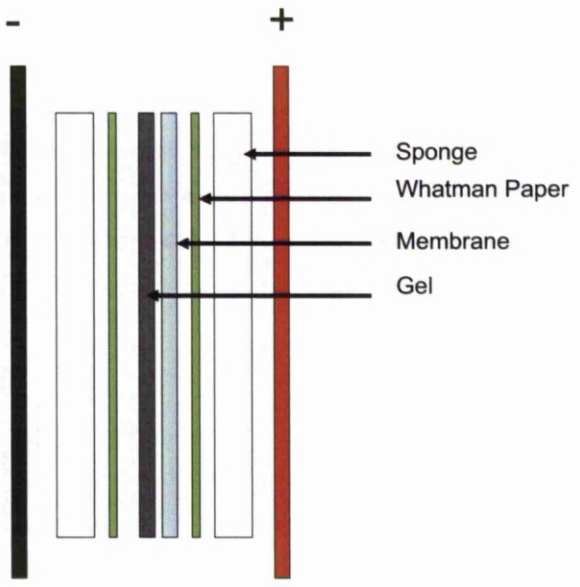


Figure 2.3.3 Diagram of the transfer "sandwich"

The membrane strips were then agitated for 1 hour in the presence of the appropriate primary antibody (see Table 2.3.4) diluted in the appropriate milk solution. The membrane strips were then washed in PBS / Tween for three 10 minute cycles. The strips were then agitated for 1 hour in the presence of the secondary antibody diluted in the milk solution (see Table 2.3.4).

The membrane strips were then covered with Enhanced Chemo-Luminescence (ECL) reagent plus (Western Lightening Chemiluminescence Plus Reagent Perkin Elmer) consisting of equal volumes of enhanced luminol reagent, and oxidizing reagent (~0.125ml of chemiluminescence reagent per cm^2 of membrane), for 1 minute. Membranes were then blotted and placed between two sheets of acetate, cut to fit into a Kodak light safe developing cassette. In a dark room, a sheet of medical x ray film (Fugi, Bedford UK) was exposed to the membrane strips, inside the cassette. The length of time of exposure varied depending on the protein being evaluated. The film was developed for 2 minutes in Kodak developer fluid and then transferred to Kodak fixer fluid for a further 2 minutes. The film was then washed in water and left to air dry. Luminescence was occasionally detected using a Kodak IS4000MM image station. A number of exposures over different time intervals were obtained to show the protein bands of interest within the linear range of the film and as far as possible with the lowest background signal.

2.3.4 Antibodies used for western blotting

Antibody	Source	Manufacturer	Antibody Concentration (µg/ml)
p53 – DO-1	Mouse Monoclonal	Oncogene/Merck	0.1 – 3
MDM2 – IF2	Mouse Monoclonal	Oncogene/Merck	3
Actin – C2	Mouse Monoclonal	Insight	0.1 – 3
β-galactosidase (Clone 200 – 193)	Mouse Monoclonal	Oncogene/Merck	3
HSP70	Mouse Monoclonal	Stressgen	0.01
HSP90α SPS-771	Rabbit Polyclonal	Stressgen	0.1
CyclinD1 – A12	Mouse Monoclonal	Santa Cruz	3
GFP –B2	Mouse Monoclonal	Santa Cruz	0.4

Table 2.3.4.1 Primary antibodies

Antibody	Source	Manufacturer	Concentration (µg/ml)
Anti-mouse IgG HRP linked	sheep	Amersham	0.4
Anti-mouse IgG HRP Linked	sheep	Jackson	0.05 – 0.1

Table 2.3.4.2 Secondary antibodies used

2.4 DNA plasmid production and quantification

2.4.1 Transformation

0.5μg of plasmid DNA was added to One Shot® *E. coli* competent cells (Invitrogen) then placed on ice for 30 minutes. Heat shock was performed by placing the tube in a 42°C water bath for 30 seconds and transferring into ice for 2 minutes. 250μl of SOC (Invitrogen) was added to the tubes. The tube is then secured at 45° in a bacterial incubator @ 37°C shaking at 225rpm for 1 hour. Over a Bunsen flame to ensure sterility, 5μl of cells were added to an apex microcentrifuge tube containing 45 μl of SOC. Using a turntable Luria-Bertani (LB) agar plates were inoculated with 5 μl of bacteria. Plates were left inverted at 37°C in a bacterial incubator overnight. Plasmids contained either an ampicillin or kanamycin resistance gene. Throughout this study the concentration of ampicillin (Sigma-Aldrich) used was 100μg/ml, and kanamycin (Merck) was used at 50μg/ml. For small scale purification of plasmid DNA, colonies were picked and inoculated separately into 5ml of LB media or for large scale purification of plasmid DNA 500ml of LB media supplemented with appropriate antibiotic was used. The inoculated culture was then incubated with shaking at 225 rpm at 37°C overnight.

2.4.2 Small scale purification of plasmid DNA

A single transformed colony was picked from a LB agar plate and added to 5ml of LB medium containing the appropriate antibiotic. Following overnight incubation in an orbital shaker incubator at 37°C at 225 rpm, plasmid DNA was purified using QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's protocol. Briefly, bacteria

were harvested by centrifugation at 3000rcf at room temperature for 10 minutes. Pelleted bacteria were thoroughly resuspended in 250µl of resuspension buffer (P1) (50mM Tris·Cl, pH 8.0; 10mM EDTA; 100µg/ml RNase A), the suspension was transferred to a fresh microcentrifuge tube, then 250µl lysis buffer (P2) (200mM NaOH, 1% SDS) was added to the suspension and the tube was inverted 4–6 times. Afterwards, 350µl of buffer N3 was added and mixed immediately and thoroughly by inverting the tube 4–6 times. The suspension was cleared by centrifugation for 10 minutes at ~17000rcf. The supernatant was applied to a QIAprep spin column, which then centrifuged for 30–60 seconds. The flow-through was discarded and the column was washed by adding 0.75 ml buffer PE and centrifuging for 30–60 seconds. After discarding the flow-through, the column was centrifuged for an additional 1 minute to remove residual wash buffer as it contains ethanol which may interfere with subsequent enzymatic reactions. DNA was eluted by adding 50µl of water to the centre of each QIAprep spin column, and centrifuging for 1 minute. Note that glycerol stocks of cultures were prepared by adding glycerol to 500µl of the growing culture to a final concentration of 25% (v/v) glycerol. These stocks were stored at -80°C until required.

2.4.3 Large scale purification of plasmid DNA

500ml of LB medium containing suitable antibiotic was inoculated with a single bacterial colony or 20µl of glycerol stock and incubated at 37°C overnight with vigorous shaking at 225 rpm. Plasmid DNA was purified using an EndoFree Plasmid Mega Kit (Qiagen) according to the manufacturer's instructions. Briefly, bacterial cells were harvested by centrifugation at 6000rcf for 15 minutes at 4°C. Pelleted bacteria were

thoroughly resuspended in 50ml of resuspension buffer (P1) and were then resuspended completely by vortexing or pipetting up and down until no visible cell clumps could be seen. 50ml lysis buffer (P2) was then added to the suspension and the tube was inverted 4–6 times to mix and left to stand for 5 minutes at room temperature. Afterwards, 50ml of pre-chilled neutralization buffer (P3) (3.0 M potassium acetate, pH 5.5) was added to the suspension and mixed immediately and thoroughly by inverting the tube 4–6 times.

The lysate was then poured into a QIAfilter Mega Cartridge and incubated at room temperature for 10 minutes. After all the liquid was passed through the filter under vacuum, 50ml of buffer FWB2 (1M potassium acetate; pH 5.0) was added to the precipitate remaining on the QIAfilter cartridge and the liquid was again passed through the filter under vacuum. 12.5ml of buffer ER was added to the filtered lysate, and mixed by inverting the bottle approximately 10 times, and then incubated on ice for 30 minutes. Afterwards, the filtered lysate was applied onto a equilibrated QIAGEN-tip and allowed to flow through the resin by gravity. The QIAGEN-tip was then washed with a total of 200ml wash buffer QC (1M NaCl; 50mM MOPS, pH 7.0; 15% isopropanol), then 35ml of buffer QN (1.6M NaCl; 50mM MOPS, pH 7.0; 15% isopropanol) was applied to elute the DNA. To precipitate DNA, 24.5ml of room-temperature isopropanol was added to the eluted DNA and the mixture was centrifuged immediately at 15000rcf for 30 minutes at 4°C. The supernatant was carefully decanted and the DNA pellet was washed with 7ml of 70% ethanol and then centrifuged at 15000rcf for 10 minutes. Then supernatant was carefully decanted without disturbing the pellet, and the pellet was air-dried for approximately 10–20 minutes. The DNA was then re-dissolved in a suitable volume of 0.1x endotoxin-free TE buffer (10mM Tris·Cl, pH 8.0; 1mM EDTA).

2.4.4 DNA quantification

DNA was quantified spectrophotometrically by measurement of absorbance at 260nm using an Eppendorf BioPhotometer (Eppendorf). The Optical density 260nm/280nm ratio was used as an indication of the DNA purity, where a ratio of 1.8 $\pm$ 0.1 was considered acceptable for DNA. Typically DNA samples were diluted 100-fold with 0.1x endotoxin-free TE buffer and pipetted into *UVette*® Eppendorf® disposable cuvettes for measurement.

2.4.5 Agarose gel electrophoresis

DNA fragments were separated by size using agarose gel electrophoresis, this technique was also used for DNA purification. Agarose gel was prepared by dissolving 0.7% - 1% (w/v) agarose – or GTG agarose for DNA purification purpose - in 1x TAE (2.1.19). Ethidium bromide was added to the molten agarose to a final concentration of 0.5µg/ml. The gel was poured into an appropriately sized casting tray containing a comb to form wells and once solidified, the gel was placed into the gel tank and covered with 1x TAE. Samples containing 10% by volume of gel loading buffer (orange G dye in 10% glycerol) were then loaded into wells, and the DNA run in comparison with 1kb DNA ladder (Invitrogen). Electrophoresis was performed at a voltage range of 20 – 90 volts depending upon the size of the gel and the size of DNA fragment being examined.

2.4.6 Restriction enzyme digestion

Restriction endonuclease digestion of DNA was carried out according to the manufacturers' recommendations. Typically 10 units of the appropriate restriction enzyme were used to digest 1μg of the DNA of interest. The enzyme and DNA were mixed in an aqueous solution containing 1x of suitable restriction enzyme buffer and BSA when indicated.

2.4.7 DNA extraction from GTG-agarose gel

DNA fragments of interest were extracted from GTG-agarose gels, and purified using a GENECLEAN® Turbo Kit (Q.Biogene) according to the manufacturer's protocol.
Briefly, DNA fragments produced in restriction endonuclease reactions were run on a GTG agarose gel (Seakem) until the DNA fragment of interest was clearly separated from the others; this band was then excised from the gel and transferred to a 1.5 ml apex microcentrifuge tube. The gel slice was melted at 55°C in 100μl of Gene clean® turbo salt solution for every 100μg of gel. A maximum of 600μl of the solution was then transferred to a Gene clean® turbo cartridge assembled in a cap less 2ml catch tube. This was then spun in a microcentrifuge for approximately 5 sec. If there was more than 600μl of solution this was repeated until all of the solution had passed through the cartridge. The cartridge was then washed twice using 500μl Gene clean® turbo wash which contains ethanol. Following the second wash the Gene clean® turbo cartridge was spun into an empty tube for 2min to remove the last of the wash solution as the ethanol it contains can inhibit subsequent reactions. The DNA was eluted by adding 30μl of Gene

clean® turbo elution solution directly onto the cartridge and allowed to soak for 5 min at room temperature. The cartridge was then spun for 1 min to transfer the eluted DNA to the tube. Note that all the above centrifugation was performed at 16000rcf.

2.4.8 DNA ligation

Based on agarose gel electrophoresis of representative samples of vector and/or insert, the concentration of each of the DNA fragments was estimated by comparing the fluorescence intensity of the band of interest to the 1.6kb band of the 1kb ladder (Invitrogen), which contains 1/10 of the marker DNA mass. The vector: insert ratio to be used in ligation reactions was estimated by the following equation:

$$\frac{\text{ng of vector x kb of insert}}{\text{kb size vector}} \times \text{molar ratio } \frac{\text{insert}}{\text{vector}} = \text{ng insert}$$

Ligation reactions were set up as shown below (Table 2.4.8) and incubated at 14°C for a minimum of 18 hours. A ligation reaction was set up without the insert to serve as a control for vector self ligation, and as a control for residual undigested vectors a ligation reaction was set up without the insert or the T4 DNA ligase.

Constituent	Amount
Vector	30-40ng
Insert DNA	Make up to volume 10µl
10x T4 ligase buffer	1µl
±10mM ATP*	0.5µl
±100mM Hexamine cobalt chloride*	0.15µl
T4 DNA ligase (1unit)	1µl
Total volume	10µl

Table 2.4.8 DNA ligation reaction

2.5 Plasmid DNA transfection experiments

2.5.1 List of expression plasmids used

pβ-galactosidase (pβ-gal)	- Boyd Lab
pCEP4	- Boyd Lab
pCEPp53	- Boyd Lab
pp53-TA-Luc	- Boyd Lab
pCMVNeoBamMDM2	- Donated by B. Vogelstein
pCMVNeoBam	- Donated by B. Vogelstein
RING finger mutant Cys464Ala:pCMVneobam3	- Donated by B. Vogelstein
pN1pbactin-rtTA2S-M2-IRES-EGFP	- Donated by B. Welman
pTre-Tight	- Clontech
pTre-Tight-Luc	- Clontech
MACSELECTK^{k}	- Mitenyi

Four different commercial methods of transient transfection were employed in this thesis.

2.5.2 GeneJuice™ (Novagen)

(For experiments in a 10cm dish)

The plasmid DNA was vortexed for 8 seconds. 500µl of serum free media was aliquoted into a 2ml Apex microcentrifuge tube. The volume of GeneJuice™ required depends on the chosen GeneJuice™ to plasmid ratio. This is normally 3:1 but different ratios were evaluated (see results section). For example for 10µg of plasmid DNA 30µl of GeneJuice™ would be used for a 3:1 ratio. The desired volume of GeneJuice™ was then transferred to the serum free media and vortexed for 10 seconds. This mixture was then left to stand for 5 minutes prior to transfer to the tube containing the DNA plasmids. The tube was then inverted and tapped repeatedly to achieve gentle mixing and was then left for 15 minutes at room temperature. The mixture was then added drop wise, in a circular pattern, onto a pre-seeded 10cm dish. The dish was then left for 24 hours in the tissue culture incubator.

2.5.3 FuGENE HD™ (Roche)

(For experiments performed in a 10cm dish)

The FuGENE HD™ containing vial was first vortexed for 1 second. The desired volume of plasmid DNA was transferred to a 2ml Apex microcentrifuge tube. To this tube a predetermined volume of serum free media was added (500µl for a 10cm dish). FuGENE HD™ was then added to this tube. Care was taken to ensure that no FuGENE HD™ contacted the sides of the tube. The tube was then inverted and tapped to mix

gently as described above. The mixture was then left for 15 minutes and then was added to the dish containing the cells, as described above. Again cells were incubated for 24 hours.

2.5.4 Lipofectamine 2000™ (Invitrogen)

For experiments in one well of a six well plate.

The desired amount of plasmid DNA was added to a 2ml Apex microcentrifuge tube. To this 250µl of serum free media was added. The tube was inverted and tapped as above. The vial containing the Lipofectamine 2000™ was gently mixed. The desired volume (again depending on the Lipofectamine: DNA ratio), was added to 250µl of serum free media in a second 2cm Apex microcentrifuge tube. The mixture was incubated at room temperature for 5 minutes and then the contents of the two tubes were mixed together by inverting and gentle tapping. This mixture was left for a further 20 minutes after which the contents were then added drop-wise to the pre-seeded well. The cells were incubated for 24 hours.

2.5.5 Polymag™ (OZbiosciences)

For experiments in one well of a six well plate.

The desired amount of plasmid DNA was added to a 2ml Apex microcentrifuge tube. To this 500µl of serum free media was added. The desired volume of polymag™ was added to a new 2ml Apex microcentrifuge tube. The contents of one tube were then added to

the other tube. The mixture was repeatedly pipetted to mix. The mixture was then left at room temperature for 25 minutes. The media covering a pre-seeded well of a six well plate was removed then DNA/polymag mixture was then added to the well. The six well plate was then placed on a magnetic mat (Ozbiosciences) for 20 minutes. 2ml of the cells' culture media was added to the well and the cells were then incubated for 24 hours.

2.5.6 Magnetic cell separation (Mitenyi Biotech)

These experiments were performed in 10cm dishes, using 117 cells and clones of cells derived from 117 cells in a tissue culture hood. Prior to transfection 10 cm dishes were seeded 24 hours before transfection. Great care was taken to prevent clumping of the cells in the dishes since this affects transfection efficiency and makes magnetic cell separation impossible. To prevent clumping the cell suspensions were vigorously pipetted and the dishes were thoroughly agitated in a figure of eight motion that optimised the even spreading of cells. Cells to be separated were co-transfected with 10μg of the plasmids of interest in this case pCMV-Neo-Bam-MDM2 +/- pβ-Gal and the MacSelectK^{k} , using FuGene HD™ (see section 2.4.2). Control dishes were co-transfected with 10 μg of the plasmids of interest and empty vector pCMV-Neo-Bam. 24 hours post transfection, the dishes were harvested and the cell suspension centrifuged for 5 minutes at 1300rcf. The supernatant was removed and discarded. The pellets were resuspended in 80μl of degassed MACS buffer (2.2.18), and repeatedly pipetted to obtain a single cell suspension. The cell suspension was then transferred to a 1ml Apex

microcentrifuge vial. 20µl of MACS microbeads (Mitenyi) were added to the cell suspension. The cell suspension was then incubated for 15 minutes at 4°C on a Nutator to agitate cells. The cells were then washed with 1ml of MACS™ buffer and re-centrifuged for 1 minute at 1300rcf. The supernatant was discarded. The cells were resuspended again in 1ml of MACS™ buffer. The centrifugation was repeated. The supernatant was discarded and the cells resuspended in 500µl of MACS buffer. The MACS™ MultiStand, MS Column and MACS™ separator unit were assembled in the tissue culture hood, as shown in Figure 2.5.7.

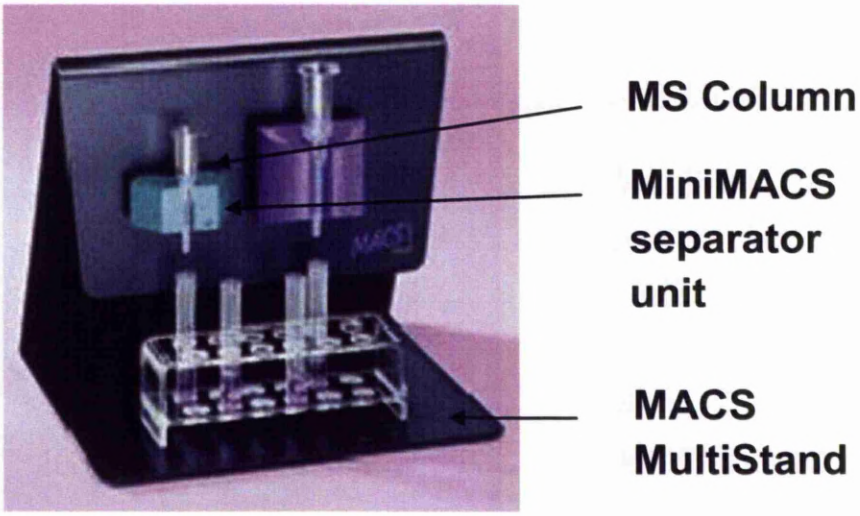


Figure 2.5.7 The assembled MACS MultiStand and components

500µl of MACS buffer was transferred into the MS column, the effluent was caught and discarded. An empty 2ml Apex microcentrifuge tube was placed under the column. This tube was labelled "negative" fraction. The column was washed with 500µl of MACS™ buffer, this was repeated until a total of 1500µl of buffer has passed over the column.

The MS column was removed from the MiniMACS™ separator unit and positioned over a 2ml Apex microcentrifuge vial, labelled "positive" fraction. 2000µl of MACS buffer was placed into the column. The supplied plunger was used to force out the buffer and cells. The fractions were recentrifuged and the supernatant discarded. When performing a β-gal assay, the cells were resuspended in 1ml of media and seeded into a 10 cm dish. The cells were left until they become adherent.

2.6 Specific methodologies

2.6.1 Proteasome inhibition using MG115

These experiments were carried out in 10cm dishes, on cells at a confluency of 70 -80%. One dish was designated the control and the cells had their media removed and replaced with 5ml of fresh media containing 5µl of DMSO. The second dish was designated treatment and cells had their media replaced with 5ml of fresh media containing MG115 (Biomol, Exeter UK) at a concentration of 1µg/ml. Dishes were incubated for 3 hours at 37°C. Cells were then harvested as described in section 2.2.3.

2.6.2 Inhibition of Heat Shock Protein 90 using geldanamycin

These experiments were carried out in 10cm dishes, on cells at a confluency of 70 -80%. For each time point, one dish was treated as follows. The cells were covered with 5ml of media containing the concentration of GA (Biomol, Exeter UK) being tested. GA was

diluted in DMSO. Concentrations used were routinely 2μM, 8μM or 20μM. The control cells were covered in 5ml of fresh media containing an equal volume of DMSO to the volume of GA used. Dishes were incubated for various time points at 37°C. Cells were then harvested as described in section 2.2.3.

2.6.3 Inhibition of de novo protein synthesis using cycloheximide

These experiments were carried out in 10cm dishes, on cells at a confluency of 70 -80%. For each time point, one dish was treated as follows. The cells were covered with 5ml of media containing cycloheximide (Biomol) at a concentration of 50μg/ml. Dishes were incubated for various times at 37°C and then harvested as described in section 2.1.3. One dish was not treated and used as a time 0 hrs control.

2.6.4 In situ β-galactosidase assay

Cells were transfected with the pβ-Gal plasmid and incubated for 24 hours at 37°C. The media was removed and the cells were then washed twice with PBS (Sigma). Next the cells were covered with β-gal fixing buffer (2.1.14) (see Table 2.6.4 for volumes) and left at room temperature for 15 minutes. The buffer was removed and the cells then washed twice with PBS. The cells were then covered with β-gal substrate buffer (2.2.15) (see Table 2.6.4 for volumes) and incubated at 37°C for 8 – 24 hours. The substrate was then removed and the plates washed gently with water. The plates were left to dry and

visualised using light microscopy. The percentage of blue (transfected) cells was estimated. A photo record was taken using a digital camera.

Culture Vessel	Volume of fixing buffer (ml)	Volume of substrate buffer (ml)
10cm dish	3	4
6 well plate	1	1

Table 2.6.4 Volumes of buffers used for in situ β-gal assay.

2.6.5 β-galactosidase enzyme assay (Promega)

Cells transfected with the β-gal plasmid and incubated for 24 hours at 37°C. The cells were then harvested and protein was extracted from the cells as previously described in section 2.3.1. To determine the amount of protein a Bradford assay was performed as described in section 2.3.2. The amount of cellular lysate containing 100μg of protein was determined. 100μg of protein was added to a test tube and 50μl of the SLIP buffer (2.2.1) used for lysis was added. 150μl of β-gal reporter lysis buffer (2.2.17) was added to the lysate/SLIP mixture. This mixture was then incubated at 37°C in a water bath for 3 hours. The chemical reaction was arrested by the addition of 500μl of 1M sodium carbonate. The mixture was then vortexed and the absorbance measured at 420nm using a spectrophotometer was determined.

2.7 Generation of inducible MDM2 expressing clones

2.7.1 Antibiotic response assay

In order to find the lowest concentration of antibiotic that caused total cell lethality following 5 days of incubation in media containing the antibiotic, the following technique was employed. Cells to be tested were seeded into 6cm dishes at a concentration low enough to give a confluency of around 10 – 20% after 24 hours incubation. Media was removed and replaced with media containing various concentrations of antibiotic. Two dishes were not treated with antibiotic and acted as controls. The cells had their media replenished (containing the appropriate concentration of antibiotic) every two days. The appearance of the cells and their confluency within the dish was recorded daily.

2.7.2 1st round cloning – Generation of 117 and 117-derived clones stably expressing rtTA2S-M2-IRES-EGFP

For each of three cell lines (117, pCEP3 & ΔNp53) the following was carried out. Two 50% confluent 10cm were transfected with 10μl rtTA2S-M2-IRES-EGFP plasmid using FuGene HD™ in a 2:1 reagent to DNA ratio as described in section (2.5.3). The second dish was used as an untransfected control. The cells were incubated for 24 hours at 37°C. The 10cm dishes were then trypsinised as previously described. The 6ml of suspended cells were then reseeded into 10 x 10cm dishes with two dishes for the following fractions of the resuspended cells 30%, 10%, 3%, 1% and 0.3%. This was repeated for

the untransfected cells. In addition to the normal culture media 800μg/ml of G418 was added. Fresh media with G418 was replenished every 2 days and the cells observed for signs of cell death. When no viable cells were visible in the control dishes, colonies were harvested from the transfected dishes.

2.7.3 Harvesting Colonies

10cm dishes containing isolated colonies of cells were taken and had their media carefully aspirated. 3ml of sterile warmed PBS was pipetted onto the dish. Isolated colonies were used that did not have multiple layers of cells giving them a "crowded" or "clumped" appearance. Using a microscope within the tissue culture hood to aid visualisation a 20μl pipette was used to place a "drop" of warmed 37°c trypsin onto the colony to be picked. Once the colony began to lose adherence with the dish surface ("lift") it was pipetted. The cells were then transferred to a single well of a 96well plate containing 80μl of warmed 37°C trypsin. This was then repeatedly pipetted to create a single cell suspension. A 200μl pipette was then used to transfer this mixture into a well of a 24 well plate containing 0.9ml of media containing the appropriate antibiotic. This was repeated until a minimum of 60 colonies were selected. This process took place over a two week period as colonies expanded at different rates.

2.7.4 Colony expansion

Throughout this process individual colonies grew at different rates, when a colony reached a confluency of 80 – 90% it was promoted to a larger dish. Until confluency was reached media was replenished every 2 days. For each step of promotion cells were washed with sterile warmed 37°C PBS. The cells were trypsinised and then an equal volume of treated media was added to the mixture. This was repeatedly pipetted to decrease cell clumping. The cells were then added to the larger receptacle containing media with appropriate concentration of antibiotic. The requirements for each step are displayed in the below table.

	24 to 12 well	12 to 6 well	6 well to 10cm dish	10cm dish to T175 flask
PBS wash (µl)	100	250	500	3000
Trypsin (µl)	100	250	500	3000
Treated media (µl)	800	2500	9000	19000

Table 2.7.4 Requirements for colony expansion

Once a colony was established in a T175 flask, flasks were split to generate frozen stocks of the clones and to allow further testing.

2.7.5 Luciferase reporter assay

The Dual-Luciferase® reporter assay system (Promega Corporation; USA) was used. The activity of firefly luciferase under rtTA control (pTre-Tight-Luc) was utilised to measure the clonal response to doxycline induced transcription of pTre-Tight–Luc. These experiments were carried out in 6 well plates seeded with cells. Briefly, Media was removed and each well washed with PBS twice. Each well was covered with 500µl of 1x Passive lysis buffer (PLB, Promega). Plates were placed on a rocker (Compact Rocker CR300, FINE PCR) at speed 9 for 15 minutes. The cell lysates were collected into pre-chilled 1.5ml microcentrifuge tubes; afterwards, the lysates were cleared by centrifugation for 1 minute at 16,000rcf at 4°C. The supernatants were kept on ice until needed. To measure the reporter activity, the supernatant and Luciferase Assay Reagent II (LAR II) and the Luciferase Assay Reagent II (LAR II) and Stop & Glo® Reagent (all three solutions provided by Promega) were thawed at 37°C in a water bath and then left to reach room temperature and just before use all components were vigorously vortexed. 20µl of the supernatant was transferred into the luminometer tube containing 100µl LAR II, after mixing by pipetting the tube was placed in a GloMax 20/20 Luminometer, which been programmed to perform a 2 second pre-measurement delay, followed by a 10 second measurement period. The protein concentration of the supernatant was calculated using a micro Bradford calculation (see section 2.3.2). The luminometer readings were normalised to the protein concentration for each sample.

CHAPTER 3 - IMMUNOHISTOCHEMICAL ANALYSIS OF P53 AND MDM2 EXPRESSION IN RENAL CELL CARCINOMA SAMPLES

3.1 Introduction

Little can be said for certain about the role of MDM2 in RCC as only four papers have reported this previously, whereas there have been over 30 papers examining p53 in RCC. What is known is that p53 and MDM2 co-expression in tumour samples is highly significantly associated[96, 105]. The main aim of this chapter is to further examine p53 and MDM2 expression in RCC and to test the significance of p53 / MDM2 expression as a putative determinant of outcome in RCC. In order to do this we constructed a tissue microarray of 90 RCC tumours to facilitate analysis.

3.2 Specific methodology

The Tissue Microarray (TMA) experiments were carried out in collaboration with the University of Liverpool's Cancer Tissue Bank Research Centre (LTB) and the Department of Pathology. Between 1992 and 2007 the LTB prospectively collected 94 nephrectomy specimens from patients undergoing radical nephrectomy for RCC with consent as described below.

3.2.1 Design and construction

94 formalin-fixed paraffin-embedded primary tumour specimens were collected by the LTB. These patients had consented for the storage and use of their tissue for research between 1993 and 2007 and study-specific ethical approval was obtained from the Liverpool Adult Research Ethics Committee. Data on the stage and grade of the tumours were collected prospectively by the LTB. All H&E-stained slides from the formalin fixed paraffin embedded (FFPE) material were evaluated by a pathologist for the presence of tissue regions optimally representative of the RCC. An appropriate slide was selected and representative regions of tumour were identified from each case. This was also performed for a slide containing adjacent non involved (non-tumour) renal tissue from each case. From each corresponding FFPE tumour block, at least duplicate cores (0.6mm in diameter) were taken from the marked area and mounted into a recipient paraffin block using a manual arrayer (Beecher Instruments Inc). Duplicate tumour cores were not placed next to each other in the recipient paraffin block to eliminate both scoring and staining biases. A single core of non-involved renal tissue was also mounted into the recipient block. Cores of normal colon, liver and testis were also included for orientation and as controls during immunohistochemistry (IHC). Serial 5μm sections were cut from the tissue microarray (TMA) and collected onto X-tra™ adhesive slides (Surgipath).

3.2.3 Preparation and staining of slides

The preparation and staining of slides was carried out by Mr Andrew Dodson (Department of Pathology, Royal Liverpool University Hospital). Sections 5μm thick were deparaffinised and pretreated in a microwave oven (25 min, 850 W) in citrate buffer [0.01 M (pH 6)]. For detection of MDM2 protein, a mouse monoclonal antibody against MDM2 (SMP-14, cat # sc-965; Santa Cruz, California, US; dilution 1:50, stock antibody concentration = 4 micrograms/ml) was used with an overnight incubation at 4°C. p53 protein was detected using monoclonal antibody DO – 7 cat # M7001 (DAKO Corp, Glostrup, Denmark; dilution, 1:2000, stock antibody concentration = 0.195 micrograms/ml) with 60 min of incubation at room temperature. The labelled-polymer/HRP detection method was used for visualization of the signal (DAKO, cat # K5007). The nuclei were finally counterstained lightly with haematoxylin, sections were dehydrated through alcohol and xylene and mounted in resinous mountant.

3.2.4 Scoring of slides

Slides of stained specimens were reviewed by two consultant histopathologists (blinded to clinical outcome) and a scoring system was determined as follows. The intensity of staining was graded 1 (weakly stained) to 3 (highly stained). The percentage of cells showing positive staining was graded as follows 1 = 0 -2%, 2 = 3 -10%, 3 = 11 -50% and 4 >50%. The TMA sections were then independently scored and results analysed. In the event of any discrepancy these specimens were jointly reviewed by the

histopathologists and a score agreed upon. For each tumour there are two samples on the TMA to be scored. For p53 and MDM2 staining tumours there were a number of specimens which stained more profoundly (intensity score 3 and percentage score 4) – for the purposes of statistical evaluation this criteria was deemed as the positive criteria for MDM2 and p53.

3.2.5 Determination of clinical outcome

Data on the stage and grade of the tumours were collected by the LTB. Periodically the LTB update their database with patient status, only death and cause of death was recorded. Evidence of disease progression was not recorded.

3.2.6 Statistical analysis

All data was entered into a database using Microsoft Office Excel 2007. Statistical analysis was performed using SPSS version 16. The association between p53 and MDM2 expression with patient and tumour factors was analysed using a two sided Fisher's exact test. Disease-specific and overall survival curves of the nephrectomy patients were estimated according to the Kaplan-Meier method. Statistical analyses of the differences between curves were performed using the log-rank test. Variables that significantly influenced survival ($P < 0.05$) in the univariate analyses were entered into a multivariate Cox regression model. In all of the analyses, the significance level was set at 0.05.

3.3 Results

3.3.1 Immunohistochemical determination of p53 expression in RCC

90 Tumours (180 separate samples) were available for scoring as detailed in section 3.2.4. A proportion of tumours (14) showed high intensity staining (graded as 3) of at least 50% of the specimen, in at least one of the two samples analysed. This pattern seemed distinct from the remainder of the samples and was therefore used to define p53 positivity. 3 separate tumours showed high intensity (grade 3) staining that only involved <2% of the specimen and these were assigned to the p53 low group for the purposes of the present analyses. Our p53 positivity rate was 14/90 (15.6%). From Table 1.4.1 the calculated mean positivity rate from published studies was 24.5%. The slightly lower positivity rate seen in these results may reflect our higher p53 cut-off criteria of 50% to assign the sample as "positive". As stated a sub-set of p53 positive tumours seemed to be distinctive due to the degree of positive staining. As discussed in section 1.4.1 differences in percentage of positive tumours may reflect differences in IHC processing and heterogeneity in the samples. For example none of our patients had metastatic disease at the time of their nephrectomy. Higher p53 positive rates in RCC have been reported with metastases or indeed where metastases themselves have been stained (please refer to section 1.4.1 and in particular the paper by Zigeuner et al[84]).

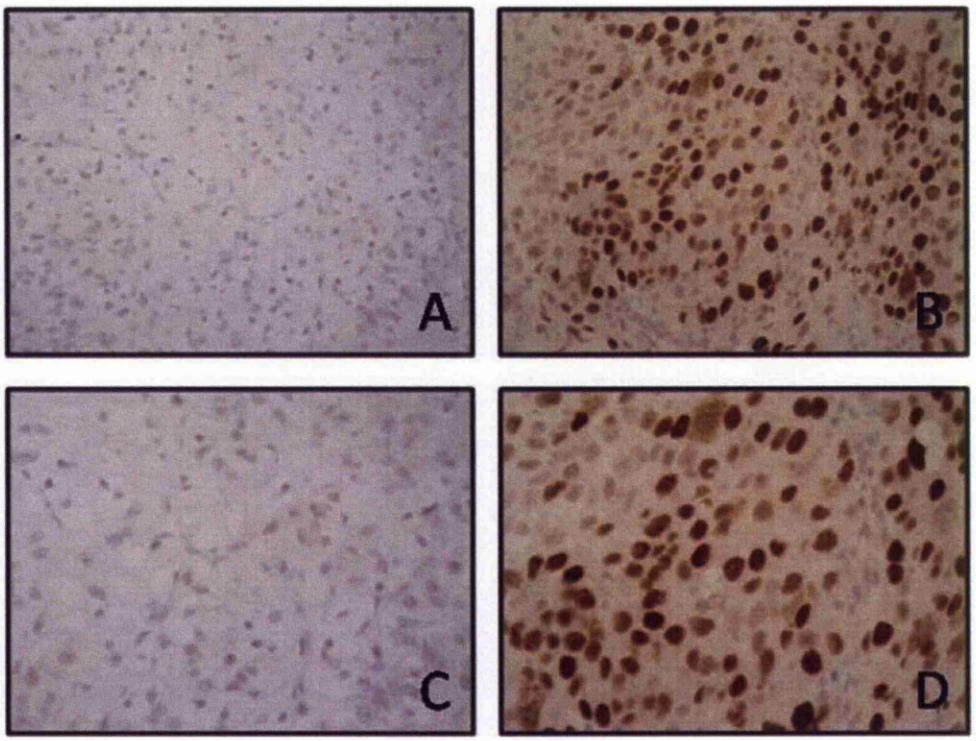


Fig 3.3.1 Photographs of selected RCC TMA samples stained for p53 using monoclonal antibody DO – 7; dilution 1:2000, antibody concentration = 0.195 micrograms/ml). The labelled-polymer/HRP detection method was used for visualization of the signal. The nuclei were counterstained with haematoxylin. **A** & **C** x40 & x60 original magnification respectively of a p53 negative staining tumour. **B** & **D** x40 & x60 original magnification respectively of a p53 positive staining tumour.

3.3.2 Immunohistochemical determination of MDM2 expression in RCC

90 Tumours (180 duplicate samples) were available for scoring as detailed in section 3.2.4. A proportion of tumours (24) showed high intensity staining (graded as 3) of at least 50% of the specimen, in at least one of the two samples stained. This pattern again appeared to distinguish a sub-set of the samples and therefore was used to define MDM2 positivity. 5 separate tumours showed high intensity (grade 3) staining but this only involved <10% of the specimen. Our MDM2 positivity was 24/90 (26.7%). IHC expression of MDM2 has been evaluated in three previous papers (see section 1.4.5) with positive percentages of 2%[94], 30%[105] & 19%[96]. All three studies used the same monoclonal antibody - IF2 that recognises an epitope in the amino-terminal portion of the human MDM2 protein. In this study, the MDM2 antibody used was SMP – 14 which recognizes residues 154-167 of MDM2. These three studies and ours all had similar mixtures of stage and grade so the differences in expression can only be explained in terms of antibody differences, IHC processing (assay sensitivity) or intra-observer variations in interpretation.

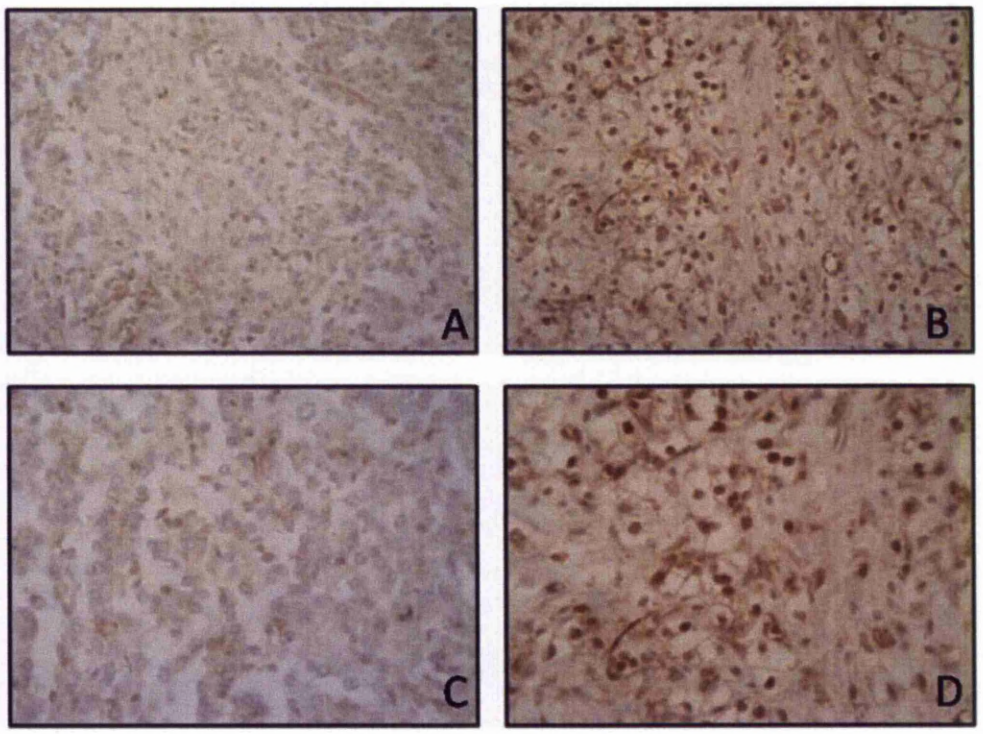


Figure 3.3.2 Photographs of selected RCC TMA samples stained for MDM2 using, a mouse monoclonal antibody against MDM2 SMP-14; dilution 1:50 , antibody concentration = 4 micrograms/ml. The labelled-polymer/HRP detection method was used for visualization of the signal. The nuclei were counterstained with haematoxylin. **A** & **C** x40 & x60 original magnification respectively of a MDM2 negative staining tumour. **B** & **D** x40 & x60 original magnification respectively of a MDM2 positive staining tumour

3.3.3 Patient and tumour characteristics

90 Tumour samples were analysed. The proportion of positive samples for p53 and MDM2 is indicated in Table 3.3.3.1. 11 tumours were dual positive for p53 and MDM2. All but three tumours were ccRCC. All of the patients underwent an attempted curative surgical procedure and not surprisingly all the tumours were Stage 3 or less with no patient having metastatic disease at the time of operation. This cohort is therefore from a potentially good prognostic group.

As stated earlier 11 patients (12.2%) were dual positive for p53 and MDM2. The association between p53 positive and MDM2 positive tumours (dual positivity) is highly significant $p < 0.0005$ using a two sided Fisher's exact test (see Table 3.3.3.3). This association of p53 and MDM2 is in keeping with the only published results examining this association - Haitel et al[96] and Moch et al[105] who found the association to be highly significant with $p = 0.0006$ and $p < 0.00004$ respectively.

	Number of patients in each category by IHC phenotype (percentage)					
	All	p53 +	p53 -	MDM2 +	MDM2 -	p53 + MDM2 +
Number of Tumours	90	14 (15.6)	76 (84.4)	24 (26.7)	66 (73.3)	11 (12.2)
Male	60 (66.7)	8	52	12	48	6
Female	30 (33.3)	6	24	12	18	5
Mean Age (24 – 82)	60.6	55.2	61.5	58.2	61.4	59.8
Died all causes	32	5	27	10	22	5
Died of RCC	19	5	14	7	12	5
Histological subtype						
Clear cell	87 (96.7)	14 (100)	73 (96.1)	24 (100)	63 (95.5)	11 (100)
Papillary	2 (2.2)	0	2 (2.6)	0	2 (3)	0
Chromophobe	1 (1.1)	0	1(1.3)	0	1 (1.5)	0
Fuhrman Grade						
1	20 (22.2)	3	17	7	13	2
2	37 (41.1)	3	34	10	27	3
3	29 (32.2)	7	22	6	23	5
4	4 (4.4)	1	3	1	3	1
Tumour Stage						
1	44 (48.4)	8	36	15	29	6
1a	6	0	6	1	5	0
1b	38	8	30	14	24	6
2	25 (29.6)	5	20	6	19	4
3	21 (22)	1	20	3	18	1
3a	17	1	16	3	14	1
3b	4	0	4	0	4	0
3c	0	0	0	0	0	0
4	0	0	0	0	0	0

Table 3.3.3.1 Patient and tumour characteristics of the TMA cohort

		MDM2		
		Negative	Positive	Total
p53	Negative	63	13	76
	Positive	3	11	14
	Total	66	24	90

Table 3.3.3.2 Distribution of p53 and MDM2 staining in the TMA

Chi – square testing revealed no significant difference in the association between p53, MDM2 and dual positivity with gender, grade (low vs high) or stage (1 & 2 vs 3) (see Table 3.3.3.3). The decision to compare between high and low grades and stage was felt to be clinically justifiable as poor prognosis has been shown to be correlated with increasing grade and stage (see 1.1.3.4 & 5). Haitel and colleagues[96] found a statistically significant higher incidence of MDM2 in high grade as compared with low grade tumours and no significant difference when MDM2 staining was compared between high and low stage tumours. The study by Haitel et al had 18 Grade 4 patients, in this study there were only 4. Moch[105] also found no correlation with MDM2 positivity and stage or grade.

	p53 + *P* =	MDM2 + *P* =	p53 + MDM2 + *P* =
Gender Male v Female	0.545	0.054	0.502
Grade 1 & 2 v 3 & 4	0.129	0.462	0.202
Stage 1 & 2 vs 3	0.174	0.170	0.447

Table 3.3.3.3 Association of patient and tumour characteristics with p53/MDM2 phenotypes

3.3.4 Survival analysis of p53 and MDM2 expression phenotypes

Kaplan – Meier survival analysis was performed to analyse differences in survival (overall and disease specific) for various p53 and MDM2 expressing phenotypes. The log rank test was used to test for differences between groups with p values less than 0.05 being considered significant. Disease specific survival (DSS) may be argued as a better outcome measure than overall survival (OS). This is because OS cannot distinguish between patients that have died because of the disease itself or from an unrelated cause (e.g. being hit by a bus). On the other hand, statistically speaking, an unrelated cause of death clinically may be due to an unknown effect of the disease or its treatment on other organs (e.g. systemic treatment for RCC may affect the ability to avoid car accidents). The results are summarised in Table 3.3.4. Only dual positivity (tumours positive for p53 and MDM2) was significantly associated with DSS. The plot of this Kaplan Meier curve is displayed in Figure 3.3.4. On their own p53 and MDM2 were not prognostic i.e.

no association was detected with decreased survival (disease specific or overall survival). There are two limitations in the present analyses which must be taken into consideration; firstly some patients have been lost to follow up and are "censored" by the Kaplan Meier method. Censored patients are considered to have the same prospect of survival as those continued to be followed up thus leading to potential bias. Secondly not all patients have been followed up for an equal amount of time. Some tumours were resected in the late 1990's and therefore these patients have had 10 years plus of follow up. A minority of patients have only had three years of follow up. Two patients died of metastatic RCC at around the ten year mark highlighting this point. Late presentation of metastases following nephrectomy is a well recognised phenomenon[158]. Another consideration is that papers which show that p53 expression is associated with poor prognosis tend to have some patients with metastases in their cohort. It may be that as this study's cohort contains no patients with metastases or T4 stage disease, the link between p53 expression and prognosis is not seen

	Disease Specific Survival $p =$	Overall Survival $p =$
p53 +	0.088	0.599
MDM2 +	0.537	0.822
p53 + MDM2+	0.027	0.323

Table 3.3.4 Kaplan-Meier survival analysis for p53 / MDM2 phenotypes

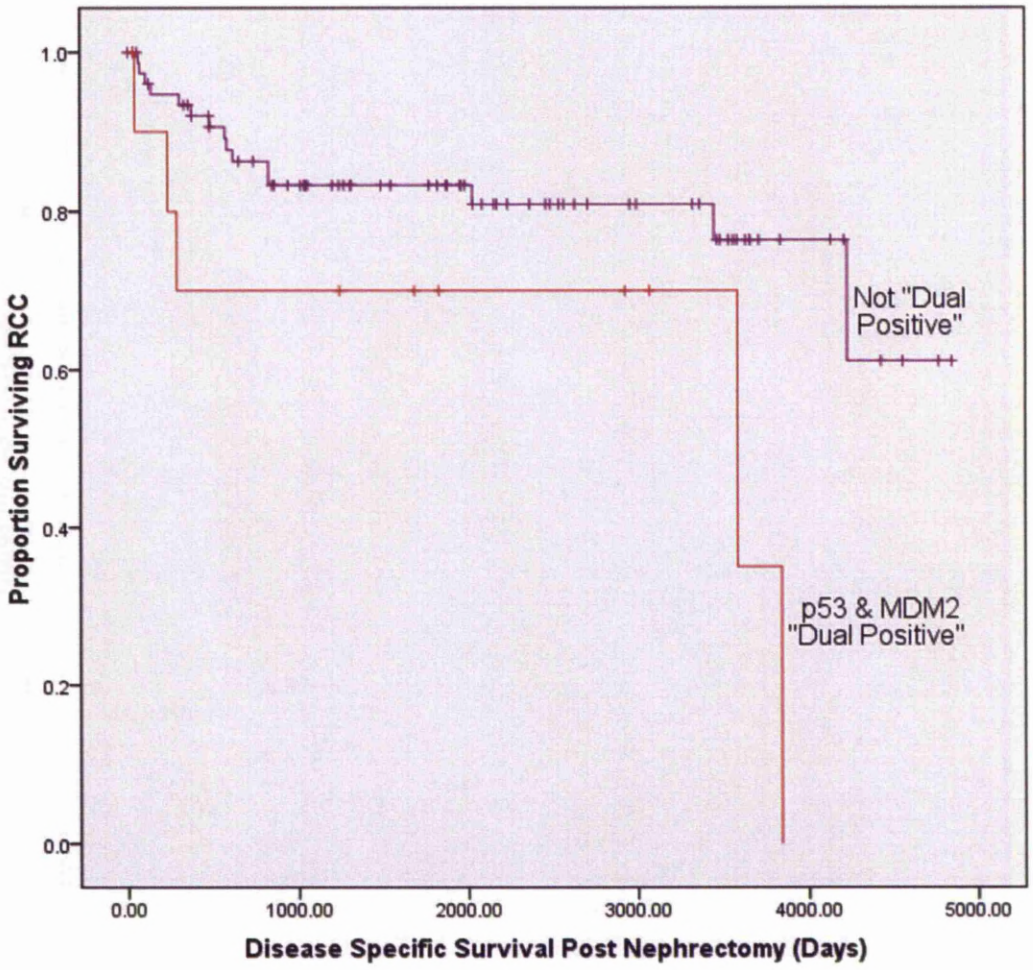


Figure 3.3.4 Kaplan-Meier disease specific survival curve for patients with both p53 and MDM2 positive staining tumors (dual positive) versus other p53 and MDM2 phenotypes (not dual positive) $p = 0.027$ log rank test.

3.3.5 Survival analysis of patient and tumour characteristics

Further Kaplan-Meier analyses were performed to test if survival (both disease specific and overall survival) was affected by various patient or tumour factors (e.g. tumour stage). The results of the log rank tests for the various characteristics analysed are displayed in Table 3.3.5. It is reassuring, given the limitations of the follow-up outlined earlier, to see that higher stage is shown to be associated with both decreased disease specific and overall survival. The Kaplan-Meier curve of stage versus specific survival is seen in Figure 3.3.5.The stage of the tumour is the most widely used clinical guide to prognosis and is found to be retained in all the published prognostic nomograms (see Rouviere et al[158], where this is reviewed). None of the other tumour or patient factors were found to be significantly associated with adverse survival. Just as higher stage showed an association with prognosis, it would have been anticipated that the same would have been found for higher Furhman grades (see section 1.1.3.5). It is possible that with a larger cohort size, as seen in the 643 nephrectomy specimens analysed by Tsui et al[22], this association would have been demonstrated.

	Disease Specific Survival *p* value	Overall Survival *p* value
Stage 3 vs 1 & 2	0.024	0.030
Fuhrman 3 & 4 vs 1 & 2	0.061	0.266
Gender Male v Female	0.288	0.406

Table 3.3.5 Survival analysis of patient and tumour characteristics

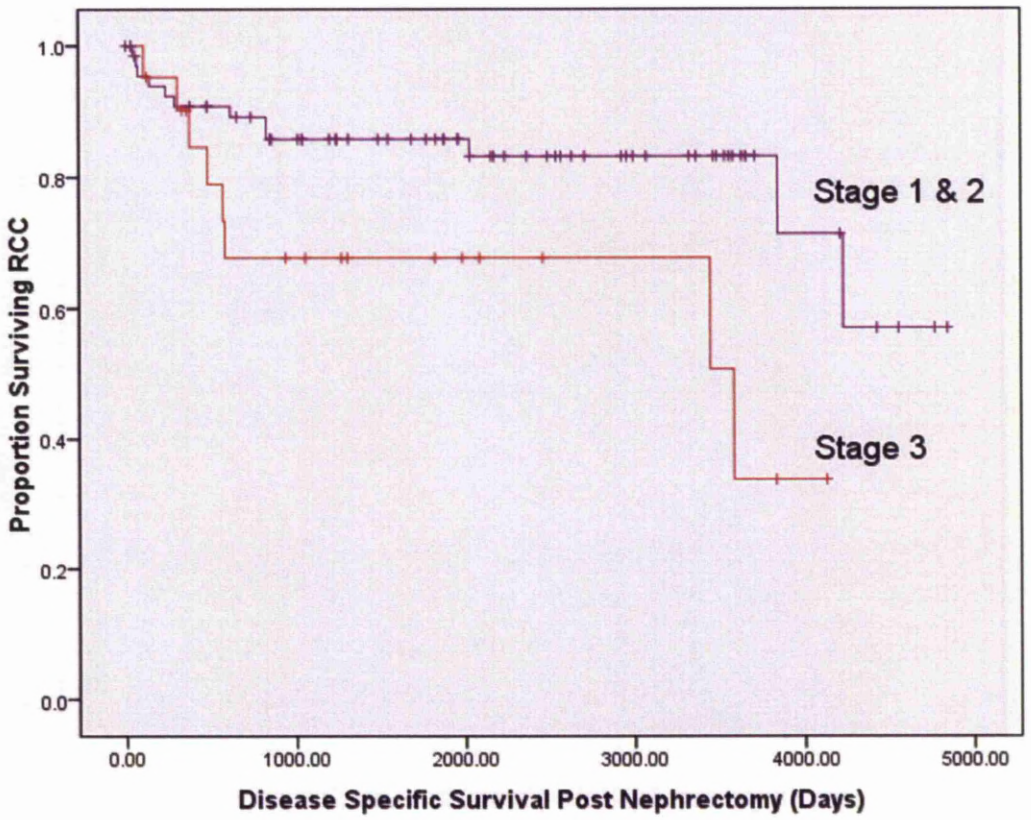


Figure 3.3.5 Kaplan Meier disease RCC specific survival curve for patients with Stage 3 versus Stages 1 & 2 ($p = 0.024$ log rank test)

3.3.6 Univariate and multiple cox proportional hazards regression analysis

Cox proportional hazards regression allows analysis of variables (covariates) to predict time to an event (in this case death). Multivariate analysis takes in to account how important a covariate is at predicting the tested event. For example if some patients are known to have metastatic disease at the time of their nephrectomy it would be expected that they would have a much poorer survival. It would be very hard for other covariates e.g. age / gender to add to the prognostication in the presence of such a strong outcome

predictor. Multivariate analysis statistically examines whether each covariate individually contributes to the prediction of the tested event. Table 3.3.6.1 shows the results of Cox regression analysis of the following tumour and patient characteristics. Only *p* values less than 0.05 were considered significant. For significant univariate factors the relative risks are also shown in table 3.3.6.1.

	All Patients N = 90 Death Events = 20	
Univariate Analysis	Relative risk (95% C.I)	*p* value
p53	N/A	0.088
MDM2	N/A	0.537
p53 + & MDM2 +	2.989 (1.075 - 8.310)	0.036
Stage 3	2.757 (1.103– 6.889)	0.030
Fuhrman grade 3 & 4	N/A	0.161
Gender	N/A	0.288

Table 3.3.6.1 Cox regression analysis of the tumour and patient characteristics

As predicted by the Kaplan-Meier analyses only dual positivity and stage 3 tumours were found to be univariate predictors of disease specific mortality. Both of these factors were then modelled using multivariate analysis and the results of this are displayed in Table 3.3.6.2.

Multiple Analysis	All Patients N = 90 Death Events = 20	
	Relative Risk (95% C.I)	*p* value
p53 + MDM2 +	3.203 (1.143 - 8.980)	0.027
Stage 3	2.897 (1.155 – 7.270)	0.023

Table 3.3.6.2 Multivariate cox regression analysis of dual positive and stage 3 tumours

Both of these factors were retained in the model. The relative risk demonstrates that patients with dual positive tumours are on average 3.2 times more likely to die of their disease than other p53/MDM2 phenotypes. As expected patients with stage 3 disease are on average 2.9 times more likely to die than lower stage patients. Only Haitel et al[96] have included this dual positive phenotype in multivariate cox regression analysis. They also found it to be retained as an independent prognostic factor even when metastases were included in the model with a hazard ratio of 2.34 (C.I not given)

3.3.7 Assesment of p53 positive tumour samples for wild type or mutated p53

A Boyd laboratory co worker (Dr R Polanski) has analysed selected tumour samples from the TMA and performed a FASAY assay to assess the p53 status (wild type or mutant). A description of the methodology can be read in Noon et al[159] (see appendix A2.3.2). This analysis has revealed that 9 of the 11 tumour samples that were positive by IHC for p53 and MDM2 contained wild type p53.

Specimen	p53 IHC Positive	MDM2 IHC Positive	p53 status by FASAY/DNA sequence analysis	Died of RCC	Died
1	YES	YES	Wild-type[1]	NO	NO
2	YES	YES	Wild-type[1]	NO	NO
3	YES	YES	Wild-type[1]	NO	NO
4	YES	YES	Wild-type[1]	NO	NO
5	YES	YES	Mutant/G245C[1]	YES	YES
6	YES	YES	Wild-type[1]	NO	NO
7	YES	YES	Wild-type[1]	YES	YES
8	YES	YES	Wild-type[1]	YES	YES
9	YES	YES	Mutant/Q136E[1]	NO	NO
10	YES	YES	Wild-type[1]	YES	YES
11	YES	YES	Wild-type[1]	YES	YES
12	NO	YES	ND	NO	NO
13	NO	YES	ND	NO	NO
14	NO	YES	ND	YES	YES
15	NO	YES	ND	YES	YES
16	NO	YES	ND	NO	NO
17	NO	YES	Wild-type[1]	NO	NO
18	NO	YES	ND	NO	NO
19	NO	YES	ND	NO	YES
20	NO	YES	ND	NO	YES
21	NO	YES	Wild-type[1]	NO	YES
22	NO	YES	ND	NO	NO
23	NO	YES	Wild-type[1]	NO	NO
24	NO	YES	ND	NO	NO
25	YES	NO	Wild-type[1]	NO	NO
26	YES	NO	Wild-type[1]	NO	NO
27	YES	NO	Wild-type[1]	NO	NO
28	NO	NO	Wild-type[1]	NO	NO

Table 3.3.7 Analysis of p53 status in selected tumour samples using FASAY / p53 genotyping. IHC, immunohistochemistry, FASAY, functional assay of separated alleles in yeast, ND = not done.[1] Sequence confirmed for at least three clones or by direct sequencing of PCR products. Sequences were compared to the *Homo sapiens*

chromosome 17 contig NT_010718.15, positions 7189581-7169068 bp, using Sequencher v 4.9 software (Gene Codes Corporation).

This finding is contrary to the popular theory that tumours express p53 as a result of p53 mutation. The underlying mechanism as to why tumours express high level (unmutated) p53 and MDM2 is unknown but intriguing and raises the same questions posed by RCC cell lines that also express high p53 and MDM2 - what mechanism exists to prevent p53 from being degraded by MDM2 which appears to be expressed in high amounts. Secondly if MDM2 is being expressed is this p53 driven? If p53 is transcriptionally active then why are its normal anti-oncogenic functions being bypassed?

3.4 Discussion

There has been a substantial body of published work demonstrating that p53 is associated with poor prognosis in RCC. However, the present study has not been able to confirm this. This difference may be due to a number of factors, not least of which is that many studies including the present one, are relatively small with n<500. Other factors that differ between the present study and those in the literature include the inclusion in the latter of patients with metastases in their study cohort, the incomplete follow-up for some patients in the present study as well as differences in interpretation/dichotomisation of p53 positivity/negativity. However the finding that tumours dual positive for p53 and MDM2 are associated with poor prognosis is in agreement with the only other study that has looked at both of these markers in RCC (Haitel et al[96]). Also in our study there is a highly significant association between p53

and MDM2 positivity again confirming previous studies by Haitel et al[96] and Moch et al[105]. The finding that in the majority of cases (9/11 see Table 3.3.7) p53 is wild type and not mutated (as in a number of RCC cell lines see 1.7.1) is novel and further supports our hypothesis that understanding the mechanism of this phenotype may allow for therapeutic intervention if wild type p53 function can be restored. The underlying mechanism of this dual expression is investigated further in this thesis. If dual p53/MDM2 positivity is a true marker of poor prognosis then it can only be speculated as to what the mechanisms leading to this association between the phenotype and outcome are. It is possible that the expression of this phenotype is coincidental and a mere marker of extreme de-differentiation from normal tissue, but given these proteins are known to be important in the prevention of and causing oncogenesis, this seems unlikely.

CHAPTER 4 – INVESTIGATING THE EFFECTS OF HIGH MDM2 EXPRESSION IN RCC CELL LINES

4.1. Introduction

As shown in the previous chapter there is evidence that RCCs that express MDM2 and p53 are associated with decreased disease specific survival compared with RCCs that do not have this phenotype. As described in section (1.3.4) MDM2 is thought to possess oncogenic properties in its own right (independent of p53) but it is not known whether MDM2 expression is directly responsible for the poor prognosis seen in patients with tumours that express p53 and MDM2. To try and answer this question a laboratory co-worker (HW) attempted, through cloning experiments, to manipulate a RCC cell line with low p53 and MDM2 (UOK-117 herein referred to as 117) to express high p53 and MDM2 (the putative poor prognosis phenotype in RCC tumours). These experiments are detailed in section (1.6). It was found to be impossible to obtain stable MDM2 expressing clones from simply transfecting MDM2 into cells and performing clonal selection. MDM2 clones could be generated after the cell line had undergone some unknown permissive change during other clonal experiments (please refer to section 1.6. where this is explained in more detail). Two 117-derived clones were established from which MDM2 expressing clones could be generated through further cloning (pCEP4 Clone 3 and ΔNp53 clone 3). These two clones are herein referred to as pCEP3 and ΔNp53and are thought to have acquired unknown molecular changes that make them permissive for high MDM2 expression.

The experiments detailed in this chapter were designed to investigate the effects of high MDM2 expression in the 117 parental cell line and the two derived clones that are permissive to high MDM2 expression pCEP3 and ΔNp53. It was hoped to make these cells express high levels of MDM2 and use a DNA microarray to evaluate changes in gene expression. By comparing the differences in gene expression between the three different cell lines and their normal MDM2 expression controls (i.e. without high MDM2 expression), groups of genes may be identified for further evaluation that would enable identification of pathways responsible for the more aggressive phenotype which we hypothesise is elicited by MDM2 expression.

There were two strategies selected as possible means for achieving high MDM2 expression in these 117 cell lines –one was to employ a commercially available transient transfection technique. Another method was to try and generate inducible (expression of gene interest after cells are treated e.g. with an antibiotic) MDM2 expressing clones using the Tet-On® Advanced inducible gene expression system (ClonTech™). In the Tet-On® Advanced system the gene of interest (GOI) expression (MDM2 in this case) is induced upon the addition of doxycycline (DOX) to the culture medium. There were pros and cons to both strategies. With transient transfection the major concern was to ensure a sufficient percentage (transfection efficiency) of cells were reliably transfected with MDM2 to enable the detection of alterations in gene expression when MDM2 was expressed. This is particularly important for detection of decreases in gene expression. Another potential problem was the detection of altered gene expression secondary to the effects (toxicity) of the transient transfection process. The major drawback of the

inducible gene system was the amount of time taken to perform this protocol compared to transient transfection. Although, if successful, a suitable MDM2 inducible clonal cell line, expressing MDM2, should allow detection of altered gene expression as a consequence of MDM2 expression. There were concerns that the further cloning needed to generate inducible MDM2 expression clones, may lead to unknown genetic alterations which would prejudice the results of gene analysis. There was also a risk of a small amount of the GOI (MDM2) still being expressed in the absence of the inducing agent (DOX). Small levels of additional MDM2 expression may be sufficient to lead to changes in the expression of other genes, obviously again affecting interpretation of the gene expression profiling. In the first instance transient transfection was employed to obtain high MDM2 expression, as discussed earlier.

4.2 Results of Transient Transfection

4.2.1 Transient Transfection of 117 and derived clones

The 117 cell line was transiently transfected with four commercially available transfection reagents - GeneJuice™ (Novagen), FuGENE HD™ (Roche), Lipofectamine™ 2000 (Invitrogen) and Magnetofection™ (Polymag) to investigate which product resulted in the highest transfection efficiency. Assessment of transfection efficiency was made using an in situ β-galactosidase (β-gal) assay. Details of these experiments can be found in Appendix (1). It was found that FuGENE HD™ at a

reagent volume (µl) to transfected DNA weight (µg) ratio of 2:1 resulted in the highest transfection efficiency of 40 - 45% (see Figure 4.2.1).

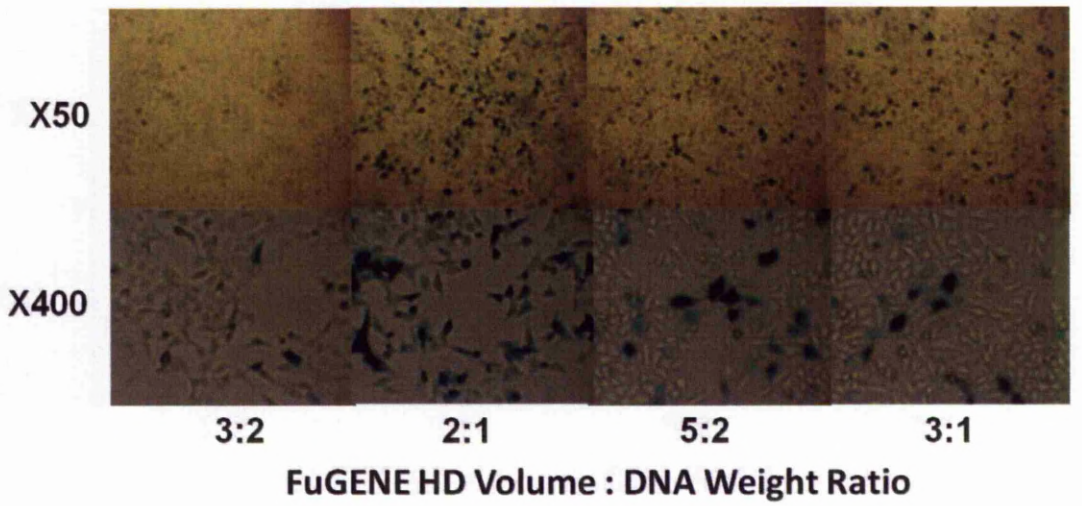


Figure 4.2.1. Estimation of the optimum FuGENE HD™ volume to plasmid DNA weight ratio for transfection of the 117 cell line using an in-situ β-gal assay. 117 cells were transfected with 5µg of β-gal plasmid and 5µg of pCEP4 plasmid using FuGene HD™ transfection reagent, at transfection reagent volume to DNA plasmid weight ratios as indicated. Cells were incubated for 24 hours before performing the in situ β-gal assay. Cells were covered with substrate buffer for 24 hours before a representative photograph was taken at 50x and 400x magnification as indicated.

4.2.2 Magnetic labelling and separation using the MACSelect™ system (Miltenyi Biotech).

The results in section 4.2.1 demonstrated that the transient transfection methods tested could only achieve 40 - 50% transfection efficiency. Although this transfection efficiency may be sufficient to detect large fold changes in gene expression by expression profiling, there is a concern that smaller fold increases may not be detected.

To try and increase the transfection efficiency a magnetic transfection enrichment technique (MACSelect™) was investigated. The MACSelect™ system requires cells to be co-transfected with the plasmid of interest and a plasmid (MACSelect K^K) responsible for the expression of a cell surface molecule (antigen) that can bind a specific antibody coupled to a magnetic particle. The transfected cells are then mixed with magnetic particles and passed through a magnetic column. This causes separation of the cell suspension into a magnetic and non magnetic fraction. The magnetic fraction is then eluted from the column and as a result of this enrichment process should contain a higher percentage of cells expressing the plasmid of interest. This is because cells successfully transfected with the MACSelect K^K plasmid are more likely to have been co-transfected with the plasmid of interest.

The MACSelect™ system was evaluated using the 117 cell line and FuGENE HD™ transfection reagent which had previously given the highest transfection efficiency. Cells were transfected with pCMVNeoBamMDM2 and the MACSelect K^K plasmid. A control group of cells were transfected with pCMVNeoBamMDM2 and pCMVNeoBam. The enrichment of MDM2 was assessed using western blot analysis. The results are shown in figure 4.2.2. As can be seen in figure 4.2.2, in the positive column fraction (cells retained on the magnetic column) there was a considerably higher expression of MDM2, compared to the negative fraction and the MDM2 transfected control. The results show that the MACSelect™ system can be used to enrich the transfected cell population but the resultant transfection efficiency still needs to be determined to ensure it is superior to existing methods tested.

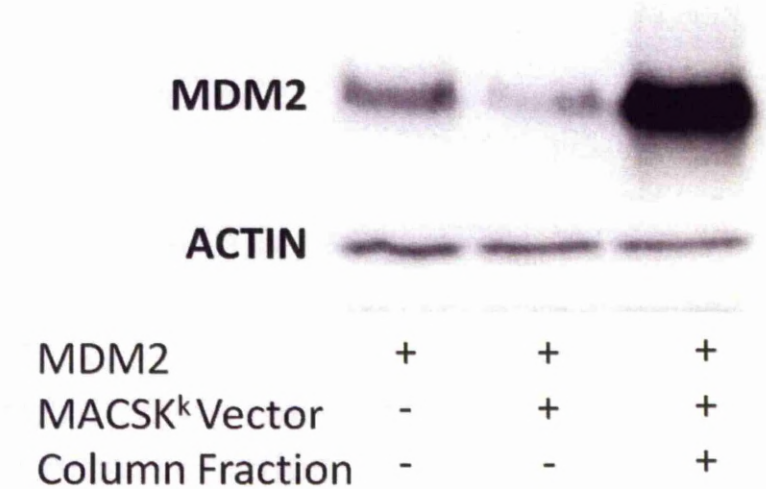


Figure 4.2.2.1 Western blot analysis of 117 cells transfected and selected using the MACSelect™ system. The 117 cell line was transfected with 10μg of MDM2 expression plasmid pCMVNeoBamMDM2 and 10 μg of the MACSelect K^K plasmid. As a control 117 cells were also transfected with 10μg of pCMVNeoBamMDM2 and 10μg of pCMVNeoBam instead of the MACSelect K^K. 24 hours after transfection cells were passed through the MAC Separation column. There was no sample of cells retained on the column (+ column fraction in figure) for 117 cells not transfected with the MACSelect K^K plasmid. Three samples were analysed by western blotting, for MDM2 levels. Actin was probed for as a loading control.

To see if this method would provide greater transfection efficiency than previous methods tested, selected cells had to be reseeded after the column separation and a β-gal assay performed. This process required cells to be out of their normal culture environment for approximately 30 minutes which could potentially "stress" the cells and

risk altering the p53/MDM2 levels. For each 117 line (117, pCEP3 and ΔNp53), cells were transfected as in 4.2.2 except that pβ-gal replaced the pCMVNeoBamMDM2. The cells transfected with the MACSelect k^K plasmid were harvested and the cell suspension transferred to the magnetic column. Cells that passed through the column ("non-magnetic") were collected and designated as the negative fraction. The column was washed with elution buffer to collect cells held in the magnetic field of the column; these were designated as the positive fraction (these are cells transfected with MACSelect k^K plasmid). The control cells (not transfected with the MACSelect k^K plasmid were treated in the same manner, however very few cells were recovered in the positive fraction as expected. The cells were then resuspended in media and seeded into 10cm dishes. 12 hours later, a β-gal assay was performed. Figure 4.2.2.2 shows the three cell lines and representative photographs for each condition. Marked cell death was observed and the viable cells available to be assessed only demonstrated a transfection efficiency of 10 – 20%. This result suggests that there is a significant reduction in cell viability as a result of this process which appears to particularly affect the transfected cells and therefore we did not pursue this approach further. It was therefore decided to try the second approach, namely to generate MDM2 inducible clones.

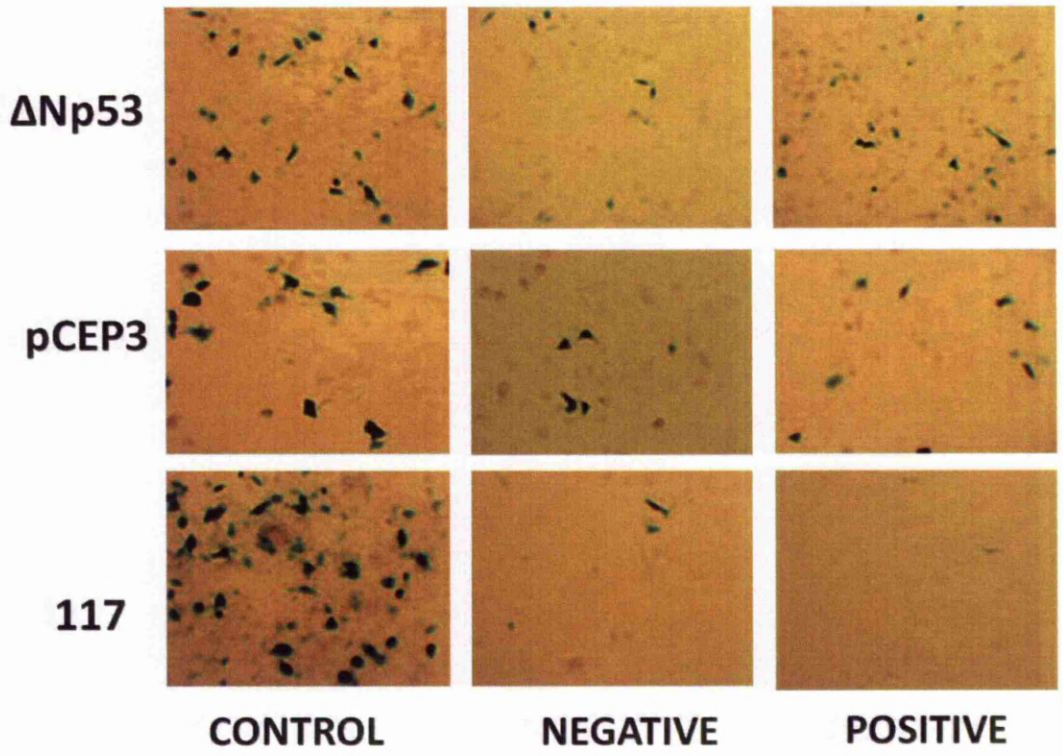


Figure 4.2.2.2 Transfection efficiency for 117 cell lines following MACSelect™ enrichment. Cells were transfected as in 4.2.1 with 10μg of β-galactosidase plasmid instead of pCMVNeoBamMDM2. Transfected cells were harvested and transferred to the magnetic column. Cells transfected with MACSELECT k^K plasmid were separated by the column into a negative fraction for cells passing through the column and a positive fraction for those cells attracted to the magnetic field of the column. The control cells were not transfected with MACSELECT k^K . The cells were then resuspended in media and seeded into 10cm dishes. 12 hours later, a β-gal assay was performed. Cells were left covered with substrate buffer for 24 hours before photographs were taken at 400x magnification.

4.3 Generating inducible expressing clones

4.3.1 Overview of the Tet-On Advanced system inducible cloning system

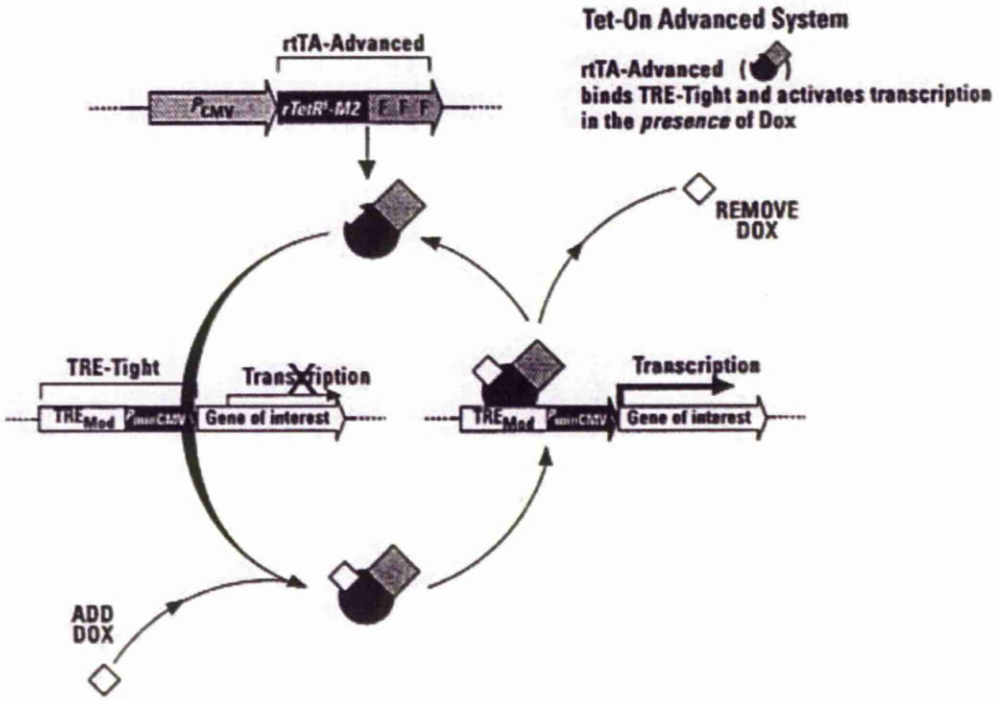


Figure 4.3.1.1 The Tet-On Advanced system schematic - adapted from the Tet-On® Advanced user manual (ClonTech™, Mountain View, CA, USA). DOX = Doxycycline, TRE = Tetracycline response element.

In this system, induction of gene expression is controlled by a "mutant" reverse Tet repressor[160]. In *E.Coli* the Tet repressor protein (TetR) negatively regulates the genes of the tetracycline-resistance operon. TetR blocks transcription of these genes by binding to the *tet* operator sequences (*tet*O) in the absence of tetracycline. The mutant reverse Tet repressor (rTetRs) bind to tetO in the presence of DOX. The second component of this

system is the Tetracycline –response element (TRE) containing seven direct repeats of a 42bp sequence containing *tetO*, located just upstream of a minimal CMV promoter (P_{minCMV}). The rTetR DNA binding domain has been fused to minimal herpes simplex virus (HSV) VP16 protein. Therefore rtTA-A activates transcription of the GOI upon addition of DOX. The GOI is controlled by the TRE-Tight control system. Welman et al[160] have developed a modification of the pTet-on plasmid in order to try and combat problems associated with epigenetic silencing of the cassette containing the transcriptional transactivator gene. This newly developed pN1pβactin-rtTA2S-M2-IRES-EGFP plasmid, combines the strong and less 'silencing-prone' chicken β-actin promoter with the advantages of an IRES (internal ribosome entry site)-based selection principle and an EGFP (red shifted variant of wild type green fluorescent protein (GFP) optimized for better fluorescence and expression in mammalian cells) for selecting clones. These authors have shown that using this vector leads to a higher number of positive clones and increased inducibility[160].

The generation of Tet-On inducible clones is a multiple step procedure illustrated in figure (4.3.2). Firstly the concentration of selection antibiotic (lowest concentration required to kill all cells not transfected with antibiotic resistance gene) G418 has to be determined (antibiotic sensitivity assay). The cell lines are then transfected with the N1pβactin-rtTA2S-M2-IRES-EGFP plasmid. G418 resistant colonies are then selected and cultured. The selected colonies are then screened by transiently transfecting them with pTRE-Tight-Luciferase and selecting clones with maximum expression of luciferase on addition of DOX and minimal expression in its absence. Following this, the

optimum concentration of hygromycin for selection) was determined. Selected clones then underwent transfection with pTRE-Tight plasmid containing the GOI. Hygromycin resistant clones were screened for GOI expression in response to DOX.

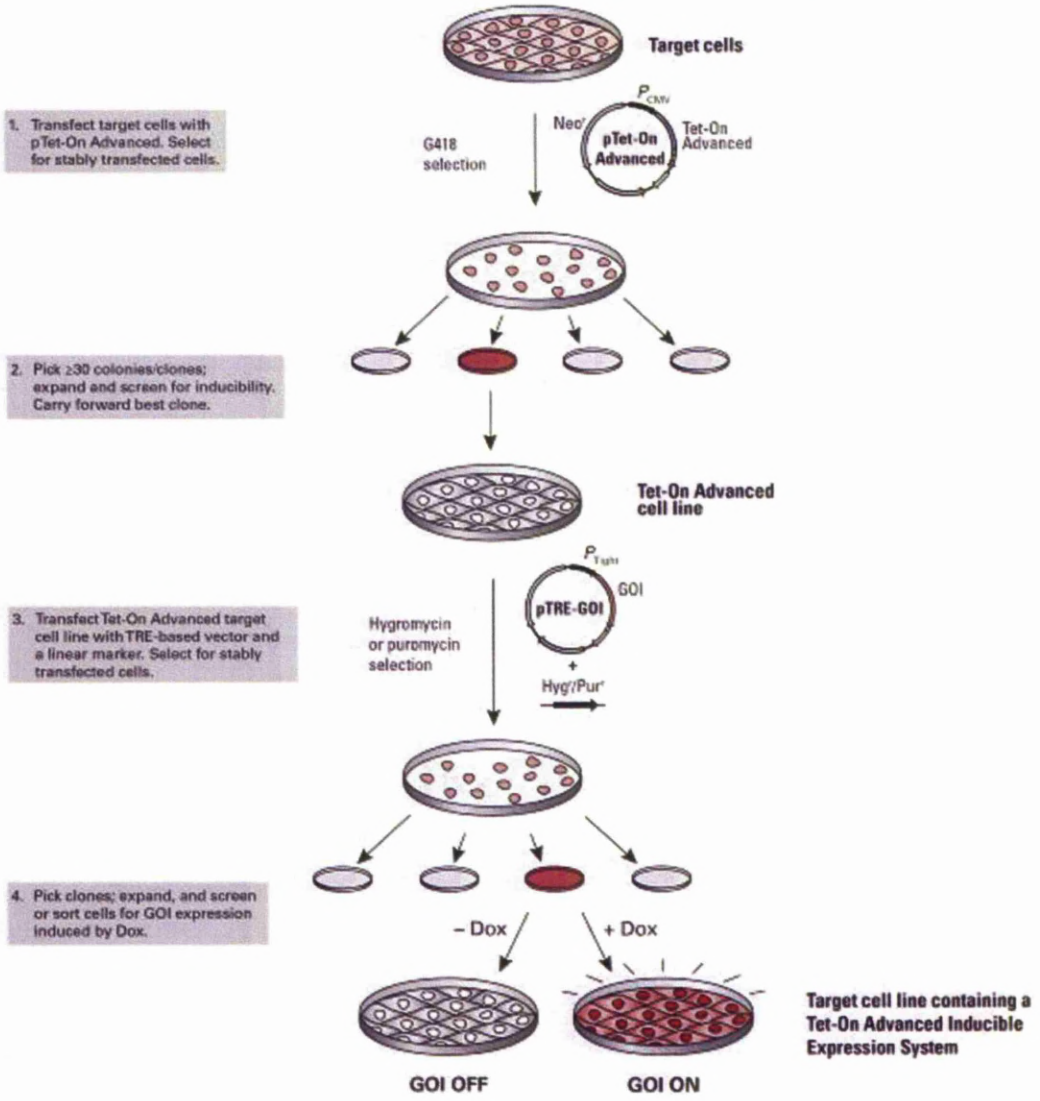


Figure 4.3.1.2 Schematic for the generation of DOX inducible MDM2 expressing clones. GOI = Gene of interest, Hyg = Hygromycin. Adapted from the Tet-On® Advanced user manual (ClonTech™, Mountain View, CA, USA)

4.3.2 Determination of G418 concentration for 1st round clonal selection – G418 antibiotic sensitivity assay

G418 is an aminoglycoside antibiotic that selects for mammalian cells expressing neomycin phosphotransferase, from the neo gene (contained within the pN1pβactin-rtTA2S-M2-IRES-EGFP vector). A dose of 800 μg/ml was found to cause cell death in all three cell lines after 5 days and therefore we selected this dose for selection of transfected cells (see table A1.1.7 in Appendix 1).

4.3.3 Generation and selection of rtTA2S-M2 expressing clones

Each cell line was transfected with N1pβactin-rtTA2S-M2-IRES-EGFP and underwent antibiotic selection, as described in section 2.7.2. After 7 days none of the control transfected cells (no neo gene transfected) were viable and after 11 days for 117 cells and 13 days for 117-derived clones, colonies were of sufficient size to be expanded. Before proceeding to colony expansion, one 117 colony was harvested and the cell suspension subjected to ultra-violet light to evaluate whether the cells fluoresced as a consequence of EGFP expression. EGFP expression would signify that the cells had been successfully transfected as planned. As can be seen in figure 4.3.2.2 a high percentage of cells were found to express EGFP.

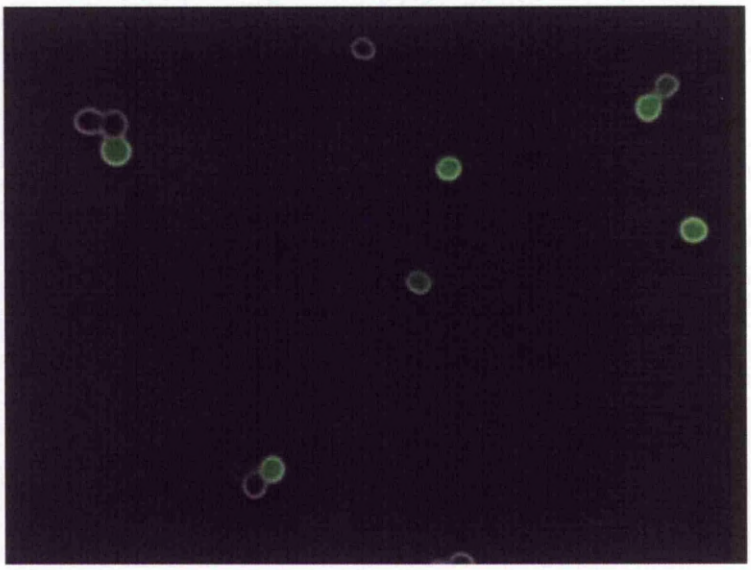


Figure 4.3.3.1 Photograph under ultraviolet light of UOK 117 cells transfected with N1pβactin-rtTA2S-M2-IRES-EGFP. Cells that have been transfected fluoresce green.

Clones were then tested to ensure they contained a functional DOX responsive element (rtTA see section 4.3.1). Testing was performed by transiently transfecting candidate clones with pTRE-Tight-Luciferase and then incubating them in media containing DOX. Clones containing a functional rtTA would express luciferase (when exposed to DOX), the activity of which was assayed using a luminometer (section 2.7.5). The "ideal clone" would have at least a 20-fold induction of luciferase and the lowest background expression of luciferase in the absence of DOX treatment. Clones of cells that met these criteria were subjected to western blot analysis to compare their expression of p53 and MDM2 to that of the original 117, ΔNp53 & pCEP3 parental cell lines. The results of these experiments are shown in figures 4.3.3.1 to 4.3.3.3 below. There is a degree of variability in the quality of the blot panels arising as a consequence of different clones

being tested at different times as they grew at different rates. As a result protein extraction was undertaken using different stocks of SLIP buffer and protein was electrophoresed on SDS gels made at different times. The presence of a p53 doublet can be seen in figures 4.3.3.2 and 4.3.3.3, this is thought to arise due to a *p53* polymorphism at codon72 giving distinct alleles encoding both proline and arginine[161]. From these results the following clones were selected for second round cloning 117 – clone 9, 117 ΔNp53 – clone 2 and 117 pCEP4 – clone 9 (indicated in figures 4.3.3.1 to 4.3.3.3 with a black arrow). An interesting observation was that a number of DOX-inducible clones were found not to express EGFP probed for using an anti GFP antibody in the western blots. This suggests that in these cell lines only the neo and rtTA gene sequences have been incorporated into the cell lines' DNA and are transcriptionally active. Selection of clones only on the basis of EGFP expression would have led to identification of cells that may have lacked the key feature of DOX inducibility and even if the pTre-Tight-MDM2 was then cloned into these cells, MDM2 would not be induced.

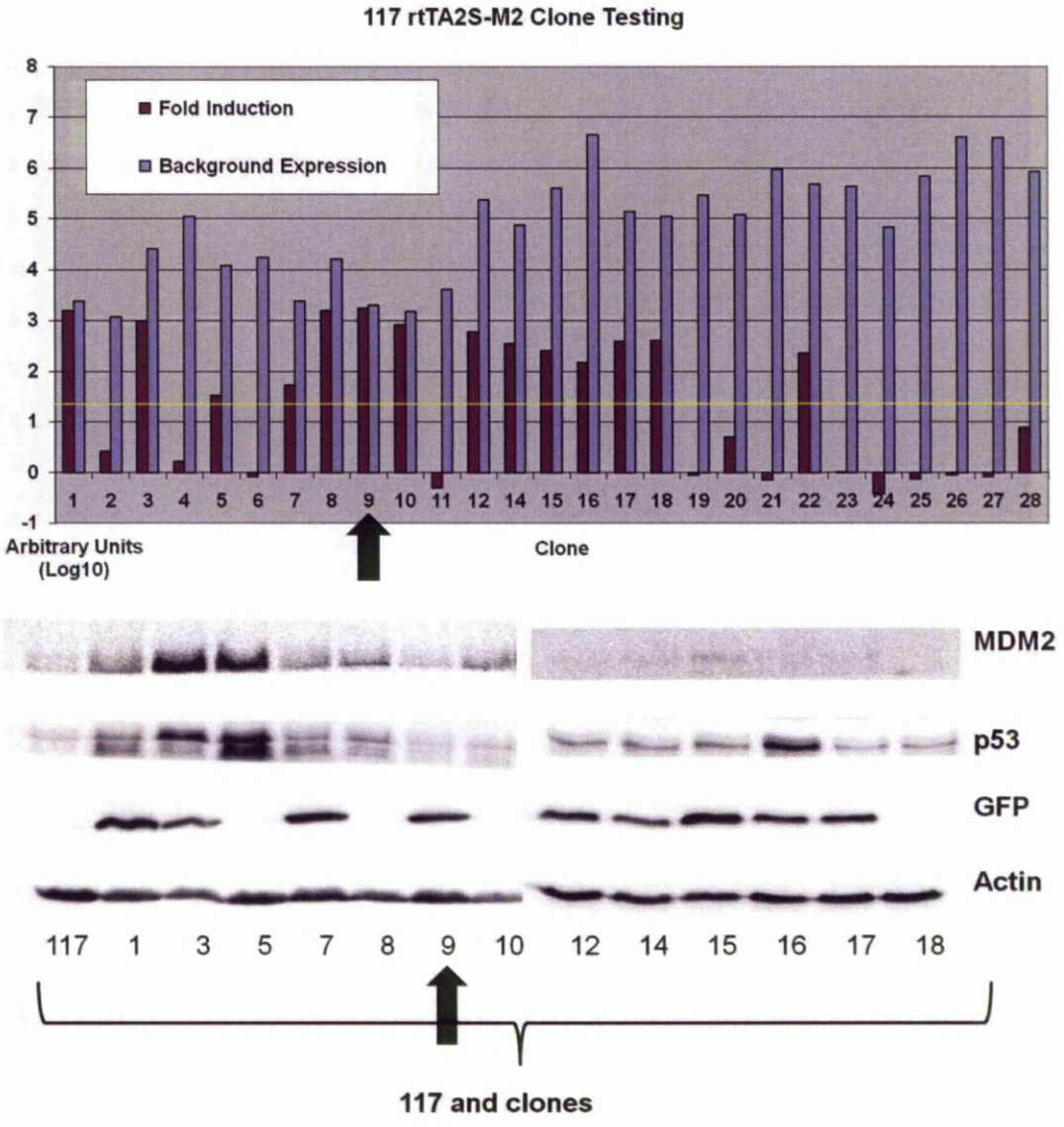


Figure 4.3.3.2 Analysis of 117 rtTA2S-M2 clones for DOX dependent induction of expression of luciferase. Top, graph showing the logarithmic fold induction upon DOX treatment (calculated by dividing the induced relative light units by the background relative light units) for each clone. Also shown is the background expression expressed logarithmically. The yellow line indicates 20-fold induction. **Bottom**, western blot analysis of selected 117 rtTA2S-M2 clones and the parental 117 cell line proteins probed for as indicated in figure.

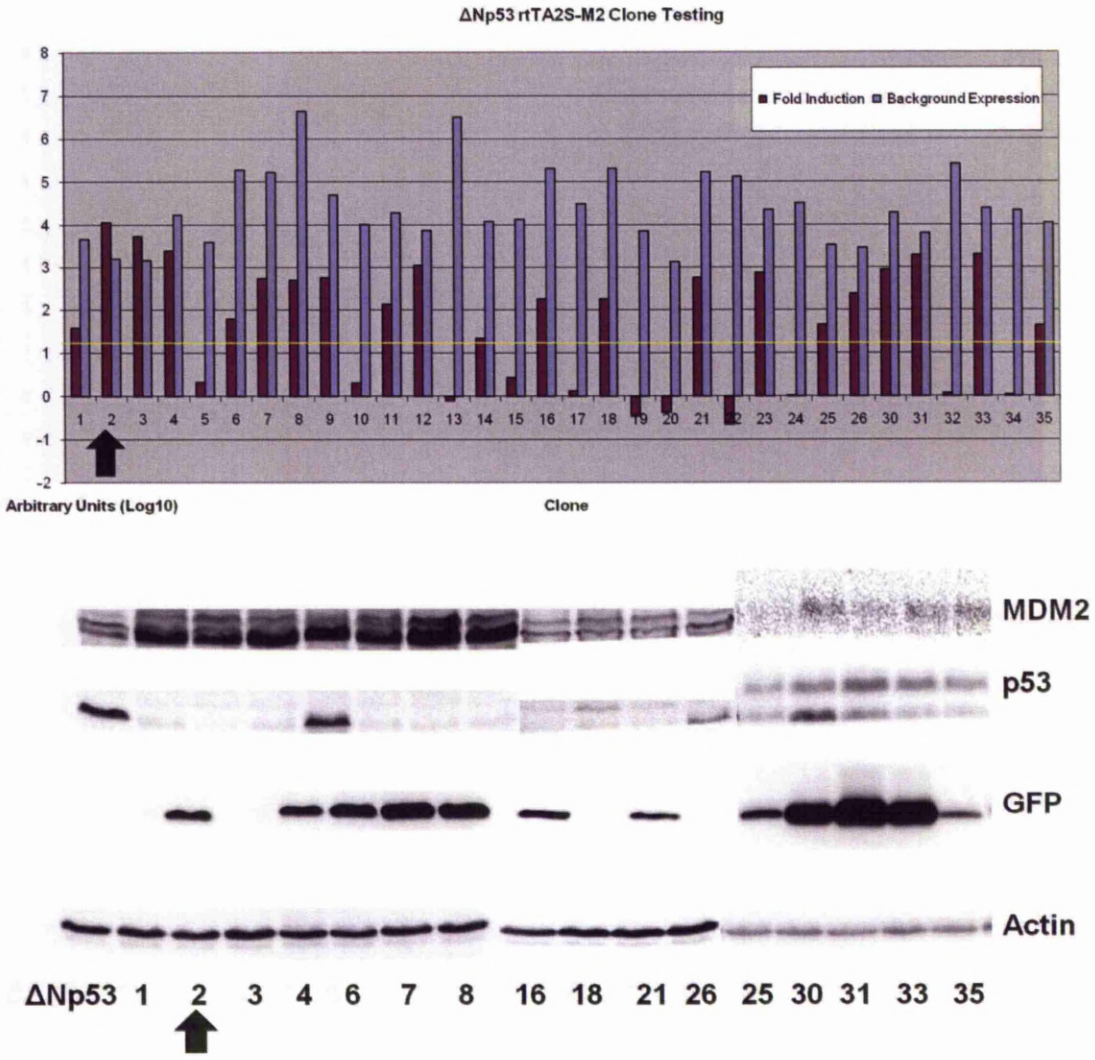


Figure 4.3.3.3 Testing of 117 ΔNp53 rtTA2S-M2 clones for DOX-dependent induction of expression of luciferase. Top panel Graph showing the logarithmic fold induction upon DOX treatment (calculated by dividing the induced relative light units by the background relative light units) for each clone. Also shown is the background expression expressed logarithmically. The yellow line indicates 20-fold induction. **Bottom panel** western blot analysis of selected 117 ΔNp53 rtTA2S-M2 clones and the parental 117 ΔNp53 cell line. Proteins probed for as indicated in figure.

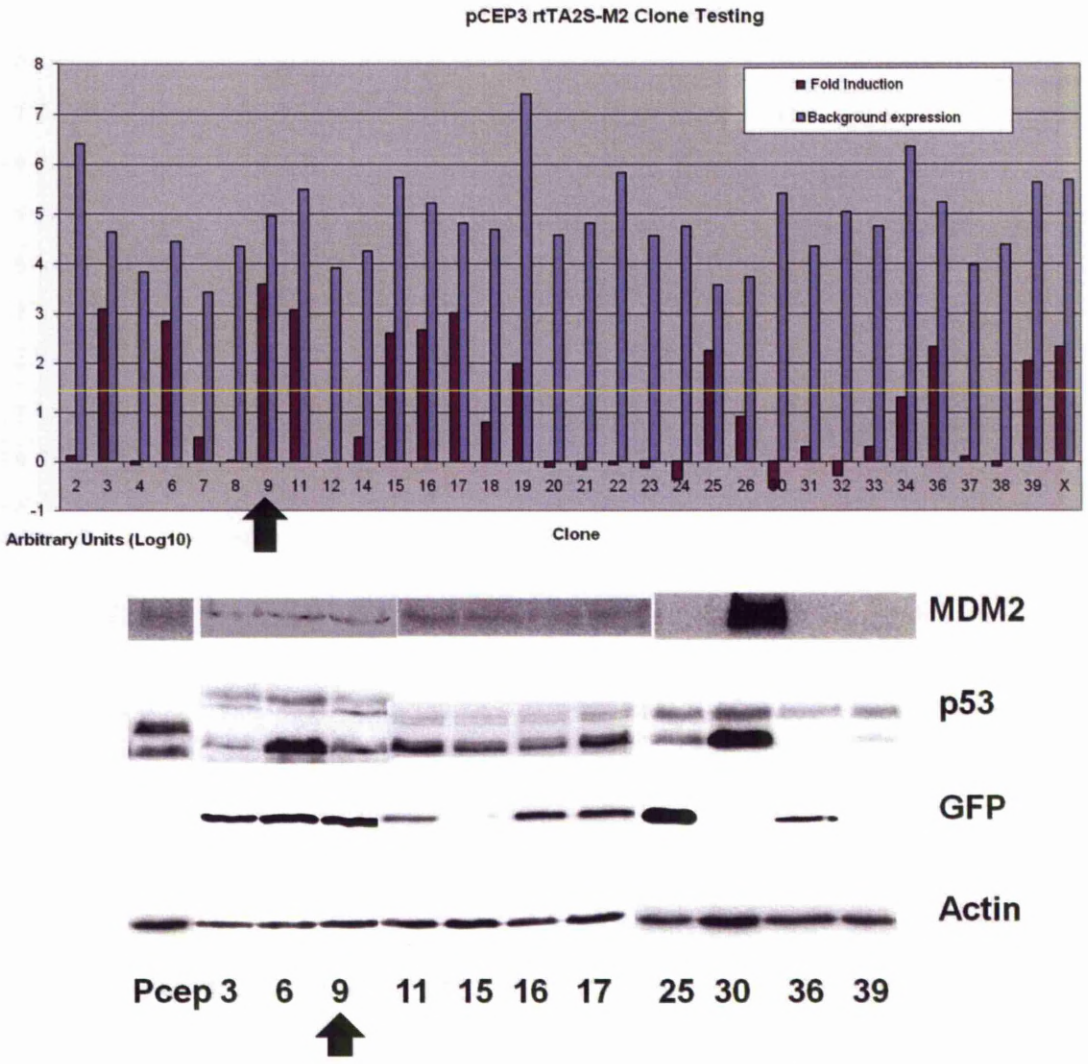


Figure 4.3.3.4 Analysis of 117 pCEP 4 clone 3 rtTA2S-M2 clones for DOX dependent induction of expression of luciferase. **Top panel** Graph showing the logarithmic fold induction upon DOX treatment (calculated by dividing the induced relative light units by the background relative light units) for each clone. Also shown is the background expression expressed logarithmically. The yellow line indicates 20-fold induction. **Bottom panel** western blot analysis of selected 117 pCEP 3 rtTA2S-M2 clones and the parental 117 pCEP3 (pCEP in figure) cell line. Proteins probed for as indicated in figure.

4.3.4 Generation of pTRE-Tight-MDM2 plasmid

The pTRE-Tight vector was obtained from ClonTech™. Human *MDM2* cDNA was obtained from pCMVNeoBamMDM2 and cloned as follows. pCMVNeoBamMDM2 was digested with BamH1 restriction enzyme and the MDM2 fragment was then sub-cloned, digested and phosphatased. Two clones were selected for further restriction enzyme digest to determine whether the ligation reaction produced the anticipated recombinant DNA. As can be seen from figure 4.2.4 below, both clones display the correct pattern anticipated for pTre-Tight-MDM2.

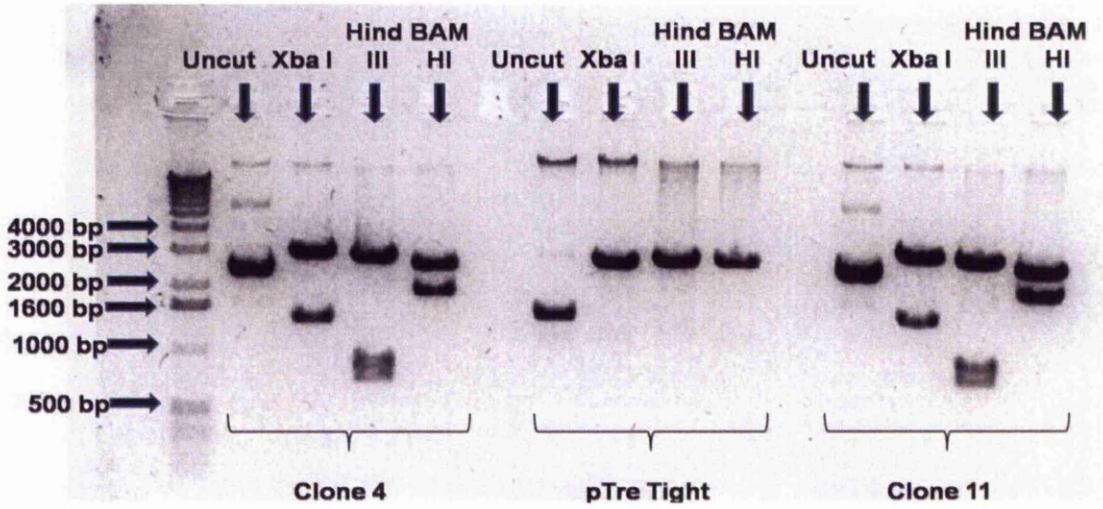


Figure 4.3.4 Agarose gel electrophoresis of a test restriction enzyme digest of candidate pTRE-Tight-MDM2 clones 4 & 11. 1.5 µg of plasmid DNA was digested with the indicated restriction enzymes. MDM2 cloned in the correct orientation into the MCS of the pTRE-Tight plasmid would give a plasmid 4469 bp in length. Restriction enzyme digests of this plasmid using XbaI would give fragments of 18, 1387 and 3064

(incorrect 18, 462 and 3989); for HindIII: 750, 884 and 2835 (incorrect 751, 884 and 2834) and for BamHI: 1869 & 2600 (incorrect 1869 & 2600). Both clone 4 and clone 11 plasmids match this indicating successful cloning of pTre-Tight-MDM2.

4.3.5 Testing the newly generated pTre-Tight-MDM2 plasmid's function

Before proceeding to perform the second stage of the cloning procedure, it was important to check that the newly generated pTre-Tight-MDM2 plasmid expressed functional MDM2 in the presence of rtTA2S-M2 and DOX. A p53 luciferase reporter assay was used to determine if the MDM2 was functional (it is expected that there would be lower p53 levels in response to increased MDM2 levels leading to a reduction in luciferase reporter activity). The 117 rTTA2S-M2 stably expressing clone 9 (see 4.3.3.1) was selected for this experiment. Cells were exposed to DOX for 24 hours and the p53 – luciferase reporter assay was used to determine the activity of the induced *MDM2*. As can be seen in figure 4.3.5.2 for both concentrations of transfected pTre-Tight-MDM2, the addition of DOX resulted in a significant reduction of p53 reporter signal (3µg/µl p = 0.0076 & 10µg/µl p = 0.0287 using a paired t-test). This is because MDM2 causes proteasomal degradation of p53 therefore reducing the amount of p53 available for transcription. The reduction in p53 signal is not as great compared to pCMVNeoBamMDM2; this may be explained by the fact that the cells in the latter are exposed to MDM2 for longer as no induction is required. The western blot (figure 4.3.5.2) shows that DOX treatment results in increased MDM2 signal and a reduction in p53 signal.

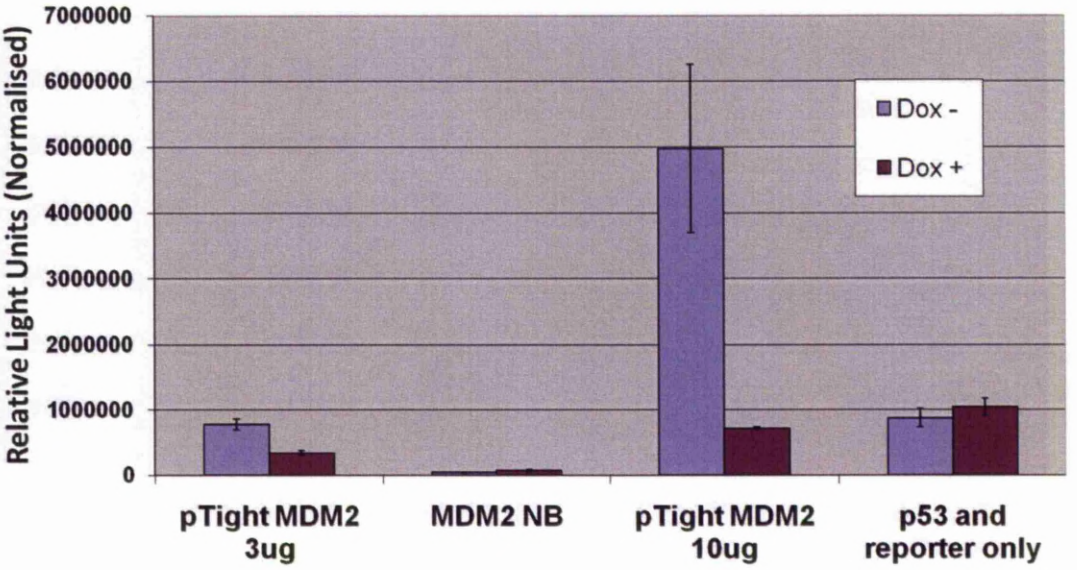


Figure 4.3.5.1 p53 luciferase reporter assay of pTre-Tight-MDM2 induction with DOX in 117 rtTA2S-M2 stably expressing clone 9 cells. 117 rtTA2S-M2 stably expressing clone 9 cells were transiently transfected with a p53 expression plasmid (pCEPp53), a p53 luciferase reporter construct (pp53-TA-Luc) and β-Gal as a transfection control. There were four test conditions as follows: transfection of pTre-Tight-MDM2 3μg/μl (labelled pTightMDM2 3μg in figure), transfection of pCMVNeoBamMDM2 1.2μg/μl (labelled MDM2 NB in figure), transfection of pTre-Tight-MDM2 10μg/μl (labelled pTightMDM2 10μg in figure) and a control (No MDM2 transfected, labelled p53 and reporter only in figure). Cells were incubated for 24 hours and then for each condition half were incubated with fresh media containing DOX at a concentration of 1μg/ml and the other half incubated with fresh media only. 24 hours later a luciferase reporter assay was carried. The normalised relative light units for each condition are displayed. Western blot analysis of this experiment is displayed in Figure 4.3.5.2.

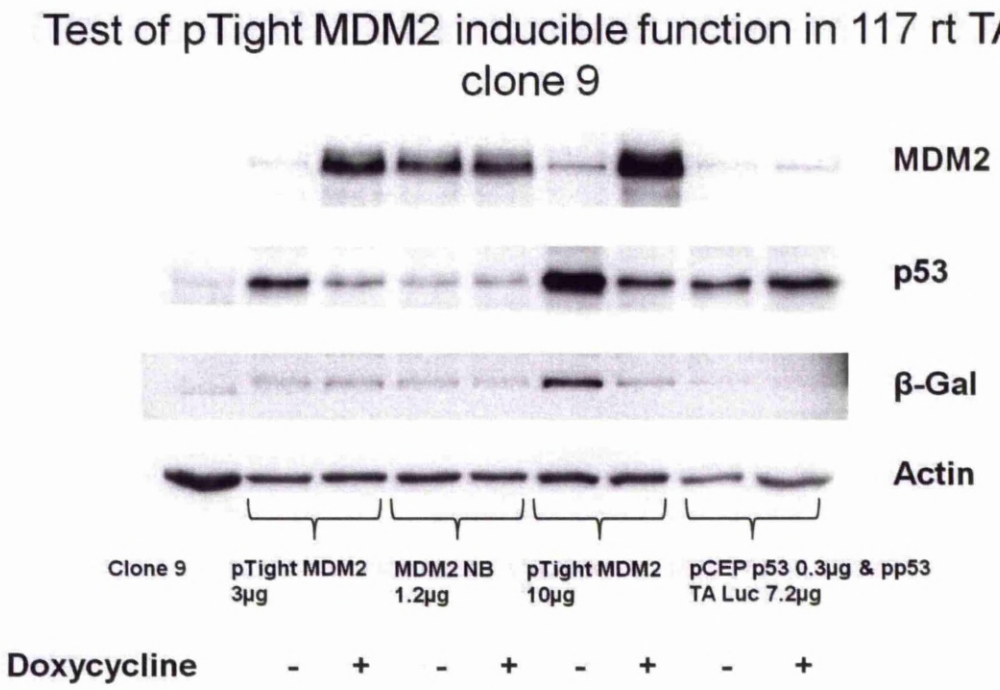


Figure 4.3.5.2 Western Blot of experiment 4.3.5.1 - 117 rtTA2S-M2 stably expressing clone 9 cells were transiently transfected with a p53 expression plasmid (pCEPp53), a p53 luciferase reporter construct (pp53-TA-Luc), β-Gal plus either pTre-Tight-MDM2 or pCMVNeoBamMDM2 (MDM2 NB in figure) as indicated below lanes on figure. Lane 1 contains an untransfected 117 rtTA2S-M2 clone 9. For each of the four conditions cells were incubated at 37°Cwith media containing DOX (+ in figure) or normal media (- in figure). Proteins probed for as indicated in the figure.

4.3.6 Second round cloning

Having identified clones that demonstrate inducibility in response to Dox, the second round of cloning required the rtTA stable clones be co-transfected with the pTre-Tight-MDM2 plasmid (See figure 4.2.x) together with a linear antibiotic selection marker to allow for selection of stably expressing clones. The 117 derived clones used at the beginning of this series of experiments pCEP4 clone 3 and ΔNp53 clone 3 have both previously been generated through selection using hygromycin. Therefore hygromycin could not be used again for clonal selection in the two cell lines. ClonTech™, the suppliers of this system, supplied a puromycin linear marker however due to production problems there was a worldwide shortage and it was not possible to obtain this. Therefore the experimental plan was revised. The main goal was always to study the consequences of MDM2 expression in a RCC cell line, therefore the experiment proceeded but only with the 117 cell line which was still hygromycin sensitive. The 117 rtTA clone 9 would also be stably transfected with a pTre-Tight construct containing a RING-finger mutant of MDM2 (pTre-Tight-RING). In the same way that the consequences of MDM2 expression were going to be compared between the 117 cell line and the now discarded pCEP3 and ΔNp53, the RING-finger mutant of MDM2 (MDM2RFM) would now be used for comparison as well. This experiment would also potentially help distinguish between genes being expressed as a consequence of the E3 ligase activity (RING finger dependent) of MDM2 and those altered through MDM2 protein-protein interactions.

The pTre-Tight-RING was generated and tested in the Boyd laboratory by a co-worker Dr M Maguire, using the same technique and strategy detailed earlier. The MDM2-RING-finger-mutant was extracted from the human RING finger mutant Cys464Ala pCMVNeoBam3plasmid.

117 rtTA clone 9 cells were transiently transfected with pTre-Tight-MDM2 and pTre-Tight-RING along with the linear hygromycin selection marker. Control dishes without the linear hygromycin selection marker were similarly transfected. The cells were cultured in media containing both G418 and Hygromycin to provide selection. After 6 days none of the control transfected dishes contained viable cells. Colonies on the experimental or test dishes were however ready to be picked after 12 days for both sets of clones. A minimum of 40 colonies were picked for each cell line.

As these cells were expanded it was noted that a number of pTight-MDM2 colonies grew poorly and a number eventually died despite attempts at promoting growth by cell passage. Of the 40 colonies that were selected for expansion from 96 wells to 24 well plates only 11 were eventually able to be tested for functionality.

4.3.7 Screening for inducible pTre-Tight-MDM2 expressing clones

The following experiment was carried out to test whether the selected clones expressed MDM2 after treatment with DOX. Clones were incubated at 37°C in media containing 1μg/ml of DOX for 24 hours, untreated controls were also cultured. The cells then underwent protein extraction and western blot analysis. Clones showing increased

MDM2 signal with DOX treatment underwent further evaluation. From Fig 4.3.7 it can be seen that no clones demonstrated increased expression of MDM2 after exposure to DOX. Clones 2, 16 and 21 were selected to undergo more detailed testing as they showed a slight increase in MDM2 signal after treatment with DOX. Clone 15 appears to show an increase in MDM2 signal after treatment with DOX however this is due to more protein being loaded (as indicated by the Actin having a high signal compared to the control lane) in the DOX treatment "+" lane compared to the non treatment "-" lane.

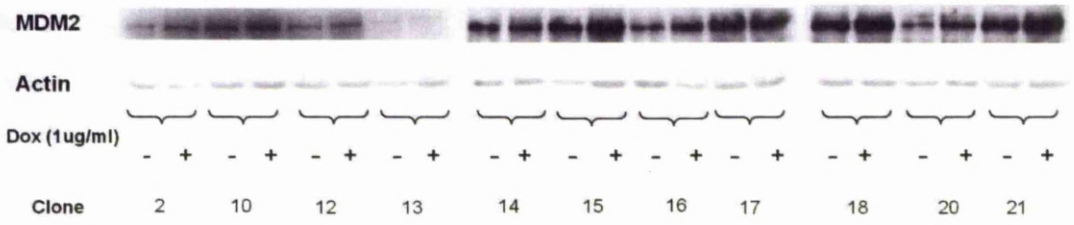


Figure 4.3.7 Western blot showing MDM2 expression in response to DOX treatment in clones generated following transfection with rtTA2S-M2 and pTre-Tight-MDM2. Experiment performed with assistance by MM. Numbered candidate clones were incubated in their normal culture media (lane labelled -) or in normal culture media containing doxycycline 1µg/ml (lanes labelled +). Cells were then harvested and underwent protein extraction which was probed for as indicated in figure.

4.3.8 Testing of potential inducible pTre-Tight-MDM2 expressing clones

The candidate inducible MDM2 clones selected from 4.3.7 (clones 2, 16 & 21) then underwent further testing. To test whether inducible and functional human MDM2 was being induced upon DOX treatment, the clones were transiently transfected with p53 (pCEPp53) and a p53 luciferase reporter (pp53-TA-Luc). A range of increasing DOX concentrations was used to increase the chances of detecting induction of MDM2. It was expected that following induction of MDM2 expression there would be a decrease in p53 function. As can be seen from figure 4.3.8.1 panel A, there is no evidence of a reduction in p53 signal after DOX treatment. In the western blot analysis of this experiment (panels B & C in same figure) there was no change in MDM2 signal or p53 signal after DOX treatment, again signifying that functional MDM2 expression has not been induced. To ensure that the DOX response element of the system was still working after the second round of cloning, the three clonal cell lines were transfected with the pTre-tight-MDM2 plasmid (used for the second round of cloning) and pCMVNeoBamMDM2 (see panel D) along with p53 and a p53 luciferase reporter. As can be seen, treatment with DOX resulted in a decrease in p53 reporter activity proving that the clones retain their rtTA function. A control for the p53 reporter assay is demonstrated in Panel E in figure 4.3.8.1. Here the three cell lines were transfected with p53, the p53 luciferase reporter and either pCMVNeoBamMDM2 (expresses functional MDM2 – pCMVNBMDM2 in figure)) or pCMVNeoBam (pCMVNB in figure) plasmid. After 24 hours, all cell lines transfected with pCMVNeoBamMDM2 demonstrate a

reduction in p53 reporter signal compared to the control (pCMVNeoBam) indicating that the p53 reporter signal changes in the presence of functional MDM2.

To ensure that the cells were not transiently or slowly expressing MDM2 after DOX treatment, the experiments were repeated using a shorter and longer time courses for clone 2 and clone 16 (see figure 4.3.8.2 & 4.3.8.3). These tests show that both clones 2 and 16 retain DOX responsive expression of rtTA but no evidence of MDM2 induction in response to DOX treatment. This suggests that for some unknown reason the plasmid that should produce MDM2 expression is either unresponsive or is not present or disrupted and thus there is no DOX induced MDM2 expression. We can only speculate as to why this situation may have arisen. One possibility is that there is still an increase in expression of MDM2 as a result of transfection with pTre-Tight-MDM2 which is not tolerated by the cells. Whatever the exact reason, there was no evidence of MDM2 inducibility in any of the tested clones.

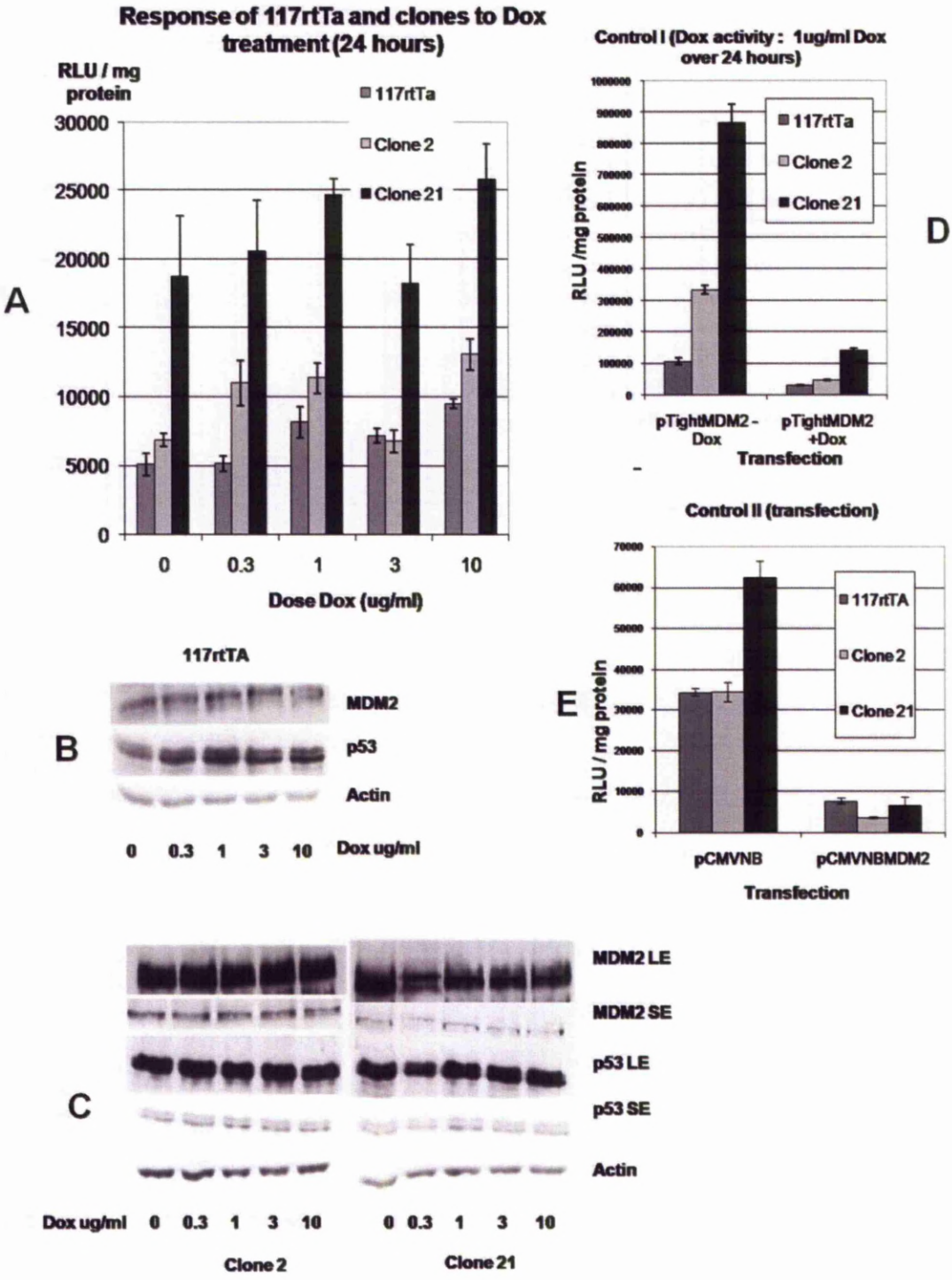


Figure 4.3.8.1 Screening of clones 2 and 21 for DOX inducible expression of MDM2. Experiment performed with assistance from MM. **A**, clones 2 and 21 and 117

rtTA clone 9 were transiently transfected with p53 and the p53 luciferase reporter. After treatment with differing concentrations of DOX for 24 hours p53 reporter activity was estimated using the luciferase reporter assay. **B&C**, western blot analysis of the same experiment probed for the indicated proteins LE = long exposure, SE = short exposure. **D,** transient transfection of the same cell lines with pTre-Tight-MDM2, p53 and the p53 luciferase reporter. After treatment with the indicated concentration of DOX for 24 hours, p53 reporter activity was estimated using the luciferase reporter assay **E**, transient transfection of the same cell lines with pcmvNEOBAMMDM2, p53 and the p53 luciferase reporter. After 24 hours, p53 reporter activity was estimated using the luciferase reporter assay.

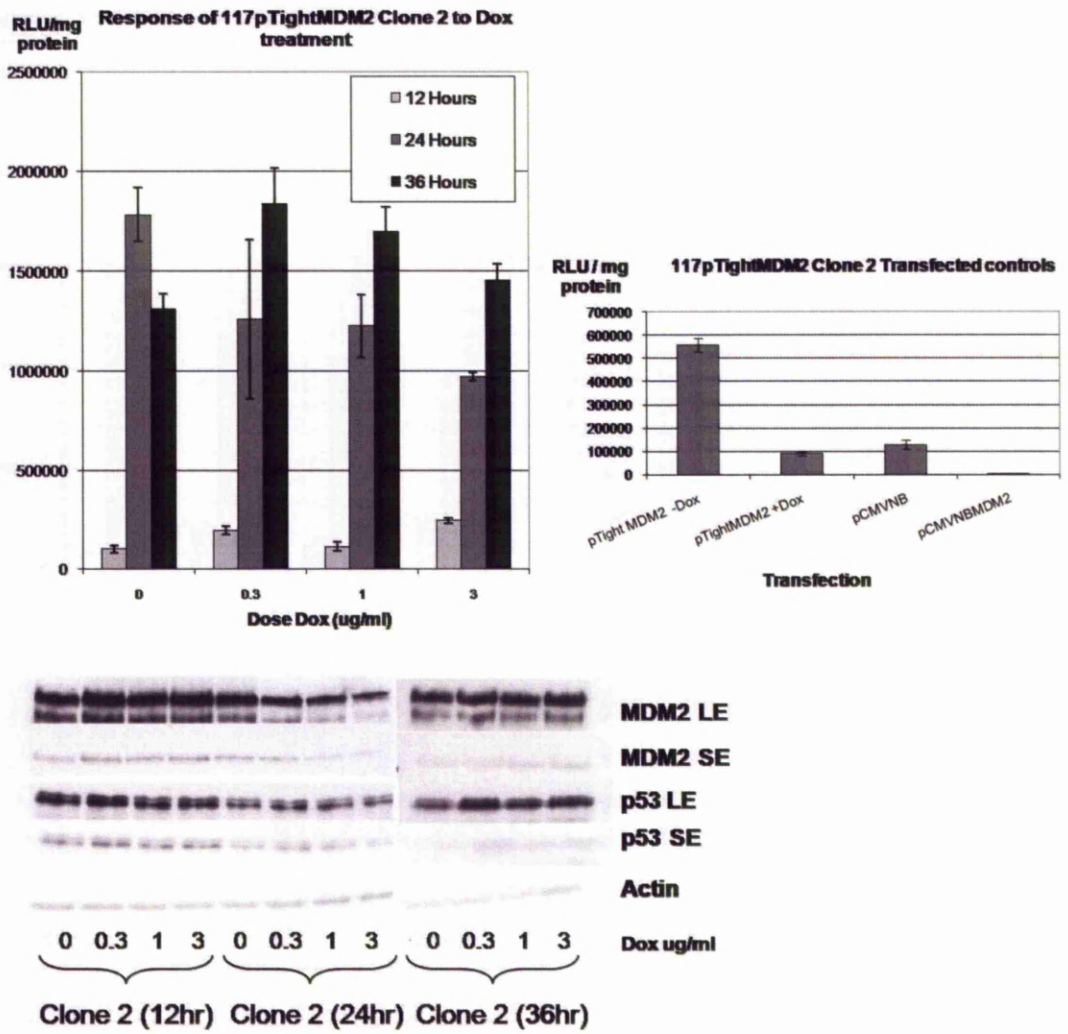


Fig 4.3.8.2 Further Screening of clone 2 for DOX inducible expression of MDM2. Experiment performed with assistance from MM.**Top left** clone 2 was transiently transfected with a p53 luciferase reporter. After treatment with differing concentrations of DOX for 12, 24 and 36 hours p53 reporter activity was estimated using the luciferase reporter assay. **Bottom** western blot analysis of the same experiment, probed for the indicated proteins (LE = long exposure, SE = short exposure). **Top right** p53 reporter assay of clone 2 after transient transfection with p53, p53 reporter plasmid and either

pTre-Tight-MDM2 (plus or minus DOX 1μg/ml) or pcmvNeoBam (pCMVNB in figure) or pcmvNeoBamMDM2 (pCMVNBMDM2 in figure).

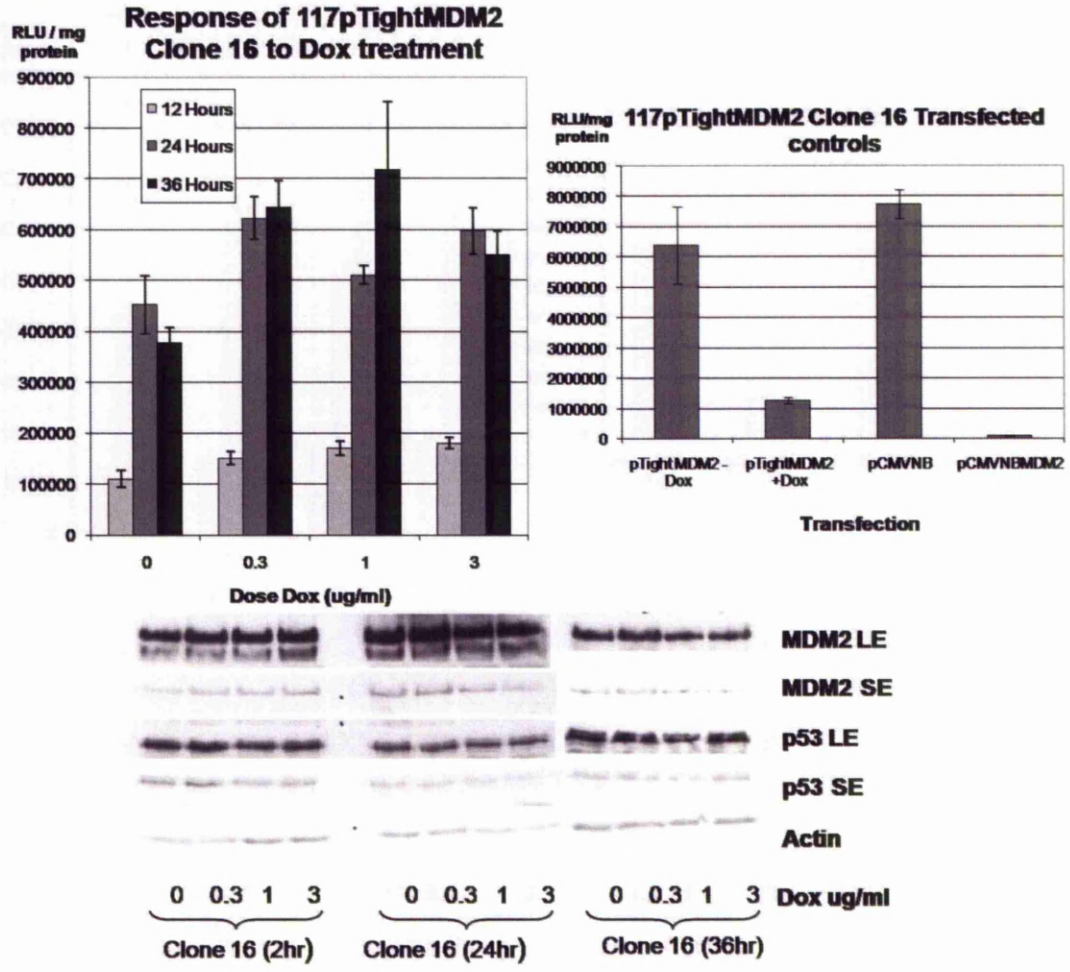


Figure 4.3.8.3 Screening of clone 16 for DOX inducible expression of MDM2. Experiment performed with assistance from MM.**Top left** clone 16 was transiently transfected with the p53 luciferase reporter. After treatment with differing concentrations of DOX for 2, 24 and 36 hours, p53 reporter activity was estimated using the luciferase reporter assay. **Bottom** western blot analysis of the same experiment probed for the indicated proteins. **Top right** p53 reporter assay of clone 16 after

transient transfection with p53, p53 reporter plasmid and either pTre-Tight-MDM2 (plus or minus DOX 1μg/ml) or pcmvNeoBam (pCMVNB in figure) or pcmvNeoBamMDM2 (pCMVNBMDM2 in figure).

4.3.9 Screening of inducible pTre-Tight-RING expressing clones

The following experiment was carried out to test whether the selected clones expressed MDM2RFM after treatment with DOX. Clones were incubated at 37°C in media containing 1μg/ml of DOX for 24 hours, untreated controls were also cultured. The cells then underwent protein extraction and western blot analysis. Clones showing increased MDM2 signal with DOX treatment underwent further evaluation. From Fig 4.3.9, clones 1, 2, 4, 16 and 25 were selected to undergo more detailed testing as they showed some induction of MDM2. Clone 25 could not be recovered from frozen stocks and was not available to undergo further testing.

Four clones 1, 2, 4 and 16 underwent further analysis to determine if they were indeed stable DOX-inducible MDM2RFM expressing clones. Clones were transiently transfected with p53 and a p53 luciferase reporter. The clones were then treated with increasing concentrations of DOX containing media for 24 hours. Controls included transfection with the pTre-Tight-RING plasmid and pCMVNeoBamMDM2.

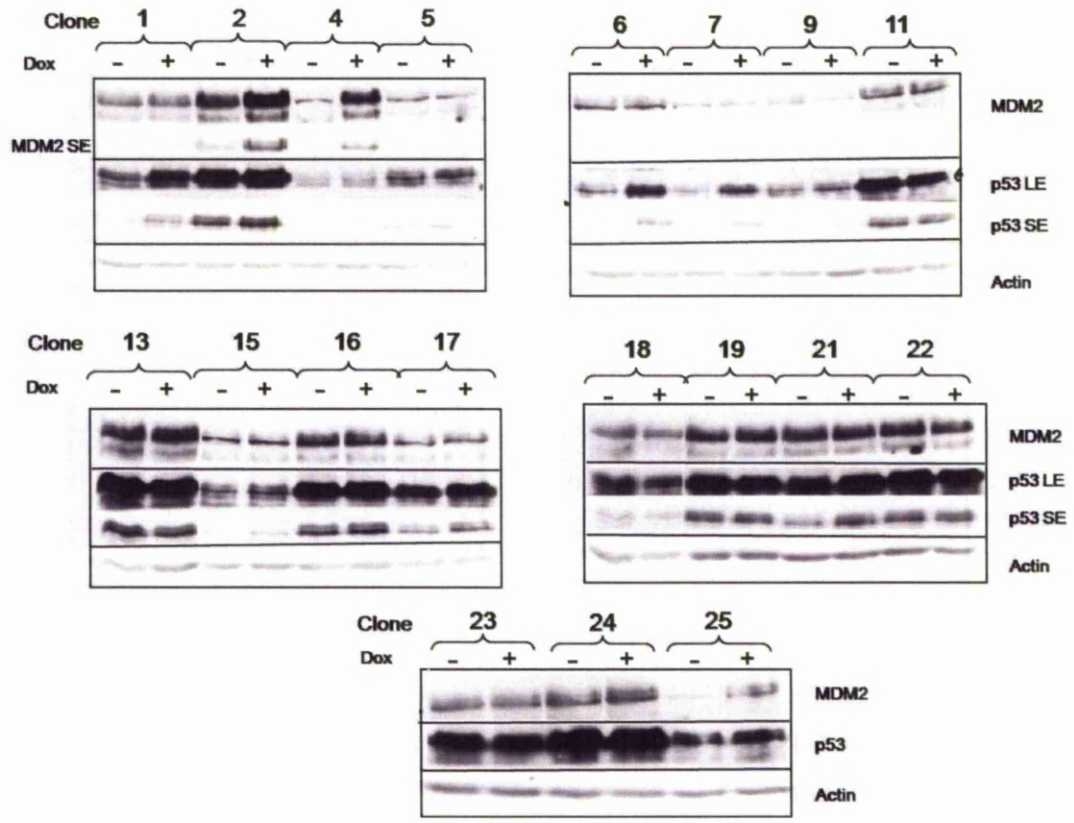


Figure 4.3.9.1 Western blot showing the results of screening for inducible pTre-Tight-RING expressing clones. Experiment performed with assistance by MM. Candidate pTre-Tight-RING clonal cell lines were incubated at 37°C with normal media or media containing 1µg/ml of DOX for 24 hours. Proteins were probed as indicated in figure. LE = long exposure, SE = short exposure.

To try and detect if a clone does inducibly express MDM2RFM in response to DOX one would expect to see an increase in MDM2 levels when probed for on western blotting. It may also be possible to detect changes in p53 transcriptional activity as a result of increasing expression of MDM2RFM, however this latter effect would be due to MDM2 inhibiting p53 transcription, as MDM2RFM is unable to ubiquitinylate and therefore bring about p53 degradation[59].

Figures 4.3.9.2 shows the results of testing candidate clone 1. Panel A of this figure shows a p53 luciferase reporter assay of clone 1 cells exposed to different concentrations of DOX. There is evidence of a very small decrease in p53 reporter signal upon DOX treatment (bars 2, 3 & 4) as compared to the control (0μg DOX – bar 1). However the last two bars of the chart show the change in p53 signal when the clone is transfected with and in the last bar, also exposed to DOX. As can be seen there is a much more dramatic decrease in p53 signal when DOX is added causing inducible expression of MDM2RFM from transfected pTre-Tight-RING. This suggests that the clone 1 cells at least retain their DOX inducibility but does not provide conclusive evidence that the clone is inducible These last two bars of the graph were calculated using a different batch of luciferase reagents (performed at a different time), for this reason bars 5 and 6 can only be compared to each other and not the bars 1 – 4. Panel C shows the control experiment for the p53 luciferase reporter assay – a reduction in p53 signal is observed in clone 1 cells transfected with an MDM2 expression plasmid (pCMVNeoBamMDM2)

compared to those transfected with the empty vector control (pCMVNeoBam) demonstrating that the p53 reporter assay is able to detect changes in p53 signal as a result of MDM2 expression. Panel B shows the western blot of p53 reporter assay experiment shown in panel A. There is no obvious increase in MDM2 signal in lanes 2, 3 and 4 compared to lane 1 (no DOX treatment) however there is a marked increase in MDM2 signal in the lane 6 (transfected pTre-Tight-RING and DOX treatment) compared to lane 5 transfected pTre-Tight-RING (note also there is no appreciable change in the p53 signal as expected). These results, especially the lack of appreciable increase in MDM2 levels on western blotting, suggest that clone 1 is not suitable for use as an inducible clone. Testing of clone 16 in exactly the same way (Figure 4.3.9.3) revealed the same pattern as that described for clone 1 and therefore clone 16 was also discarded for further experimentation.

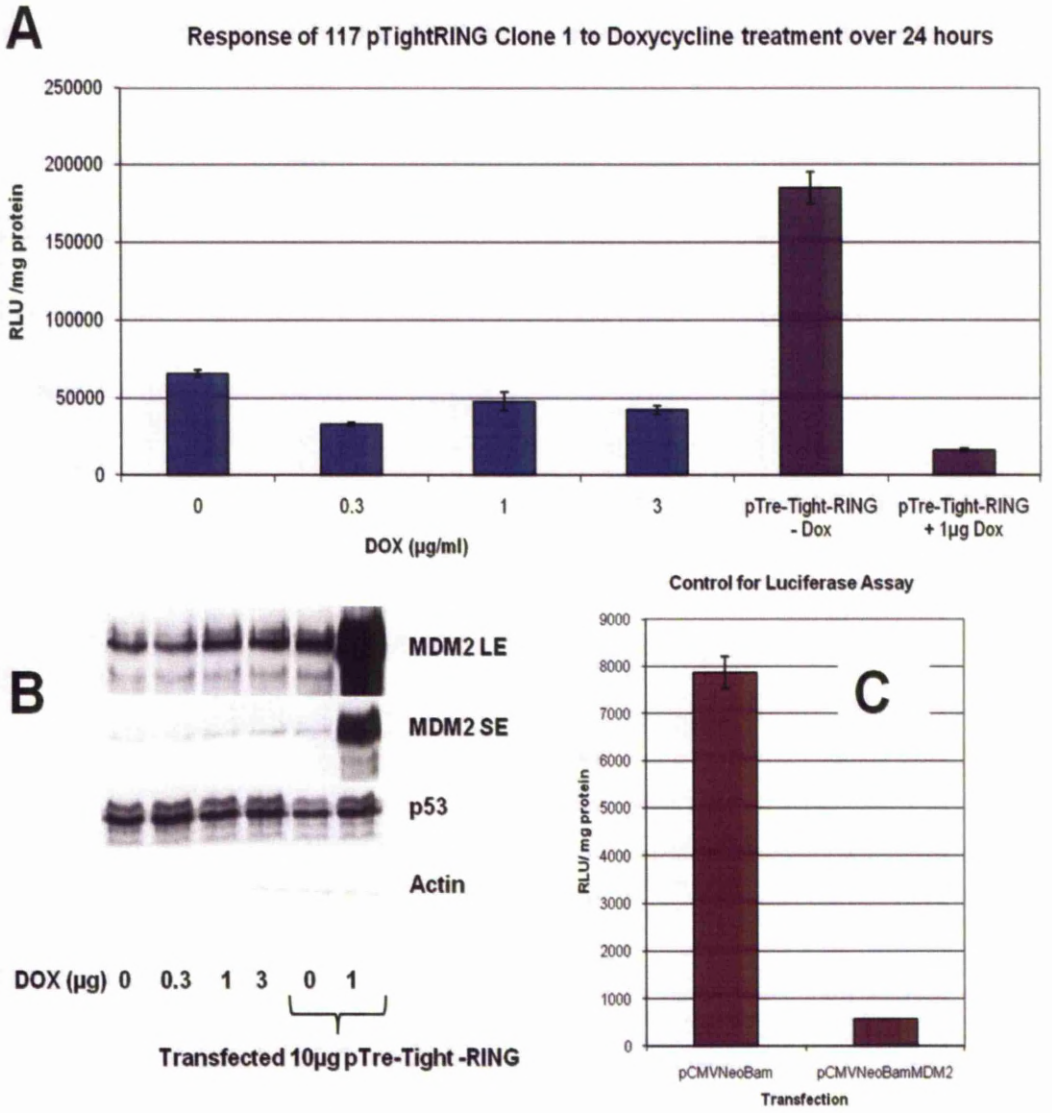


Figure 4.3.9.2 Screening of candidate clone 1 for DOX inducible MDM2 RING mutant expression. Experiment performed with assistance from MM. **Panel A**, clone 1 was transiently transfected with pCEPp53 and pp53TALuc (luciferase reporter). After treatment with differing doses of DOX for 24 hours p53 reporter activity was estimated using a luciferase reporter assay. The pTre-Tight-RING plasmid was also transiently transfected as a control for DOX inducibility. **Panel B**, western blot analysis of same

experiment proteins probed for as indicated in figure (LE = long exposure, SE = short exposure). **Panel C**, luciferase reporter assay control. Transient transfection of clone 2 with pp53TALuc, pCEPp53 and either pCMVNeoBam or pCMVNeoBamMDM2.

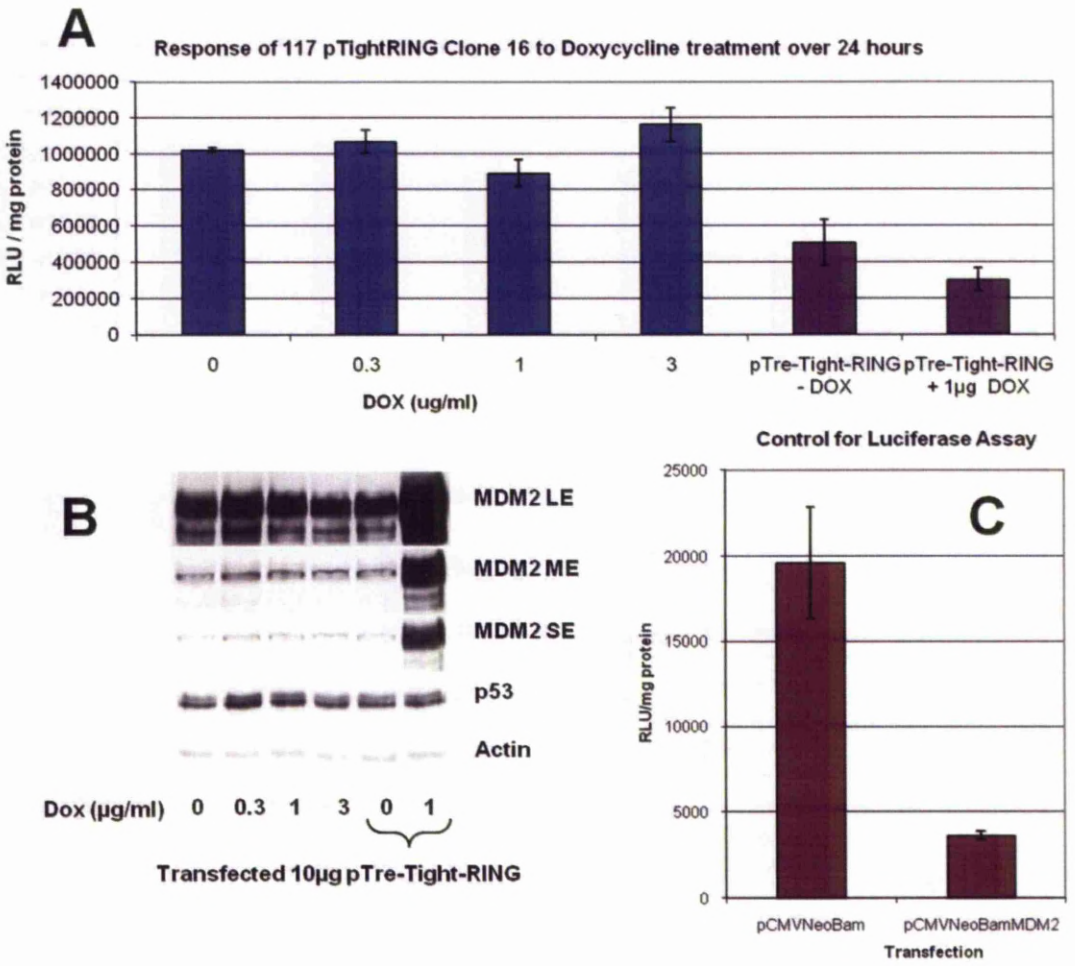


Figure 4.3.9.3 Screening of candidate clone 16 for DOX inducible MDM2 RING mutant expression. Experiment performed with assistance from MM. **Panel A**, clone 16 was transiently transfected with pCEPp53 and pp53TALuc (luciferase reporter). After treatment with differing doses of DOX for 24 hours p53 reporter activity was estimated using a luciferase reporter assay. The pTre-Tight-RING plasmid was also

transiently transfected as a control for DOX inducibility. **Panel B**, western blot analysis of same experiment proteins probed for as indicated in figure (LE = long exposure, ME = medium exposure , SE = short exposure). **Panel C**, luciferase reporter assay control - transient transfection of the clone 1 with pp53TALuc, pCEPp53 and either pCMVNeoBam or pCMVNeoBamMDM2.

Figure 4.3.9.4 shows the same experimental tests this time performed on candidate clone 2. This time, as can be seen in the western blot (panel B), there is a marked increase in MDM2 signal in lanes 2 – 4 (Dox treatment) compared to lane 1 (control i.e. no DOX). The level of MDM2 signal in the DOX treatment lanes is almost the same as that seen in lane 6 (cells have been transfected with pTre-Tight-Ring and treated with DOX). This finding demonstrates that clone 2 is a DOX inducible MDM2RFM expressing clone. As expected the marked increase in MDM2RFM levels has not altered the p53 levels on the western blot for reasons mentioned earlier. In the p53 luciferase reporter assay (panel A), allowing for increased protein loading into lanes 3 & 4 (evidenced by uneven Actin signal), there is a slight reduction in p53 reporter signal in all DOX treated lanes. However as this pattern was also seen in clone 2 (Figure 4.3.9.2 panel A) where there was no evidence of increased MDM2RFM levels on western blot (Figure 4.3.9.2 panel B) it must be concluded that the p53 luciferase reporter assay is not capable of detecting changes in MDM2RFM levels. Testing of clone 4 in the same manner as just described (figure 4.3.9.5), shows the same pattern of results as clone 2 suggesting it is also an inducible MDM2RFM expressing clone.

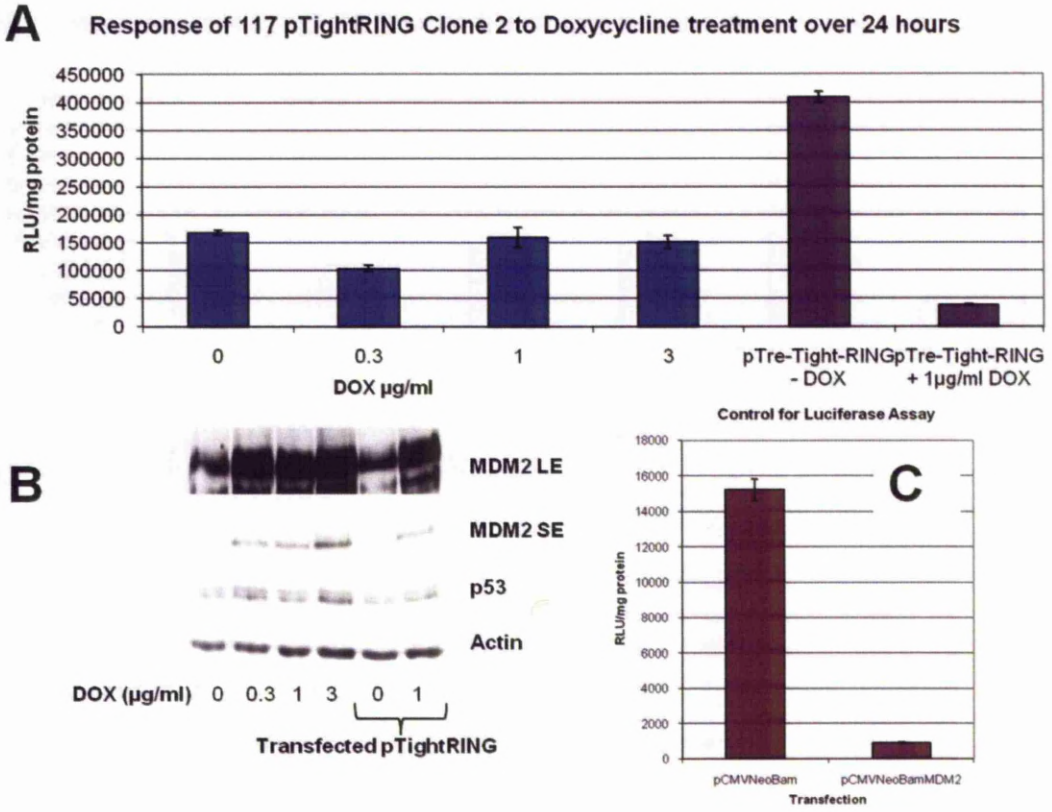


Figure 4.3.9.4 Screening of candidate clone 2 for DOX inducible MDM2 RING mutant expression. Experiment performed with assistance from MM. **Panel A**, clone 1 was transiently transfected with pCEPp53 and pp53TALuc (luciferase reporter). After treatment with differing doses of DOX for 24 hours p53 reporter activity was estimated using a luciferase reporter assay. The pTre-Tight-RING plasmid was also transiently transfected as a control for DOX inducibility. **Panel B**, western blot analysis of same experiment proteins probed for as indicated in figure (LE = long exposure, ME = medium exposure , SE = short exposure). **Panel C**, luciferase reporter assay control. Transient transfection of the clone 2 with pp53TALuc, pCEPp53 and either pCMVNeoBam or pCMVNeoBamMDM2.

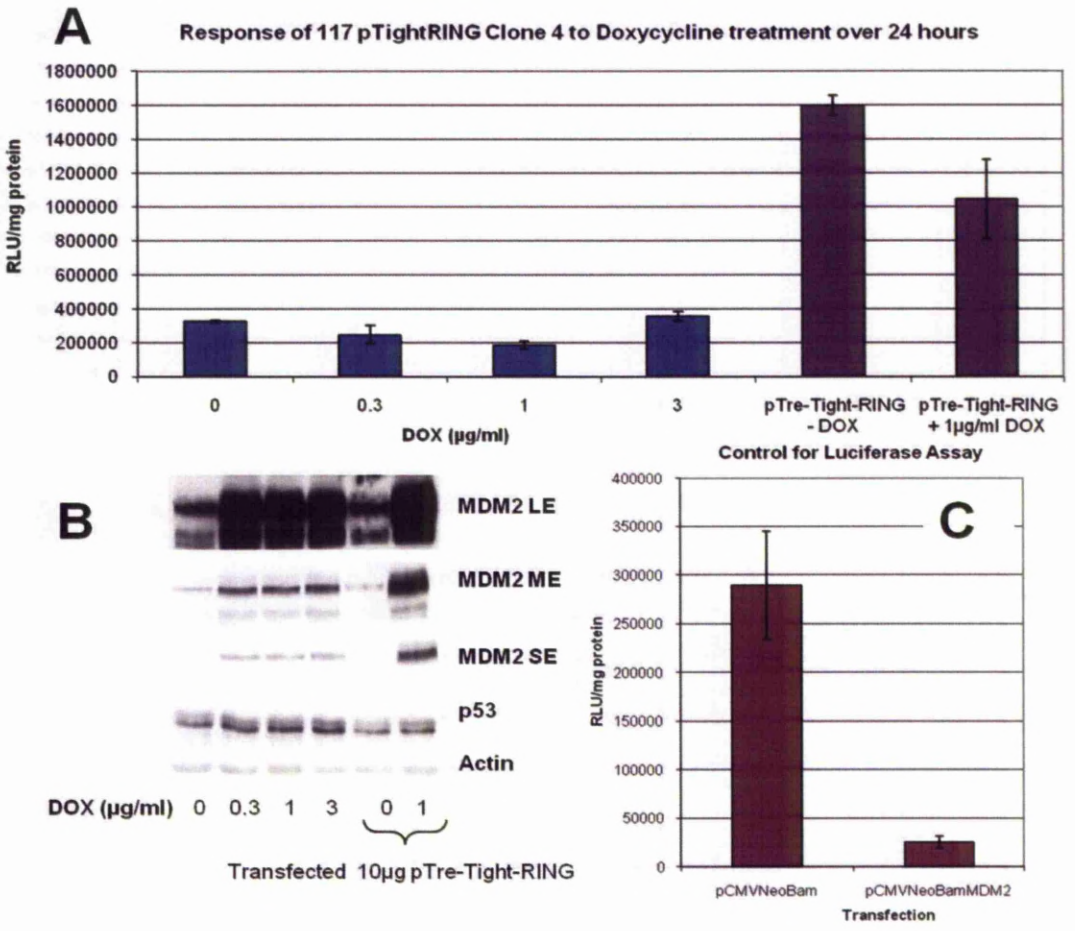


Figure 4.3.9.5 Screening of candidate clone 4 for DOX inducible MDM2 RING mutant expression. Experiment perfomed with assistance from MM**. Panel A**, clone 4 was transiently transfected with pCEPp53 and pp53TALuc (luciferase reporter). After treatment with differing doses of DOX for 24 hours p53 reporter activity was estimated using a luciferase reporter assay. The pTre-Tight-RING plasmid was also transiently transfected as a control for DOX inducibility. **Panel B**, western blot analysis of same experiment proteins probed for as indicated in figure (LE = long exposure, ME = medium exposure , SE = short exposure). **Panel C**, luciferase reporter assay control - transient transfection of the clone 4 with pp53TALuc, pCEPp53 and either pCMVNeoBam or pCMVNeoBamMDM2.

4.4 Discussion

The main aim of this chapter was to produce a model for examining the effects of high MDM2 expression in RCC cell line UOK 117, using gene expression profiling. In simplistic terms the model must be able to contrast a low MDM2 expression state (seen in UOK 117) with a high MDM2 expression state (achieved through transient transfection or inducible expressing 117 clones). The first series of experiments detailed in this chapter were designed to evaluate and select a commercially available transient transfection system that could reliably achieve high transfection efficiency, in the 117 and 117 derived clones (pCEP3 & ΔNp53), for the planned microarray gene expression profiling experiment. Optimised conditions for Lipofectamine™ 2000 and FuGENE HD™ achieved the highest transfection efficiency of around 40 - 45%. This transfection efficiency was far below that desired and therefore a new approach was taken – to try and produce inducibly expressing MDM2 clones using the Tet-On® Advanced inducible gene expression system (ClonTech™).

In order to produce MDM2 inducible expressing clones, the Tet-On® Advanced inducible gene expression system requires two rounds of cloning. The first round aims to produce clones that stably express rtTA. The second round of cloning allows the gene of interest (MDM2 or MDM2RFM) to be expressed in response to rtTA and DOX; it is probable that a relatively small amount of GOI is expressed in the absence of DOX. The three cell lines (117, ΔNp53 & pCEP3) all underwent the first round of cloning producing a number of new rtTA clones that could be evaluated. A new rtTA clone from

each of the parental cell lines was selected that demonstrated a high level of DOX inducible expression, minimum expression (of the test plasmid pTre-Tight-Luc) in the absence of DOX ("background expression") and levels of p53 & MDM2 expression on western blotting as similar as possible to the parental cell lines.

The second round cloning of these three clones could not be performed as planned due to the unavailability of the puromycin antibiotic selection marker. The 117 rtTA clone could still undergo second round cloning with an alternative and available antibiotic selection marker (hygromycin). Using the 117 rtTA clone 9, second round cloning took place with the aim of producing two inducible expressing clones (MDM2 and MDM2RFM), to allow the consequences of high MDM2 expression to be contrasted with that of the MDM2RFM (which cannot act as a E3 ligase) by gene expression profiling. The second round cloning initially resulted initially in a similar number of candidate clones to be tested as seen after cloning round 1. It was observed that the second round clones seemed to grow less well with fewer colonies being able to survive colony expansion. Testing of the pTightMDM2 clones failed to show any that demonstrated inducible MDM2 expression whereas two pTightRING clones were found that inducibly expressed MDM2RFM.

It can only be speculated as to why the second round of cloning produced so few desired clones compared to the first round of cloning. It is possible that small amounts of background expression of MDM2 may be causing cell death. Colonies surviving may have silenced MDM2 expression or were only successfully transfected with the

hygromycin selection marker alone and not the MDM2 sequence. Some support is leant to the first hypothesis by the fact that two mutant MDM2 clones were generated, where it is possible the effects of MDM2RFM are less deleterious for the cells. It would have been very interesting to see if more clones were generated from the pCEP3 and ΔNp53 117 clones, which previously have produced high MDM2 expressing clones as a result of cloning experiments. To fully test whether the pTre-Tight results in lethal MDM2 background expression a larger scale repeat of the experiment would be needed which would include the 117 derived clones from which stable MDM2 expressing clones can be made (ΔNp53 & pCEP3).

CHAPTER 5 – INVESTIGATING THE ROLE OF PROTEASOMAL DEGRADATION AND HEAT SHOCK PROTEIN 90 IN THE "HIGH" STEADY STATE LEVELS OF P53 AND MDM2 SEEN IN RCC CELL LINES

5.1 Introduction

It has been shown that some RCC cells in culture express relatively high levels of p53 and MDM2 with intact p53 function[128] (see also section 1.6.5). As described earlier in chapter 1, and demonstrated in chapter 3, there is evidence that this phenotype (i.e. high expression of p53 & MDM2 determined by tumour immunohistochemistry) is associated with poor prognosis in patients with RCC[96].

The mechanism responsible for high levels of p53 and MDM2 protein levels seen in some of the RCC cell lines is still to be discovered. In very simple terms the levels of p53 and MDM2 within the cell depend upon the rates of protein expression and degradation. p53 and MDM2 undergo proteasomal degradation as a result of ubiquitylation (see section 1.3.3), by inhibiting the function of the proteasome with an inhibitor, one would expect to see an increase in p53 and MDM2 protein levels. Comparison between the different RCC cell lines in their degree of p53 and MDM2 stabilisation upon proteasome inhibition may reveal differences brought about by a variation in the susceptibility of p53 and MDM2 to proteasomal degradation (although

there are of course other reasons that may explain any observed differences), for example if within a given cell line a proportion of p53 and MDM2 is shielded from undergoing proteasomal degradation, one would expect less p53 and MDM2 stabilisation (upon proteasome inhibition) compared to a cell line where all of the available p53 and MDM2 undergo proteasomal degradation. Such cell lines in which a proportion of p53 and MDM2 is resistant to proteasomal degradation could also be expected to have increased levels of these proteins compared to cell lines with "normal" p53 and MDM2 proteasomal degradation. One process that allows cells to stabilise a protein, by preventing proteasomal degradation, is through interaction with the HSP90 chaperone complex (see section 1.7). As detailed in section 1.7.5 there is evidence that p53 may be a client protein of the HSP90 chaperone complex[155]. This raises the possibility that some of the RCC cell lines may exhibit high p53 and MDM2 protein levels due to a reduction in proteasomal degradation through the action of the HSP90 chaperone complex. This hypothesis can be tested by inhibiting the function of the HSP90 to see if this does result in any reduction in p53 and MDM2 steady state levels.

5.2 Results

5.2.1 Effects of proteasome inhibition on p53/MDM2 steady state levels in RCC cell lines.

To examine the effects of proteasome inhibition on p53 and MDM2 protein levels in RCC cell lines, the following experiment was carried out. The panel of RCC cell lines

(see section 1.7.1), the osteosarcoma cell line U2OS, and the p53 null - non small cell lung carcinoma cell line H1299 were treated for three hours with either a DMSO control or the proteasome inhibitor MG115. Cellular lysate was then subjected to western blot analysis and the proteins p53 and MDM2 were detected. Actin was used as a loading control. Cyclin D1, a member of the cyclin family of CDK (Cyclin dependent kinase) regulators, known to be degraded by the proteasome[162], was used as an internal control of proteasome inhibition. In all figures, comparison can only be made between the DMSO control and the adjacent treatment lane. For comparisons of relative levels of MDM2 or p53 in each cell line please refer to figure 1.7.1 in the introduction. Results of the western blots are shown in figure 5.2.1. A summary of the effects on p53 and MDM2 stabilisation are shown in table 5.2.1

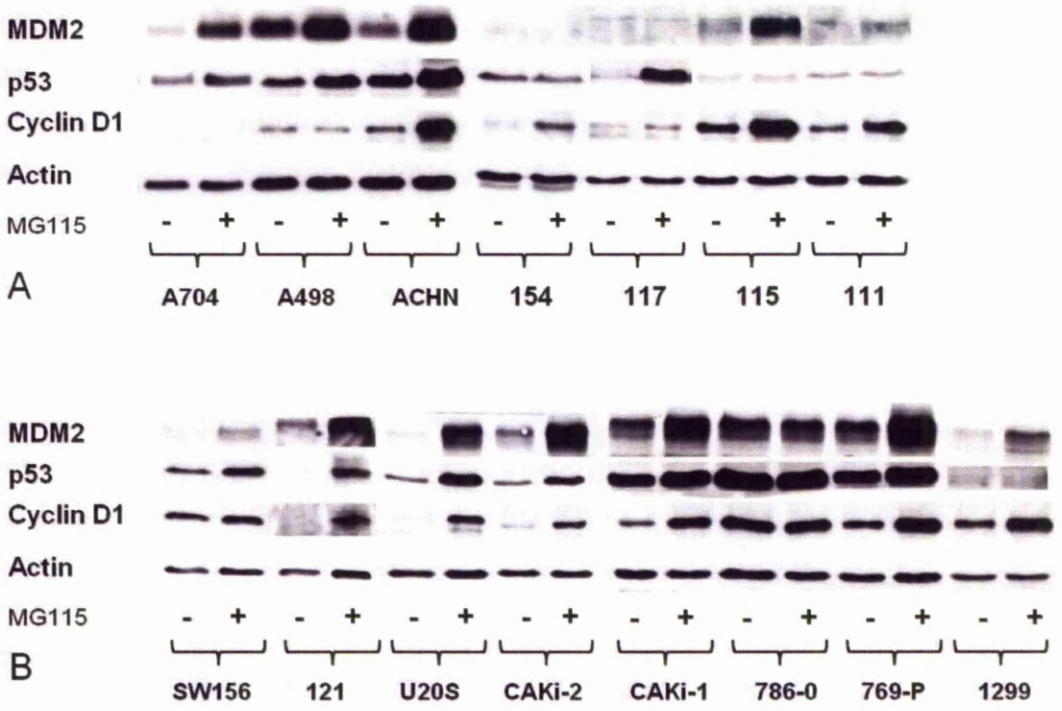


Figure 5.2.1. Western blots showing MDM2 and p53 protein levels in RCC cell lines before and after inhibition of the 26S proteasome using MG115. Cells were cultured for 3 hours in their normal media containing either 10μmol/L of MG115 (lanes labelled "+") or an equal volume of DMSO (lanes labelled "-"). Proteins were probed for as indicated on the figure.

Cell line	Panel	P53 status	p53 Stabilised	MDM2 Stabilised	Cyclin D1 stabilised	Relative expression*
111	A	MT	NO	NO	YES	High p53 moderate MDM2
115	A	MT	NO	YES	YES	High p53 Moderate MDM2
117	A	WT	YES	NO	YES	Low p53 Low MDM2
121	D	MT	YES	YES	YES	High p53 Moderate MDM2
154	A	MT	NO	NO	YES	High p53 Low MDM2
A704	B	MT	YES	YES	YES	Low p53 Moderate MDM2
A498	B	WT	YES	YES	YES	Moderate p53 High MDM2
ACHN	B	WT	YES	YES	YES	High p53 Moderate MDM2
Caki1	C	WT	YES	YES	YES	Moderate p53 High MDM2
Caki2	D	WT	YES	YES	YES	Moderate p53 High MDM2
786-O	C	MT	NO	NO	NO	High p53 High MDM2
769-P	C	?	YES	YES	YES	Low p53 Moderate MDM2
SW156	D	WT	YES	YES	YES	Moderate p53 Moderate MDM2
U20S	D	WT	YES	YES	YES	Moderate p53 Moderate MDM2
1299	C	p53 null	N/A	YES	YES	P53 null Moderate MDM2

Table 5.2.1 Analysis of changes in p53 and MDM2 protein levels (stabilisation) after inhibition of the proteasome. WT = wild type p53, MT = mutant p53. *Relative expression refers to the levels of p53 or MDM2 protein expression by western blot figure 1.7.1.

As can be seen from table 5.2.1, treatment of RCC cell lines with MG115 resulted in the anticipated increase in cyclin D1 expression in all cell lines except 786-O. The 786-O cell line showed no increase in p53 or MDM2 expression either. Since none of these three proteins (p53, MDM2 & Cyclin D1) were stabilised in 786-O this might either represent increased resistance to MG115 or reduced proteasomal degradation in this cell line. To further investigate this, the experiment should be repeated using increasing concentrations of MG115 and/or an alternative proteasome inhibitor in the first instance. The A498 cell line showed stabilisation of p53 and MDM2 (implying MG115 was inhibiting the proteasome) but no appreciable stabilisation of cyclin D1. The reasons for this effect are not clear but cyclin D1 has been reported to be mutated in human cancers resulting in post translational stabilisation[163].It is possible that a cell line specific effect is responsible for the failure of MG115 to stabilise cyclin D1.

With the exception of H1299 (p53 null), 111, 115, 117 and 154 the remaining cell lines tested demonstrated stabilisation of p53 and MDM2 suggesting the ubiquitylation / proteasome degradation pathway is actively degrading p53 and MDM2 in these cell lines. The 111, 115 and 154 cell lines showed stabilisation of cyclin D1 but no appreciable stabilisation of p53, the reasons for this can only be speculated upon but may be due to a consequence of p53 mutation, for example a mutation resulting in an inability to up-regulate MDM2 thus causing an imbalance in the autoregulatory loop. This would prevent p53 from undergoing proteasomal degradation after ubiquitylation brought about by the action of MDM2. Cell line 154, has a p53 mutation (248 R – Q), 111 (173 V – Q) and 115 (280 R – T) a search of the IARC database of p53 mutations

does not show published evidence of these mutations preventing MDM2 interaction or ubiquitylation. The R248Q mutation does result in complete loss of p53 function in yeast p53 reporter gene assays[132] this finding may explain the cell lines very low MDM2 levels (MDM2 is not being transcribed by the mutant p53) and no stabilisation upon proteasome inhibition as MDM2 is not present in sufficient quantities to lead to ubiquitylation and degradation by the proteasome. No specific consequences of the V173G p53 mutation, present in the 111 cell line, have been reported. However 111 has a higher steady state level of MDM2 on WB (figure 1.5) compared to 154 which suggests MDM2 may still be upregulated by p53. The 115 mutation (R280T) is a missense core domain mutation which has shown complete loss of wt p53 function and dominant p53 inhibition in a yeast assay[132]. Unlike 154 and 111 cell line 115 shows a very impressive stabilisation of MDM2 upon inhibition of the proteasome. It is hard to explain the differences in MDM2 stabilisation upon proteasome inhibition between 115 and 111. Both cell lines have similar steady state levels of p53 and MDM2, neither show p53 stabilisation upon proteasome inhibition however 115 shows marked stabilisation of MDM2. If the R280T p53 mutation present in 115 is transcriptionally inactive and also dominant negative, the MDM2 present in the cell must be constitutively expressed. It is possible that in 115 and not 111, MDM2 undergoes a higher rate of proteasomal degradation making its stabilisation more obvious after proteasome inhibition. The exact mechanism underlying the observed differences in MDM2 stabilisation in 111 and 115 will require further investigation.

The cell line 117 (wild type p53) showed stabilisation of p53 but not of MDM2. Interestingly along with 117, both 111 and 154 also failed to demonstrate stabilisation of MDM2 upon proteasome inhibition. The 117 and 154 cell lines both have a very low steady state level of MDM2 as demonstrated in figure 1.7.1. The lack of stabilisation of MDM2 in these cell lines may be explained by a hypothesis described in Clegg et al[66]. In this paper it is proposed that MDM2 does not autoubiquitylate unless it is present in high concentrations, such as those seen following transient transfection. The low levels of MDM2 expression in 117 and 154 may allow an unknown protein (such as HSP90 as hypothesised earlier) to prevent ubiquitylation that can be overcome when MDM2 is present at higher levels. The second mechanism proposed by these authors is that MDM2 trans-ubiquitylates another molecule of MDM2 rather than one molecule causing self or cis-autoubiquitylation. Within cells with very low levels of MDM2 the molecules may rarely interact to bring about ubiquitylation. Although lower rates of MDM2 degradation by the proteasome may be due to low relative concentrations of MDM2 in these cell lines, one may expect lower p53 degradation as well given MDM2 ubiquitylates p53. In the 117 cell line there is less expressed MDM2 relative to the other RCC cell lines (see figure 1.5.1) and yet there is still an impressive stabilisation of p53 but not of MDM2 on proteasomal inhibition. Low MDM2 concentrations may explain what is occurring with MDM2 however unless p53 is being ubiquitinylated by another protein (which is unlikely from the work by Itahama et al[164] where it was shown that fully functional MDM2 was needed to rescue p53 embryonal lethality) - further studies are needed to explain the observations in the 117 cell upon proteasome inhibition.

Whitesell et al[149] showed that wild type, but not mutant, p53 cell lines showed stabilisation of p53 in response to proteasome inhibition however only 4 non-RCC cell lines were studied. These authors pre-treated the cell lines with the transcription inhibitor cycloheximide – demonstrating that increased p53 signal seen, was not due to an increase in p53 transcription as a result of cellular stress / stabilisation of other proteins in response to proteasomal inhibition. Furthermore these studies demonstrated that p53 was stabilised in the mutant p53 cell lines when these cells were treated with the HSP90 inhibitor GA and a proteasome inhibitor MG115. These authors' last observation supports the hypothesis that p53 can be "shielded" from degradation by the proteasome by interaction with other proteins in this case HSP90. Our finding that two mutant p53 cell lines show stabilisation of p53 upon proteasome inhibition in the absence of GA, suggest that the majority of p53 must be free to undergo degradation by the proteasome in these lines and that Whitsell et al's observations do not extend to all p53 mutant RCC cells.

5.2.2 Inhibition of de novo protein synthesis in 121 and ACHN cell lines using cycloheximide

As discussed earlier high MDM2 expression in RCC seems to be due to post translational stabilisation, as opposed to enhanced protein synthesis. In order to test this hypothesis, the following experiment was undertaken. RCC cell lines ACHN and 121 were selected as they both have similar expression of MDM2 and p53 (see figure 1.7.1), and demonstrate p53 and MDM2 stabilisation after inhibition of proteasomal

degradation. They differ in regards to their p53 status; 121 harbours mutant p53 and ACHN has functional wild type p53. Both cells were treated with 50μg/ml of cycloheximide, for time points spanning 0 to 8 hours. Figure 5.2.2 shows a western blot analysis of expression of p53 and MDM2 at the indicated time points. The half life of p53 in non-cancerous (normal) cells is typically approximately 15 to 30 minutes; the half life of MDM2 is similarly short at around 20 minutes[78]. In the ACHN and 121 cell lines, p53 is shown to be stable with a half-life (assessed semi-quantitatively) in excess of 8 hours. MDM2 is shown to have a half-life (assessed semi-quantitatively) of approximately 30 minutes in 121 and 1 hour in ACHN. Given that both of these cell lines have p53 and MDM2 that are degraded by the proteasome the question remains as to why these cells lines have such an extended p53 and MDM2 half life. Could it be that the HSP90 chaperone or another unknown mechanism is preventing a large amount of cellular p53 and MDM2 from coming into contact with the ubiquitylation / proteasome pathway? The increase in p53 levels following proteasomal degradation may represent stabilisation of the p53 not in complex with HSP90 for example.

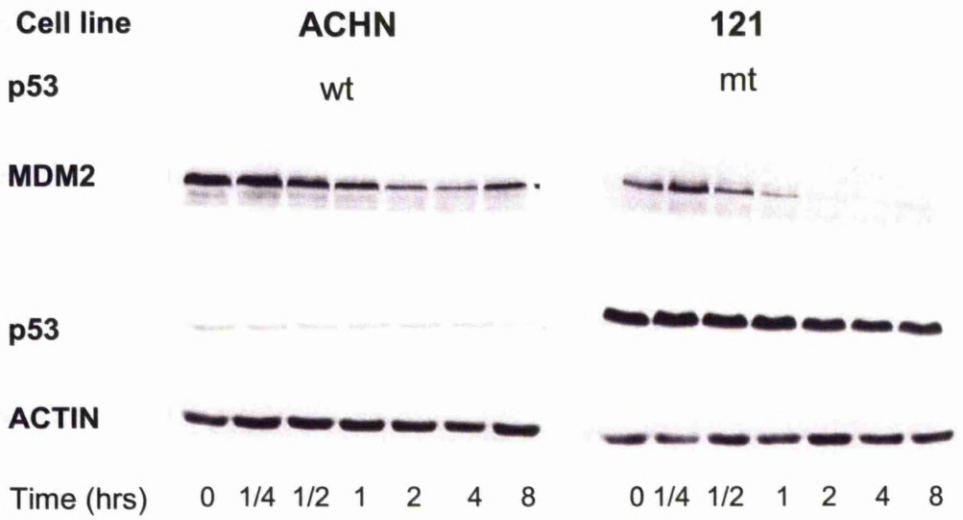


Figure 5.2.2 Western blot of p53 and MDM2 expression following cycloheximide treatment in ACHN and 121 RCC cell lines. p53 status is represented as wild type (wt) or mutant (mt) in the figure. Cells were incubated for the indicated time points in 5ml of normal media treated with 50μg/ml of cycloheximide. Cells were then harvested at the indicated time points and western blot analysis performed with proteins probed for as indicated in the figure.

5.2.3 Detecting HSP90 inhibition by geldanamycin using HSP70 as a control

Throughout this chapter we have postulated that some mechanism, possibly the HSP90 chaperone complex for example, may exist to promote post translational stabilisation of p53 and MDM2. As described in the introduction (see section 1.8.4) the HSP90 chaperone can be inhibited with the ansamycin antibiotic geldanamycin (GA). The following experiments were designed to evaluate if p53 and MDM2 steady state levels

were altered after inhibition of the HSP90 complex with GA. Before the effects of GA could be evaluated in the RCC cell lines a suitable control of HSP90 inhibition was needed. As discussed in the introduction, inhibition of the HSP90 chaperone complex by GA results in release of HSF1 and subsequent transcription of Heat shock proteins, including HSP70. HSP70 was selected as a control with increased levels expected if HSP90 has been inhibited. The required concentration of GA needed to bring about this increased transcription of HSP70 in the RCC cell lines was unknown, as was the optimum amount of time taken to leave the cells exposed to GA. The expression of HSP70 in the RCC cell lines and therefore the optimum concentration of primary antibody for western blotting were also unknown. The 121 cell line was chosen to be initially investigated as it has high (mutant) p53 and MDM2 expression and undergoes proteasomal degradation. It was felt that once the control was optimised this would be a good cell line to use to evaluate the effects of GA on p53 and MDM2. Figure 5.2.3 shows the effects of incubating 121 cells for 2 and 8 hours in the presence of 2μmol/L or 8μmol/L concentrations of GA. A 2 hour DMSO treatment was used as a control. The Stressgen™ anti HSP70 antibody was used at 1, 0.1 and 0.01μg/ml concentrations. The results shown are using 0.01 ug/ml, as other concentrations resulted in gel burn out at very low exposures. Actin was detected as a loading control. Both concentrations of GA caused an increase in HSP70 levels.

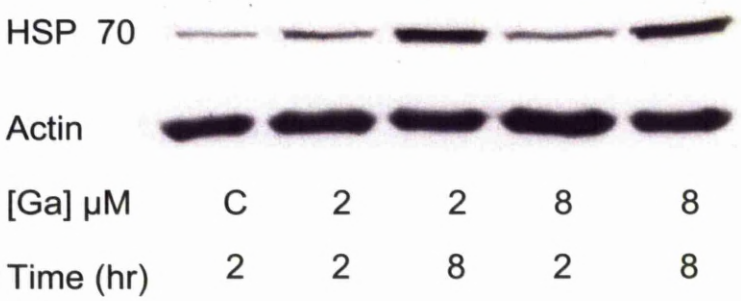
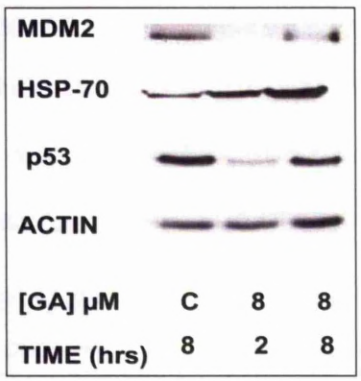


Figure 5.2.3 Western blot of the 121 RCC cell line treated with the HSP90 inhibitor geldanamycin. Cells were treated for the indicated time points with media containing geldanamycin (GA) at a concentration of 2μmol/L or 8μmol/L. The control (C in figure) comprised 121 cells treated for 2 hours with the equivalent volume of DMSO as the volume of geldanamycin used to give a concentration of 2μmol/L. Proteins were probed for as indicated in the figure.

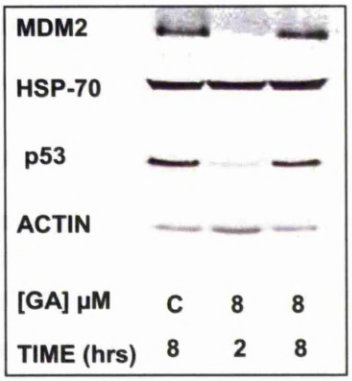
5.2.4 The effect of inhibition of HSP90 on expression of p53 and MDM2 in RCC cell lines

To investigate whether the steady state levels of p53 or MDM2 were altered after HSP90 inhibition with GA, a selection of RCC cell lines with high p53 and MDM2 protein expression profiles were treated with GA at a concentration of 8μmol/L for 2 and 8 hours. Western blotting was used to detect changes in protein levels (see Figure 5.2.4.1). In figure 5.2.4.1, cell lines A498, ACHN, CAKi-1 and CAKi-2 harbour wild type p53 whereas 121 harbours a mutant p53. These five cell lines all show evidence of a

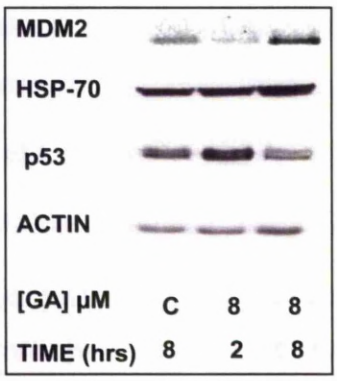
reduction in the steady state level of MDM2 at 2 hours which has recovered at 8 hours. This is a new finding as previously GA has only been shown to reduce the steady state levels of MDM2, in the presence of mutant p53[156]. The 121 mutant p53 line in panel E shows a small reduction in the steady state level of p53 at 2 hours and further at 8 hours. This is in contrast to the other 4 cell lines where any change in p53 steady state levels are only seen at 2 hours. The RCC cell lines tested here have high steady state levels of MDM2 (relative to other cell RCC cell lines see Figure 1.5) approaching those seen after transfection of MDM2 (see figure 4.3.5.2). This relative high abundance of MDM2 may be a factor for the reduction in p53 steady state levels seen when HSP90 is inhibited with GA, as Burch et al[165] reported MDM2/HSP90 complexes can unfold p53 and degrade it in the presence of GA, with transfected MDM2. Caki-1 cell line demonstrated a decrease in MDM2 but an increase in p53 steady state levels at 2 hours and no change in either protein after 8 hours. The effect seen in this experiment seem to be an anomaly as repeat testing (Figure 5.2.5) shows Caki-1 to fit with the earlier model proposed. In summary this experiment shows cell line specific effects of GA on p53 and MDM2 levels.



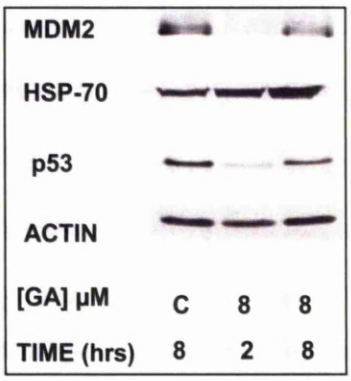
A – ACHN



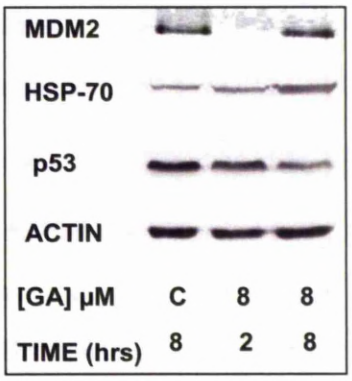
B – A498



C – Caki-1



D – Caki-2



E – 121

Figure 5.2.4.1 – Western blot of p53 and MDM2 levels in 5 RCC cell lines treated with geldanamycin. Each RCC cell line was treated for 2 or 8 hours with 8µmol/L of geldanamycin (GA). The control (C in panels) was the cells treated with an equal volume of DMSO to that used for to give 8µmol/L of geldanamycin, again for 8 hours. HSP70 was used as a control for HSP90 inhibition. Proteins were probed for as indicated in the figure.

To further evaluate the effects of HSP90 inhibition with GA treatment, cell lines were treated with GA at a concentration of 8μmol/L for various time points ranging from 0 to 8 hours. A498 was treated with GA at a concentration of 20μmol/L to see if a higher dose would have more of an effect. Figure 5.2.4.2 shows cell lines 121, ACHN, A498 and Caki-1 treated with GA over a time course. From this experiment it can be seen that the mutant p53 line, 121, shows a reduction in p53 steady state levels starting at 1 hour and maximising at 6 hours. In the other three (wild type) cell lines, p53 levels are reduced maximally at 2 hours and are returned to base line levels at 6 hours. In all cell lines the decreases in MDM2 levels mirror the changes in p53. There does appear to be a clear difference between the 121 and the three "wild type p53" cell lines. 121 shows a continual reduction in p53 and MDM2 steady state levels over the time course 1 hour to 6 hour time points, whereas the three wild type lines do not show further reductions in protein levels after the initial decrease seen at 2 hours. Although only one mutant p53 cell line had been evaluated, these results led us to ask the following questions - was the p53 and MDM2 reductions in steady state levels due to degradation by the proteasome? Secondly is the reduction in steady state levels maintained if protein synthesis is inhibited with the translation inhibitor cycloheximide? Could the transient down regulation be due to loss of GA efficacy over time? These points are addressed in the next section.

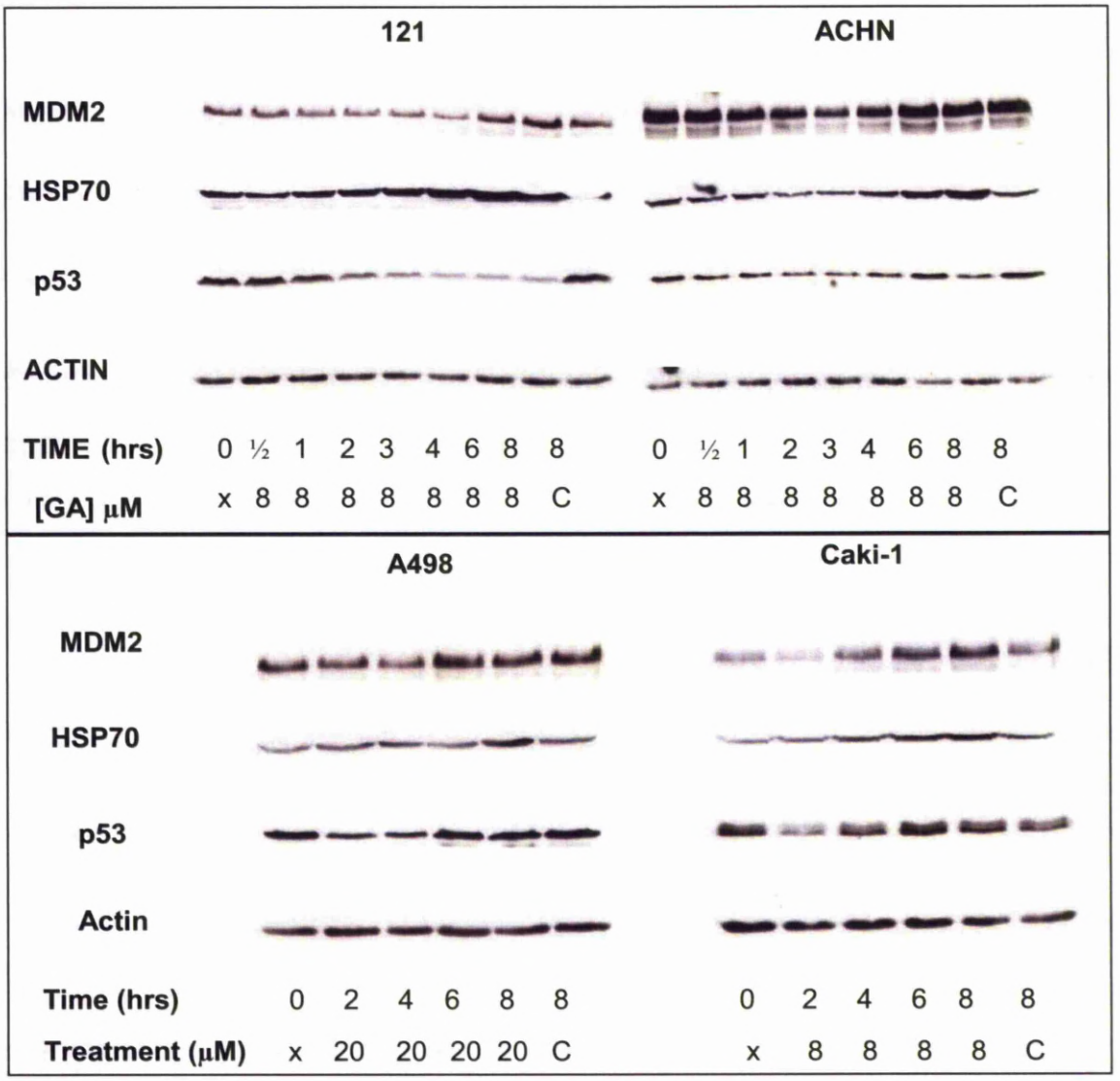


Figure 5.2.4.2 Western blot showing p53 and MDM2 protein expression in RCC cells treated with geldanamycin. Three RCC cell lines were treated for 2 or 8 hours with geldanamycin (GA) at a concentration of 2µmol/L, A498 (bottom panel) was treated with geldanamycin (GA) at a concentration of 20µmol/L. Each cell line had two controls, firstly no treatment (lane labelled X in figures) and secondly (lane labelled C in figures) the cell line treated with an equal volume of DMSO to that used for the treatment dose of GA, for 8 hours. Proteins were probed for as indicated in the figure.

5.2.5 MDM2 and p53 levels after treatment with Geldanamycin and MG115 in UOK – 121

If the decrease in p53 and MDM2 protein levels seen after HSP90 inhibition with GA is due to degradation by the proteasome then it would be expected that this effect would be reduced by proteasome inhibition using MG115. To test this hypothesis 121 cells were treated with GA at a concentration of 2μmol/L, MG115 at a concentration of 10μmol/L or both. The cells were harvested after 2, 4, 6 and 8 hours of treatment and underwent protein extraction and western blotting to assess the levels of p53 and MDM2 (see figure 5.2.5). For each time point the levels of p53 in the double treated (GA and MG115) are intermediate to those in the GA (lowest) and MG115 (highest) and approaching the untreated levels (Time 0 in figure 5.2.5). The same trend was also observed for MDM2 and in exactly the same way as experiment 5.2.4.2, the maximum reduction of MDM2 steady state levels was seen at 2 and 4 hours with GA treatment. It would have been expected that if p53 and MDM2 are chaperoned by HSP90 and the decrease in their steady state levels upon HSP90 inhibition is due to proteasomal degradation, then treatment with both GA and MG115 would lead to the same levels of protein stabilisation as treatment with MG115 alone. The fact that dual treatment (MG115 & GA) led to an intermediate level (i.e. between the lowest - GA alone and highest MG115 alone) is intriguing. One possible explanation is that some of the chaperoned p53 or MDM2 is being degraded by a non proteasomal mechanism, for example caplain[166].

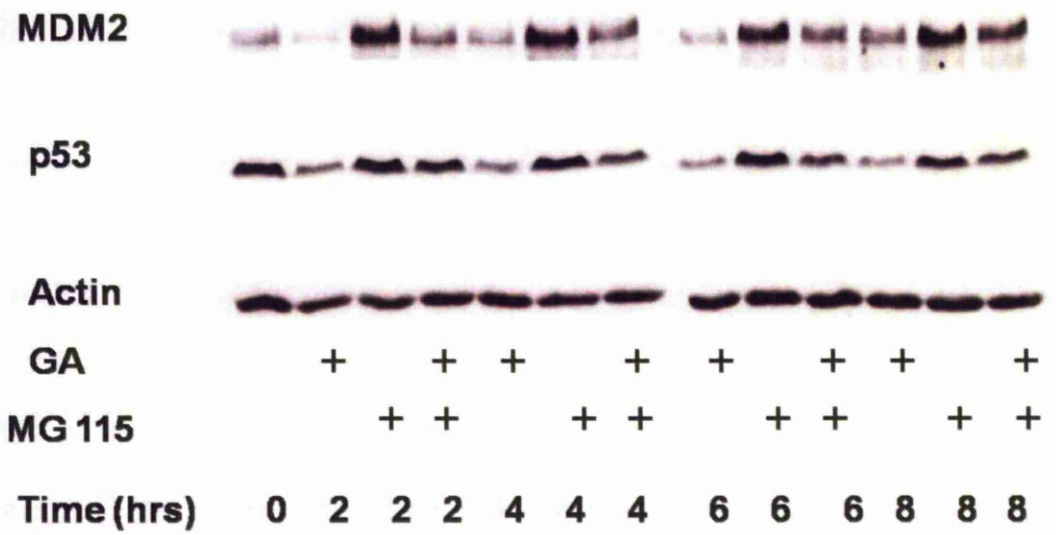


Fig 5.2.5 Western blot showing p53 and MDM2 protein levels in 121 RCC cells treated with geldanamycin and the proteasome inhibitor MG115. The cells were treated (indicated by an X in figure) with geldanamycin (GA) at a concentration of 2μmol/L or MG115 at a concentration of 10μmol/L5 or for 2, 4, 6 & 8 hours. Proteins were probed for as indicated in the figure.

5.2.6 Analysis of potential in vitro interactions between MDM2 and HSP90 by affinity chromatography

It has been observed that steady state levels of p53 and MDM2 decrease after HSP90 inhibition with GA. This suggests that these molecules are chaperoned by HSP90 however it is possible that the effects seen on p53 and MDM2 levels may be indirect, possibly due to the action of another HSP90 client protein being liberated from its interaction with the chaperone for example. Therefore it was decided to investigate whether HSP90 does form a complex with MDM2. A laboratory co-worker (MM) had previously constructed and tested a His6-tagged recombinant human MDM2 (His6-rMDM2) column to investigate possible MDM2 binding partners[167]. In this experiment the His6-rMDM2 column was first washed with 20 column volumes of SLIP buffer and blocked with 1 mg/ml bovine serum albumin (BSA) in 12 ml of SLIP buffer for 16 h before application of 15 mg of filtered human embryonal kidney (HEK) 293 cell cleared lysate also for 16 h. The column was washed with 50 column volumes of SLIP buffer and exposed to a 0 to 1mol/L imidazole gradient, and the eluate was collected in 3-mL fractions. Proteins associated with MDM2 would be eluted in the same fraction as the histidine tagged recombinant MDM2. This is because as the imidazole gradient increases, imidazole (histidine analogue) competes with the histidine tagged MDM2 for column binding causing displacement of the latter from the column. A second (BSA control) column (using BSA as both binding and blocking agent) was treated in the same way. The various eluates from this experiment were probed for HSP90 and HSP70 using the primary antibodies anti HSP90α 1:250 and anti HSP70 1μg/ml.

HSP90 was detected strongest in eluate fractions 5 & 6 along with MDM2 and known MDM2 interacting proteins MDMX and p53. In the same way – western blotting of the eluates from the BSA control column reveals HSP90 in fractions 2 & 3 again the same as for MDM2, MDMX and p53. In contrast HSP70 (not part of the HSP90 chaperone complex) is detected in most of the eluate fractions from the MDM2 column suggesting is does not co-purify. This experiment suggests that from this cell line MDM2 and HSP90 can form an in vitro complex and may interact directly. To test if there is an in vivo association co-immunoprecipitation experiments would need to be conducted.

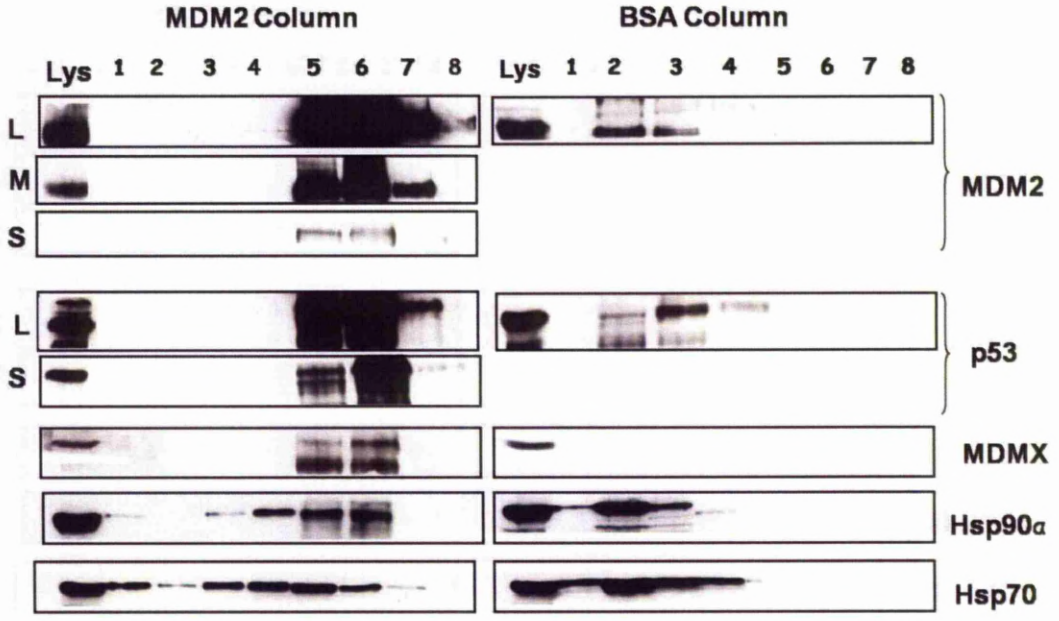


Fig 5.2.6 Western blot of MDM2 and BSA affinity chromatography column fractions to evaluate if MDM2 co-purifies with HSP90 in Human Embryonal Kidney cell line 293. Left hand side of figure represents immunoblotting of lysates from the His6-tagged recombinant human MDM2 column and the right side immunoblotting of lysates from the BSA control column. Each column was washed with 50 column volumes of SLIP buffer and exposed to a 0 to 1mol/L imidazole gradient, and the eluate was collected in 3-mL fractions. Lysates from each fraction (labeled 1 to 8) underwent immunoblotting to detect the proteins indicated in the figure. Lys = lysate, L = long exposure, M = medium exposure and S = short exposure.

5.3 Discussion

In order to try and explain the high levels of p53 and MDM2 expressed in our RCC cell line panel the role of proteasomal degradation and the heat shock 90 chaperone complex have been investigated. As has been shown, inhibition of the proteasome leads to stabilisation of p53 and MDM2 in the majority of cell lines tested and thus it seems that reduced degradation is an unlikely explanation for increased expressed levels of p53 and MDM2. All p53 wild type cell lines showed p53 stabilisation. It can be seen from experiment 5.2.1 it can be seen that 121 and A704 cell lines harbour mutant p53 that is degraded by the proteasome and therefore the mechanism of their high p53 levels remain unknown but in the case of 121 there is evidence that the HSP90 chaperone may act to stabilise p53 and MDM2 (see below).

The HSP90 chaperone complex was known to "chaperone" / stabilise mutant p53 and transfected i.e. over-expressed MDM2[156]. It has been demonstrated that in some RCC cell lines p53 and MDM2 protein levels decrease following HSP90 inhibition and undergo proteasomal degradation. In one mutant p53 line (121) the decrease in p53 steady state levels is more pronounced lasting up to 8 hours. If high levels of p53 or MDM2 or both are responsible (directly or indirectly) for the oncogenic properties of the cell, HSP90 inhibition may be useful therapeutically, particularly in cancers with a dominant negative mutant p53.

This series of experiments has shown the degree of variation that exists between cancer cell lines in culture even when they are propagated from clinically "similar" tumours. Potential therapeutic options may have to be tailored to individual patient tumours rather than based traditionally on histological typing and stage.

CHAPTER 6 – DISCUSSION

6.1 Summary of findings

The original hypothesis for this study was that p53 and MDM2 up-regulation in RCC predicates poor prognosis. Since this idea is based upon only one clinical study and some in vitro data we therefore set out to investigate this question in a local cohort of patients and if confirmed, to perform in vitro studies to investigate this at the cell and molecular level. The list below summarises the findings:

1) In the first instance this study has confirmed that p53 and MDM2 are up-regulated in a subset of RCCs. Interestingly, there is a highly significant correlation between RCC tumours that express p53 and MDM2

2) It has been demonstrated that patients with tumours that express both p53 and MDM2 have a poorer prognosis compared to those tumours that do not express both p53 and MDM2.

3) The mechanisms underlying the observed up-regulation of p53 and MDM2 in RCC were investigated. The up-regulation of p53 and MDM2 is not due to a complete failure of proteasome-mediated degradation, since inhibition of proteasomal function in RCC cell lines in culture leads to an appropriate increase in p53 and MDM2 levels in the majority of cases. Similarly inhibition of the HSP90 chaperone complex with GA, leads to a transient reduction in steady state levels of wild type and mutant p53 and MDM2, in

certain RCC cell lines. The HSP90 chaperone complex may therefore have a role in the up-regulation of p53 and MDM2 in RCC

4) In an attempt to understand the role MDM2 up-regulation may play in the poor prognosis seen in RCC that express it in high levels, the UOK-117 RCC was manipulated in a series of experiments in order to generate clones that express high levels of MDM2. It was not possible to generate these clones. Further work is needed to understand the physiological reasons why these cells are will not tolerate high MDM2 levels.

6.2 General discussion

In order for further progress to be made into treating renal cell carcinoma, it is desirable to understand the molecular mechanisms predisposing to poor prognosis and why some patients with tumours of small size and therefore lower stage, still develop metastases despite apparent complete surgical excision? It seems likely that such tumours must have acquired metastatic capability at a very early stage in their development, before surgical treatment. Clearly this is not the case for all RCC tumours, so one must conclude that either the time at which tumours develop metastatic potential is variable (dependent on when specific mutations in key regulatory pathways occur) or, and this seems less likely, there are other unknown patient factors (for example host/tumour immune system interaction) which exist to suppress metastases until the tumour is mutated in further ways to promote metastases. Some patients may lack this putative "patient factor" and could be more susceptible to metastases from lower stage tumours.

As detailed in section (1.1.4.2.1) the VHL molecule and its associated pathways are known to play an important role in RCC. Despite the use of new molecular therapies inhibiting VHL downstream targets e.g. kinases, patients with metastatic RCC are still not cured. This fact supports the need for further research into other oncological molecular pathways in RCC for additional potential therapeutic possibilities. Our study was designed to evaluate what role the p53/MDM2 pathway played in RCC. The p53/MDM2 pathway was chosen as it has oncological importance in other malignancies (see section 1.2), observations that p53 expression in RCC may be prognostically useful (see section 1.4.1) and the paper by Haitel et al[96] which had evaluated RCC tumours expressing both p53 and MDM2 and found this to confer a poorer prognosis. p53 positive staining occurred in 15.6% of the RCC specimens evaluated in our cohort (Haitel et al[96] reported 36%) , which is slightly lower than the majority of published large series looking at p53 staining in RCC (see section & Table). However, our cohort of patients all had low stage disease (T1 – T3) since only specimens taken when surgery with curative intent was performed were included. Thus, no samples were analysed from tumours in which metastases were present at the time of nephrectomy. Based purely on those studies summarised in section (3), it could be hypothesised that p53 positivity is higher in tumours from patients with metastatic disease.

The MDM2 positive staining tumours represented 26.7% of the samples analysed (18.6% of samples were MDM2 positive in the Haitel paper[96]). There was a highly statistically significant correlation between RCC specimens that expressed p53 and

MDM2. This correlation was also reported by Haitel[96] and Moch[105] suggesting a subset of RCCs can be defined by this IHC description.

The RCC specimens that expressed both p53 and MDM2 came from patients with a statistically significant poorer disease specific survival than those that did not express both these proteins. In fact in our analysis patients with tumours that were p53 and MDM2 positive were 3 times more likely to die compared to the rest of the cohort. Haitel et al[96] also showed patients with tumours that were "dual p53 and MDM2 positive", were associated with poorer prognosis on multivariate analyses. In another IHC study of only 50 patients, Moch et al[105] did not find p53 and MDM2 positive staining RCC tumours to be associated with poorer patient prognosis.

The major drawback of studies (including that described in this thesis) evaluating p53 immunohistochemical expression arises from uncertainty in interpreting what is meant by negative or normal p53 staining. For example tumours expressing low or normal p53 levels cannot be differentiated from tumours that may have deleted *p53* or be possibly inhibiting normal p53 expression by a hitherto unknown mechanism. Tumours with deleted or inhibited p53 function could also result in poorer patient prognosis[168] but will not be differentiated from "normal" p53 expressing RCC. As with most immunohistochemical analysis of protein expression only the finding of high, low or absent expression can be clinically correlated. It is impossible to infer the protein's function or structure unless specific antibodies are used that target mutated p53 or downstream targets of functional p53 for example.

Having confirmed that p53 and MDM2 expression occurs in a subset of RCC tumours, it was important to try and understand the consequence of up-regulation of these proteins in RCC. Previous attempts (From HW and the Boyd laboratory) to "manipulate " (through cloning) RCC cell lines with low steady state levels of p53 and MDM2 expression (e.g. UOK 117) into clones that stably express high p53 and MDM2 were not successful. The reasons for this failure may be because RCC cells require some permissive mutational event to occur (during disease progression) in order to acquire co-up-regulated p53 and MDM2, this event is not trivial and is absent when cells are forced to express p53 and MDM2 through cloning. Nevertheless, we found to our surprise that even though the UOK- 117 RCC cell line displays low levels of p53 and MDM2 and could not be made to stably express high levels of MDM2, when these cells were re-cloned , we obtained stable cell clones that spontaneously acquired up-regulated p53 and MDM2 (no MDM2 was transfected - please refer to section 4.xx). In order to try and discover what changes had taken place to allow this up-regulation of p53 and MDM2, seen in these two types of clones - the changes in gene expression between "parental" UOK – 117 and in the newly generated 117 clones that were "permissive" to the generation of high MDM2 stably expressing clones were to be investigated. Comparisons in gene expression between the three cell lines may reveal candidate genes responsible for allowing stable high MDM2 expression. If each of the three cell lines was manipulated to first express high levels of MDM2 and then undergo gene expression analysis – consequences of high MDM2 expression could also be evaluated by analysing changes in gene expression. It was hoped, as a result of this investigation that genes may be identified that, upon further research, could explain either the

consequences of high MDM2 expression or the mechanisms allowing RCC cell lines to acquire the poor prognostic features of high p53 and MDM2 up-regulation. For this experiment, a reproducible method of manipulating the experimental 117 cell lines to express high MDM2 had to be discovered. The cells only had to express high MDM2 levels transiently for the gene expression analysis experiment. Stable expression of MDM2 in these cells has not been possible therefore transient transfection was employed. RCC cell lines are notoriously hard to transfect to the same efficiency as other cancer cell lines. Unfortunately despite trying different commercially available methods of transient transfection, a reliable means of achieving high level transfection efficiency, could not be found. A new strategy for obtaining transient high MDM2 expression was therefore required. Clones could not be made that would stably express high MDM2 levels - however it may be possible to generate clones that will inducibly express high MDM2 levels. The commercially available Tet-on inducible expression system from ClonTech™ was employed with the substitution of a modified rtTA plasmid which encodes a mutated form of the reverse transcriptional transactivator that is more sensitive to doxyxcycline, more stable and displays a lower background expression of the gene of interest (kindly supplied by, P Welman of the Paterson Institute, Manchester (see section 4.3.1 for explanation). To generate clones using this system which would inducibly express MDM2, two rounds of cell cloning are required. The first stage involves generating clones expressing the doxycycline responsive element "modified rtTA". This process was successfully carried out for each of the three experimental cell lines (determined functionally using a transient assay to show that doxycycline treatment did indeed lead to rtTA activation of a pTight reporter construct).

The second round of cloning involved generating clones that had incorporated the pTre-Tight-MDM2 plasmid. The pTre-Tight-MDM2 plasmid does not contain an antibiotic resistance sequence. Instead a linear antibiotic resistance marker is co-transfected with pTre-Tight-MDM2 to allow clonal selection to take place when cells are cultured in an antibiotic treated medium. The derived 117 clones were already resistant to Hygromycin and G418 – therefore the only other available linear antibiotic marker produced from the manufacturer (Clontech™) that could be used on the cell lines of interest was puromycin. Unfortunately no puromycin selection marker could be obtained from ClonTech™ due to a manufacturing problem. Due to time constraints rather than waiting for the marker to become available or attempting to clone a puromycin resistance sequence into the pTre-Tight-MDM2 plasmid the experiment was modified. The parental UOK-117 cell line could still undergo the second round cloning to become MDM2 inducible because unlike the other two 117 derived clones, it was not resistant to Hygromycin (from previous cloning) and a linear Hygromycin selection marker was available from ClonTech™. Therefore comparison between gene expression profiling in the UOK-117 cell line upon induction of MDM2 would be undertaken, if an inducible MDM2 expressing 117 clone could be made. A pTre-Tight-RING (inducible MDM2RFM) plasmid had been cloned previously. The opportunity was taken to attempt to generate 117 clones that would also inducibly express MDM2RFM. It was hoped that by comparing gene expression before and after induction of high MDM2 levels in the 117 cell line - a subset of genes would be identified, some of which would be a consequence of MDM2 expression. When 117 cells were made to express high levels of MDM2RFM another set of genes would be identified. By comparing this set of genes

with those upon unmutated MDM2 expression it may be easier to identify those genes expressed as a consequence of MDM2 and perhaps identify genes that require the ubiquitin ligase function of MDM2 (absent in the ring finger mutant).

Stably expressing inducible ring finger mutant MDM2 clones were successfully generated, the "normal" MDM2 inducible counterparts were not. Why this is occurred is not clear but there are two possible explanations; firstly there were simply not enough candidate colonies selected for testing. It is possible that if a greater number of colonies were selected a MDM2 inducible clone may have been identified. The second explanation may be that even under "pTight" control, enough additional MDM2 is still constitutively expressed and this results in cell death (from an unproven mechanism (likely to depend upon the ubiquitin ligase activity of MDM2) and prevents stable colonies from being generated. This is in keeping with HW's original finding that 117 cells could not be made to stably express MDM2. Were it possible to have used the MDM2 tolerant 117 derived clones, some positive clones may have been generated. This intriguingly leads back to one of the original questions as to why some RCC can tolerate and express such high MDM2 levels.

Our hypothesis is that p53 and MDM2 expression is associated with poor prognosis in RCC and therefore how this event occurs was also investigated. Experiments were carried out in RCC cell lines to try and explain how some RCC cell lines in culture demonstrate high steady state levels of p53 and MDM2 expression (see section 1.5), the hope being the same mechanism applies to RCC cell lines in culture to that occurring in

RCC tumours. Previous work in the Boyd laboratory has shown that high steady state levels of p53 and MDM2 are not explained by p53 mutation or by amplification of the MDM2 gene which has been identified as a cause of high MDM2 expression in other types of tumours[72]. One possible explanation for the observed high levels of p53 and MDM2 seen in some RCC cell lines is that in these cell lines both proteins are not undergoing "normal" degradation by the proteasome. RCC cell lines were treated with a proteasome inhibitor if p53 and MDM2 were not undergoing proteasomal degradation, it would be expected that their steady state levels would be unchanged. As demonstrated in chapter 5 (section 5.2.1) the majority of cell lines exhibited an increase in expressed levels of p53 and MDM2 after proteasome inhibition, indicating that p53 and MDM2 are degraded by the proteasome. Therefore another explanation for high levels of p53 and MDM2 steady state levels was needed.

It was hypothesised that the HSP90 chaperone complex was responsible for the high steady state levels of p53 and MDM2. HSP90 has been shown to have a mutated p53 as a client protein (please refer to section 1.7.3) for an overview of HSP90 function. Could HSP90 interaction with p53 and MDM2 result prevent degradation of these proteins and lead to higher steady state levels? To test this hypothesis the HSP90 chaperone was inhibited in a series of experiments and changes in p53 and MDM2 steady state levels were observed. Inhibition of HSP90 by geldanamycin resulted in cell-line dependent effects on p53 and MDM2 steady state levels. A number of cell lines showed a transient reduction in p53 and MDM2 steady state levels and this effect was seen in both wild type and mutated p53 lines. From these experiments it is not possible to say whether the

changes seen in p53 steady state levels are due directly to the effects of "chaperoning" by HSP90 or indirectly from other (unknown) HSP90 client protein interactions with p53/MDM2 after inhibition of HSP90 Further experimentation is needed to establish the exact mechanisms of the effect seen on p53 and MDM2 when HSP90 is inhibited. Despite our ignorance of the exact mechanisms responsible to the reductions in p53 and MDM2 levels upon HSP90 inhibition, it may still be of clinical interest if it can be shown that restoring normal p53 levels in RCC cells is therapeutic. HSP90 is known to chaperone other proteins important in oncogenesis, therefore inhibiting its action in RCC may have other beneficial anti-cancer effects and HSP90 should be further researched.

One observation from this thesis and from that of my predecessor (HW) is the heterogeneity of RCC cell lines in culture. Cell lines not only look very different when viewed under the microscope but show marked variations in expression of proteins (MDM2 and p53) and respond in different ways to HSP90 and 26S proteasome inhibition although the cell lines were established from the same histological types of RCC (ccRCC in most cases). When one considers that clinically RCC tumours are grouped on histological type (e.g. ccRCC, chromophobe etc) alone, it is obvious that this may be an oversimplification as it does not inform us as to what is happening at the molecular level. This may have important clinical implications as we do not know all the molecular changes that are important in responding to currently available systemic treatments. As a result of this we may be exposing patients to the side effects of systemic therapy when they are unlikely to achieve a survival benefit, because they do not have the necessary molecular "signature" for them to respond. In the UK healthcare system, where access to these drugs is limited, it is desirable for economic reasons to target

appropriate patients. There might be real benefits for using targeted therapies even if the target population is small, providing we can identify it with some confidence. What is needed, as is often the case in medicine, is for a prospective clinical trial to "molecularly characterise" tumours and follow these patients up to investigate if certain molecular traits do predict altered outcome and response to systemic treatment. It is hoped that any such trial would include p53 and MDM2.

6.3 Clinical implications

6.3.1 Inhibiting HSP90

HSP90 is known to have a number of client proteins, thought to be important in promoting malignancy (see section 1.7.1). The findings presented in this thesis, that HSP90 inhibition can lead to a decrease in p53 and MDM2 levels, may have potential for clinical benefit. Ronnen et al[169] found no clinical effect in treating 20 RCC patients using 17-AAG (an analogue of GA); this is the only published study evaluating HSP90 in RCC, but these authors did not examine the p53/MDM2 status of the patients. Our findings would suggest that the effects of HSP90 inhibition might be specific to certain tumours. Grouping patients by the molecular characterisation of their tumours (e.g. identifying tumours that expressing high levels of p53/MDM2 or HSP90), may allow better identification of therapeutic drug benefit in clinical trials.

6.3.2 MDM2 and p53 in RCC prognostication

At least 10% of patients undergoing nephrectomy for apparently localised RCC will unfortunately develop metastases during their follow up[170]. The results of the data presented in this thesis suggest that patients with tumours staining positive for p53 and MDM2 may be at higher risk of death from metastatic RCC. Immunostaining nephrectomy specimens for these markers may help target high risk tumour patients suitable for adjuvant treatment.

6.4 Conclusions

The results of this study demonstrate that:-

1) Nephrectomy specimens with positive immunohistochemical staining for both p53 and MDM2 – identify a sub group of patients with poor prognosis. This is independent of other clinical factors such as tumour stage. There is an extremely high association of tumours staining for both p53 and MDM2.
2) RCC cell lines in culture differ in response to treatment with substances that inhibit the 26S proteasome and the HSP90 chaperone. Some RCC cell lines appear to have wild type p53 that is chaperoned by HSP90, this is a new observation and may be of clinical implication.

In addition, we have also attempted to investigate the consequences of MDM2 expression in RCC cells and to this end have generated cells that could potentially be used to generate inducible MDM2 expressing cells. However an initial attempt at

this latter was unsuccessful and thus further work will be required to determine whether this can be accomplished.

6.5 Future research

A larger, prospective and appropriately powered study to evaluate whether immunohistochemical staining for p53 and MDM2 does predict poor prognosis post nephrectomy would be valuable. This study would need to be designed with a clear definition for what constitutes p53 and MDM2 positivity with validated intraobserver reliability. If p53 and MDM2 are confirmed to provide prognostic information for patients, then this "marker" should be evaluated alongside existing RCC prognostic nomograms to determine if this description adds further prognostic information. It is important to demonstrate that the poor prognosis seen in patients with tumours that express p53 and MDM2 is a direct effect of these proteins. It is possible that high p53 and MDM2 expression in RCC is a consequence of some other molecular pathway change that is responsible for poor prognosis. This is obviously important if a molecular therapy is to be developed for patients with tumours expressing p53 and MDM2.

If an inducible MDM2 expressing UOK-117 cell line clone can be generated and also in the MDM2 tolerant clones, then a gene expression profiling experiment could be performed (as originally intended at the beginning of this study) that may detect changes in gene expression when MDM2 is expressed. Specific genes or pathways may be identified that can explain how tolerance to MDM2 can arise. Experiments could be

performed to see if the acquisition of high MDM2 expression conferred a tumour advantage such as invasion or motility. The acquired high MDM2 UOK – 117 clones would be suitable for such an experiment with low MDM2 expressing parental cell line acting as a control.

HSP90 should be further researched in the setting of RCC. It would be desirable to confirm if p53 and MDM2 are HSp90 client proteins. Existing or novel drugs targeting HSP90 may be able to restore normal levels of p53 and MDM2 which may be of therapeutic potential.

REFERENCES

1. Cancer_Research, UK. UK Kidney Cancer Incidence Statistics. Available from http://info.cancerresearchuk.org/cancerstats/types/kidney/incidence/ [accessed January 11th, 2011]

2. Office for National Statistics. Cancer. Registrations of cancer diagnosed in 2007, England. Series MB1 no.38. 2010, National Statistics: London.

3. Ferlay J, Autier P, Boniol M, Heanue M, Colombet M, Boyle P. Estimates of the cancer incidence and mortality in Europe in 2006. *Ann Oncol* 2007;18(3):581-92.

4. Motzer RJ, Bander NH, Nanus DM. Renal-cell carcinoma. *N Engl J Med* 1996;335(12):865-75.

5. Lindblad PaAH. Textbook of Cancer Epidemiology: Oxford University Press: New York, 2002:467 - 85.

6. American_Cancer_Society. Cancer Facts & Figures 2010. Available from http://www.cancer.org/Research/CancerFactsFigures/CancerFactsFigures/cancer-facts-and-figures-2010 [accessed January 11th, 2011]

7. Registrations of cancer diagnosed in 2002, England. In: Office for National Statistics London, 2005.

8. Chow WH, Devesa SS, Warren JL, Fraumeni JF, Jr. Rising incidence of renal cell cancer in the United States. *JAMA* 1999;281(17):1628-31.

9. Eble JN SG, Epstein JI, Sesterhenn IA. . Pathology and genetics. Tumours of the urinary system and male genital organs. . Lyon: IARC Press 2004.

10. Vira MA, Novakovic KR, Pinto PA, Linehan WM. Genetic basis of kidney cancer: a model for developing molecular-targeted therapies. *BJU Int* 2007;99(5 Pt B):1223-9.

11. Lopez-Beltran A, Scarpelli M, Montironi R, Kirkali Z. 2004 WHO classification of the renal tumors of the adults. *Eur Urol* 2006;49(5):798-805.

12. Noon AP, Smith DJ, McAndrew P. Magnetic Resonance Imaging Characterization of a Mucinous Tubular and Spindle Cell Carcinoma of the Kidney Detected Incidentally During an Ectopic Pregnancy. *Urology* 2009: 75(2): 247 - 248.

13. Oda H, Nakatsuru Y, Ishikawa T. Mutations of the p53 gene and p53 protein overexpression are associated with sarcomatoid transformation in renal cell carcinomas. *Cancer Res* 1995;55(3):658-62.

14. Perret AG, Clemencon A, Li G, Tostain J, Peoc'h M. Differential expression of prognostic markers in histological subtypes of papillary renal cell carcinoma. *BJU Int* 2008: 102(2): 183 - 187

15. Patard JJ, Leray E, Rioux-Leclercq N, et al. Prognostic value of histologic subtypes in renal cell carcinoma: a multicenter experience. *J Clin Oncol* 2005;23(12):2763-71.

16. Cheville JC, Lohse CM, Zincke H, Weaver AL, Blute ML. Comparisons of outcome and prognostic features among histologic subtypes of renal cell carcinoma. *Am J Surg Pathol* 2003;27(5):612-24.

17. Motzer RJ, Bacik J, Mariani T, Russo P, Mazumdar M, Reuter V. Treatment outcome and survival associated with metastatic renal cell carcinoma of non-clear-cell histology. *J Clin Oncol* 2002;20(9):2376-81.

18. de Peralta-Venturina M, Moch H, Amin M, et al. Sarcomatoid differentiation in renal cell carcinoma: a study of 101 cases. *Am J Surg Pathol* 2001;25(3):275-84.

19. Eggener S. TNM staging for renal cell carcinoma: time for a new method. *Eur Urol*;58(4):517-9; discussion 19-21.

20. Frank I, Blute ML, Leibovich BC, Cheville JC, Lohse CM, Zincke H. Independent validation of the 2002 American Joint Committee on cancer primary tumor classification for renal cell carcinoma using a large, single institution cohort. *J Urol* 2005;173(6):1889-92.

21. Fuhrman SA, Lasky LC, Limas C. Prognostic significance of morphologic parameters in renal cell carcinoma. *Am J Surg Pathol* 1982;6(7):655-63.

22. Tsui KH, Shvarts O, Smith RB, Figlin RA, deKernion JB, Belldegrun A. Prognostic indicators for renal cell carcinoma: a multivariate analysis of 643 patients using the revised 1997 TNM staging criteria. *J Urol* 2000;163(4):1090-5; quiz 295.

23. Lipworth L, Tarone RE, McLaughlin JK. The epidemiology of renal cell carcinoma. *J Urol* 2006;176(6 Pt 1):2353-8.

24. McLaughlin JK, Lipworth L. Epidemiologic aspects of renal cell cancer. *Semin Oncol* 2000;27(2):115-23.

25. Bergstrom A, Hsieh CC, Lindblad P, Lu CM, Cook NR, Wolk A. Obesity and renal cell cancer--a quantitative review. *Br J Cancer* 2001;85(7):984-90.

26. Gago-Dominguez M, Castelao JE, Yuan JM, Ross RK, Yu MC. Lipid peroxidation: a novel and unifying concept of the etiology of renal cell carcinoma (United States). *Cancer Causes Control* 2002;13(3):287-93.

27. Calle EE, Kaaks R. Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. *Nat Rev Cancer* 2004;4(8):579-91.

28. Kleihues P, Schauble B, zur Hausen A, Esteve J, Ohgaki H. Tumors associated with p53 germline mutations: a synopsis of 91 families. *Am J Pathol* 1997;150(1):1-13.

29. Bond GL, Hu W, Bond EE, et al. A single nucleotide polymorphism in the MDM2 promoter attenuates the p53 tumor suppressor pathway and accelerates tumor formation in humans. *Cell* 2004;119(5):591-602.

30. Linehan WM, Walther MM, Zbar B. The genetic basis of cancer of the kidney. *J Urol* 2003;170(6 Pt 1):2163-72.

31. Gnarra JR, Tory K, Weng Y, et al. Mutations of the VHL tumour suppressor gene in renal carcinoma. *Nat Genet* 1994;7(1):85-90.

32. Semenza GL. VHL and p53: tumor suppressors team up to prevent cancer. *Mol Cell* 2006;22(4):437-9.

33. Baba M, Hong SB, Sharma N, et al. Folliculin encoded by the BHD gene interacts with a binding protein, FNIP1, and AMPK, and is involved in AMPK and mTOR signaling. *Proc Natl Acad Sci U S A* 2006;103(42):15552-7.

34. Pavlovich CP, Walther MM, Eyler RA, et al. Renal tumors in the Birt-Hogg-Dube syndrome. *Am J Surg Pathol* 2002;26(12):1542-52.

35. Volpe A, Panzarella T, Rendon RA, Haider MA, Kondylis FI, Jewett MA. The natural history of incidentally detected small renal masses. *Cancer* 2004;100(4):738-45.

36. Berger A, Crouzet S, Canes D, Haber GP, Gill IS. Minimally invasive nephron-sparing surgery. *Curr Opin Urol* 2008;18(5):462-6.

37. Russo P. Partial nephrectomy for renal cancer: Part I. *BJU Int*;105(9):1206-20.

38. Kutikov A, Kunkle DA, Uzzo RG. Focal therapy for kidney cancer: a systematic review. *Curr Opin Urol* 2009;19(2):148-53.

39. Klatte T, Marberger M. High-intensity focused ultrasound for the treatment of renal masses: current status and future potential. *Curr Opin Urol* 2009;19(2):188-91.

40. Coppin C, Le L, Porzsolt F, Wilt T. Targeted therapy for advanced renal cell carcinoma. *Cochrane Database Syst Rev* 2008(2):CD006017.

41. NICE, Bevacizumab (first-line), sorafenib (first- and second-line), sunitinib (second-line) and temsirolimus (first-line) for the treatment of advanced and/or metastatic renal cell carcinoma. (TA178). London: National Institute for Health and Clinical Excellence., 2009.

42. Motzer RJ, Hutson TE, Tomczak P, et al. Sunitinib versus interferon alfa in metastatic renal-cell carcinoma. *N Engl J Med* 2007;356(2):115-24.

43. Eisen T. Adjuvant Therapy in Renal Cell Carcinoma: Where Are We? 2007;6(7):492-98.

44. Biswas S, Kelly J, Eisen T. Cytoreductive nephrectomy in metastatic clear-cell renal cell carcinoma: perspectives in the tyrosine kinase inhibitor era. *Oncologist* 2009;14(1):52-9.

45. Lane DP, Crawford LV. T antigen is bound to a host protein in SV40-transformed cells. *Nature* 1979;278(5701):261-3.

46. Linzer DI, Levine AJ. Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. *Cell* 1979;17(1):43-52.

47. Linzer DI, Maltzman W, Levine AJ. The SV40 A gene product is required for the production of a 54,000 MW cellular tumor antigen. *Virology* 1979;98(2):308-18.

48. Hollstein M, Rice K, Greenblatt MS, et al. Database of p53 gene somatic mutations in human tumors and cell lines. *Nucleic Acids Res* 1994;22(17):3551-5.

49. Balint EE, Vousden KH. Activation and activities of the p53 tumour suppressor protein. *Br J Cancer* 2001;85(12):1813-23.

50. Nakamura Y. Isolation of p53-target genes and their functional analysis. *Cancer Sci* 2004;95(1):7-11.

51. Prives C, Hall PA. The p53 pathway. *J Pathol* 1999;187(1):112-26.

52. Wahl GM. Gatekeepers of the guardian: p53 regulation by post-translational modification, Mdm2 and Mdmx. In: Wiman PHKG, ed. 25 Years of p53 Research: Springer, 2005:96-99.

53. Sakaguchi K, Herrera JE, Saito S, et al. DNA damage activates p53 through a phosphorylation-acetylation cascade. *Genes Dev* 1998;12(18):2831-41.

54. Robins H AG, Harris S & Levine AJ. The first twenty five years of p53 research. In: Wiman PHKG, ed. 25 Years of p53 Research: Springer, 2005:96-99.

55. Wu X, Bayle JH, Olson D, Levine AJ. The p53-mdm-2 autoregulatory feedback loop. *Genes Dev* 1993;7(7A):1126-32.

56. Hardcastle I. Inhibitors of the MDM2-p53 interaction as anticancer drugs. *Drugs of the Future* 2007;20(8):511.

57. Iwakuma T, Lozano G. MDM2, an introduction. *Mol Cancer Res* 2003;1(14):993-1000.

58. Momand J, Zambetti GP, Olson DC, George D, Levine AJ. The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell* 1992;69(7):1237-45.

59. Fang S, Jensen JP, Ludwig RL, Vousden KH, Weissman AM. Mdm2 is a RING finger-dependent ubiquitin protein ligase for itself and p53. *J Biol Chem* 2000;275(12):8945-51.

60. Haupt Y, Maya R, Kazaz A, Oren M. Mdm2 promotes the rapid degradation of p53. *Nature* 1997;387(6630):296-9.

61. Kubbutat MH, Jones SN, Vousden KH. Regulation of p53 stability by Mdm2. *Nature* 1997;387(6630):299-303.

62. Argentini M, Barboule N, Wasylyk B. The contribution of the acidic domain of MDM2 to p53 and MDM2 stability. *Oncogene* 2001;20(11):1267-75.

63. Lindstrom MS, Jin A, Deisenroth C, Wolf GW, Zhang Y. Cancer-Associated Mutations in MDM2 Zinc Finger Domain Disrupt Ribosomal Protein Interaction and Attenuate MDM2-Induced p53 Degradation. *Mol Cell Biol* 2006.

64. Montes de Oca Luna R, Wagner DS, Lozano G. Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. *Nature* 1995;378(6553):203-6.

65. Gu W, Roeder RG. Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. *Cell* 1997;90(4):595-606.

66. Clegg HV, Itahana K, Zhang Y. Unlocking the Mdm2-p53 loop: ubiquitin is the key. *Cell Cycle* 2008;7(3):287-92.

67. Mani A, Gelmann EP. The ubiquitin-proteasome pathway and its role in cancer. *J Clin Oncol* 2005;23(21):4776-89.

68. Thrower JS, Hoffman L, Rechsteiner M, Pickart CM. Recognition of the polyubiquitin proteolytic signal. *Embo J* 2000;19(1):94-102.

69. Gu J, Kawai H, Nie L, et al. Mutual dependence of MDM2 and MDMX in their functional inactivation of p53. *J Biol Chem* 2002;277(22):19251-4.

70. Jones SN, Hancock AR, Vogel H, Donehower LA, Bradley A. Overexpression of Mdm2 in mice reveals a p53-independent role for Mdm2 in tumorigenesis. *Proc Natl Acad Sci U S A* 1998;95(26):15608-12.

71. Ganguli G, Wasylyk B. p53-independent functions of MDM2. *Mol Cancer Res* 2003;1(14):1027-35.

72. Onel K, Cordon-Cardo C. MDM2 and prognosis. *Mol Cancer Res* 2004;2(1):1-8.

73. Cordon-Cardo C, Latres E, Drobnjak M, et al. Molecular abnormalities of mdm2 and p53 genes in adult soft tissue sarcomas. *Cancer Res* 1994;54(3):794-9.

74. Hirata H, Hinoda Y, Kikuno N, et al. MDM2 SNP309 polymorphism as risk factor for susceptibility and poor prognosis in renal cell carcinoma. *Clin Cancer Res* 2007;13(14):4123-9.

75. Bartel F, Taubert H, Harris LC. Alternative and aberrant splicing of MDM2 mRNA in human cancer. *Cancer Cell* 2002;2(1):9-15.

76. Fridman JS, Hernando E, Hemann MT, de Stanchina E, Cordon-Cardo C, Lowe SW. Tumor promotion by Mdm2 splice variants unable to bind p53. *Cancer Res* 2003;63(18):5703-6.

77. Schlott T, Reimer S, Jahns A, et al. Point mutations and nucleotide insertions in the MDM2 zinc finger structure of human tumours. *J Pathol* 1997;182(1):54-61.

78. Noon AP, Vlatkovic N, Polanski R, et al. p53 and MDM2 in renal cell carcinoma: biomarkers for disease progression and future therapeutic targets? *Cancer* 2010: 116(4) 780 -790

79. Nocito A, Kononen J, Kallioniemi OP, Sauter G. Tissue microarrays (TMAs) for high-throughput molecular pathology research. *Int J Cancer* 2001;94(1):1-5.

80. Klatte T, Seligson DB, LaRochelle J, et al. Molecular signatures of localized clear cell renal cell carcinoma to predict disease-free survival after nephrectomy. *Cancer Epidemiol Biomarkers Prev* 2009;18(3):894-900.

81. Kim HL, Seligson D, Liu X, et al. Using protein expressions to predict survival in clear cell renal carcinoma. *Clin Cancer Res* 2004;10(16):5464-71.

82. Olumi AF, Weidner N, Presti JC. p53 immunoreactivity correlates with Ki-67 and bcl-2 expression in renal cell carcinoma. *Urol Oncol* 2001;6(2):63-67.

83. Ljungberg B, Bozoky B, Kovacs G, et al. p53 expression in correlation to clinical outcome in patients with renal cell carcinoma. *Scand J Urol Nephrol* 2001;35(1):15-20.

84. Zigeuner R, Ratschek M, Rehak P, Schips L, Langner C. Value of p53 as a prognostic marker in histologic subtypes of renal cell carcinoma: a systematic analysis of primary and metastatic tumor tissue. *Urology* 2004;63(4):651-5.

85. Chemeris G, Loktinov A, Rempel A, Schwarz M, Bannasch P. Elevated content of p53 protein in the absence of p53 gene mutations as a possible prognostic marker for human renal cell tumors. *Virchows Arch* 1995;426(6):563-9.

86. Kanamaru H, Li B, Miwa Y, Akino H, Okada K. Immunohistochemical expression of p53 and bcl-2 proteins is not associated with sarcomatoid change in renal cell carcinoma. *Urol Res* 1999;27(3):169-73.

87. Shvarts O, Seligson D, Lam J, et al. p53 is an independent predictor of tumor recurrence and progression after nephrectomy in patients with localized renal cell carcinoma. *J Urol* 2005;173(3):725-8.

88. Phuoc NB, Ehara H, Gotoh T, et al. Immunohistochemical analysis with multiple antibodies in search of prognostic markers for clear cell renal cell carcinoma. *Urology* 2007;69(5):843-8.

89. Kankuri M, Soderstrom KO, Pelliniemi TT, Vahlberg T, Pyrhonen S, Salminen E. The association of immunoreactive p53 and Ki-67 with T-stage, grade, occurrence of metastases and survival in renal cell carcinoma. *Anticancer Res* 2006;26(5B):3825-33.

90. Kramer BA, Gao X, Davis M, Hall M, Holzbeierlein J, Tawfik O. Prognostic significance of ploidy, MIB-1 proliferation marker, and p53 in renal cell carcinoma. *J Am Coll Surg* 2005;201(4):565-70.

91. Langner C, Ratschek M, Rehak P, Tsybrovskyy O, Zigeuner R. The pT1a and pT1b category subdivision in renal cell carcinoma: is it reflected by differences in tumour biology? *BJU Int* 2005;95(3):310-4.

92. Cho DS, Joo HJ, Oh DK, et al. Cyclooxygenase-2 and p53 expression as prognostic indicators in conventional renal cell carcinoma. *Yonsei Med J* 2005;46(1):133-40.

93. Uzunlar AK, Sahin H, Yilmaz F, Ozekinci S. Expression of p53 oncoprotein and bcl-2 in renal cell carcinoma. *Saudi Med J* 2005;26(1):37-41.

94. Uchida T, Gao JP, Wang C, et al. Clinical significance of p53, mdm2, and bcl-2 proteins in renal cell carcinoma. *Urology* 2002;59(4):615-20.

95. Girgin C, Tarhan H, Hekimgil M, Sezer A, Gurel G. P53 mutations and other prognostic factors of renal cell carcinoma. *Urol Int* 2001;66(2):78-83.

96. Haitel A, Wiener HG, Baethge U, Marberger M, Susani M. mdm2 expression as a prognostic indicator in clear cell renal cell carcinoma: comparison with p53 overexpression and clinicopathological parameters. *Clin Cancer Res* 2000;6(5):1840-4.

97. Rioux-Leclercq N, Turlin B, Bansard J, et al. Value of immunohistochemical Ki-67 and p53 determinations as predictive factors of outcome in renal cell carcinoma. *Urology* 2000;55(4):501-5.

98. Sejima T, Miyagawa I. Expression of bcl-2, p53 oncoprotein, and proliferating cell nuclear antigen in renal cell carcinoma. *Eur Urol* 1999;35(3):242-8.

99. Vasavada SP, Novick AC, Williams BR. P53, bcl-2, and Bax expression in renal cell carcinoma. *Urology* 1998;51(6):1057-61.

100. Sinik Z, Alkibay T, Ataoglu O, et al. Nuclear p53 overexpression in bladder, prostate, and renal carcinomas. *Int J Urol* 1997;4(6):546-51.

101. Papadopoulos I, Rudolph P, Weichert-Jacobsen K. Value of p53 expression, cellular proliferation, and DNA content as prognostic indicators in renal cell carcinoma. *Eur Urol* 1997;32(1):110-7.

102. Zhang XH, Takenaka I, Sato C, Sakamoto H. p53 and HER-2 alterations in renal cell carcinoma. *Urology* 1997;50(4):636-42.

103. Gelb AB, Sudilovsky D, Wu CD, Weiss LM, Medeiros LJ. Appraisal of intratumoral microvessel density, MIB-1 score, DNA content, and p53 protein expression as prognostic indicators in patients with locally confined renal cell carcinoma. *Cancer* 1997;80(9):1768-75.

104. Shiina H, Igawa M, Urakami S, Shirakawa H, Ishibe T, Kawanishi M. Clinical significance of immunohistochemically detectable p53 protein in renal cell carcinoma. *Eur Urol* 1997;31(1):73-80.

105. Moch H, Sauter G, Gasser TC, et al. p53 protein expression but not mdm-2 protein expression is associated with rapid tumor cell proliferation and prognosis in renal cell carcinoma. *Urol Res* 1997;25 Suppl 1:S25-30.

106. Hofmockel G, Wittmann A, Dammrich J, Bassukas ID. Expression of p53 and bcl-2 in primary locally confined renal cell carcinomas: no evidence for prognostic significance. *Anticancer Res* 1996;16(6B):3807-11.

107. Lipponen P, Eskelinen M, Hietala K, Syrjanen K, Gambetta RA. Expression of proliferating cell nuclear antigen (PC10), p53 protein and c-erbB-2 in renal adenocarcinoma. *Int J Cancer* 1994;57(2):275-80.

108. Kamel D, Turpeenniemi-Hujanen T, Vahakangas K, Paakko P, Soini Y. Proliferating cell nuclear antigen but not p53 or human papillomavirus DNA correlates with advanced clinical stage in renal cell carcinoma. *Histopathology* 1994;25(4):339-47.

109. Bot FJ, Godschalk JC, Krishnadath KK, van der Kwast TH, Bosman FT. Prognostic factors in renal-cell carcinoma: immunohistochemical detection of p53 protein versus clinico-pathological parameters. *Int J Cancer* 1994;57(5):634-7.

110. Uhlman DL, Nguyen PL, Manivel JC, et al. Association of immunohistochemical staining for p53 with metastatic progression and poor survival in patients with renal cell carcinoma. *J Natl Cancer Inst* 1994;86(19):1470-5.

111. Orita M, Iwahana H, Kanazawa H, Hayashi K, Sekiya T. Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proc Natl Acad Sci U S A* 1989;86(8):2766-70.

112. Petitjean A, Mathe E, Kato S, et al. Impact of mutant p53 functional properties on TP53 mutation patterns and tumor phenotype: lessons from recent developments in the IARC TP53 database. *Hum Mutat* 2007;28(6):622-9.

113. Soussi T, Beroud C. Assessing TP53 status in human tumours to evaluate clinical outcome. *Nat Rev Cancer* 2001;1(3):233-40.

114. Gad S, Lefevre SH, Khoo SK, et al. Mutations in BHD and TP53 genes, but not in HNF1beta gene, in a large series of sporadic chromophobe renal cell carcinoma. *Br J Cancer* 2007: 96; 336–340.

115. Kawasaki T, Bilim V, Takahashi K, Tomita Y. Infrequent alteration of p53 pathway in metastatic renal cell carcinoma. *Oncol Rep* 1999;6(2):329-33.

116. Contractor H, Zariwala M, Bugert P, Zeisler J, Kovacs G. Mutation of the p53 tumour suppressor gene occurs preferentially in the chromophobe type of renal cell tumour. *J Pathol* 1997;181(2):136-9.

117. Dahiya R, Deng G, Selph C, Carroll P, Presti J, Jr. A novel p53 mutation hotspot at codon 132 (AAG-->AGG) in human renal cancer. *Biochem Mol Biol Int* 1998;44(2):407-15.

118. Dijkhuizen T, Van den Berg E, Van den Berg A, et al. Chromosomal findings and p53-mutation analysis in chromophilic renal-cell carcinomas. *Int J Cancer* 1996;68(1):47-50.

119. Kuczyk MA, Serth J, Bokemeyer C, et al. Detection of p53 gene alteration in renal-cell cancer by micropreparation techniques of tumor specimens. *Int J Cancer* 1995;64(6):399-406.

120. Uchida T, Wada C, Wang C, Egawa S, Ohtani H, Koshiba K. Genomic instability of microsatellite repeats and mutations of H-, K-, and N-ras, and p53 genes in renal cell carcinoma. *Cancer Res* 1994;54(14):3682-5.

121. Kikuchi Y, Kishi T, Suzuki M, Furusato M, Aizawa S. Polymerase chain reaction-single strand conformation polymorphism analysis of the p53 gene in paraffin-embedded surgical material from human renal cell carcinomas. *Virchows Arch* 1994;424(3):229-33.

122. Imai Y, Strohmeyer TG, Fleischhacker M, Slamon DJ, Koeffler HP. p53 mutations and MDM-2 amplification in renal cell cancers. *Mod Pathol* 1994;7(7):766-70.

123. Reiter RE, Anglard P, Liu S, Gnarra JR, Linehan WM. Chromosome 17p deletions and p53 mutations in renal cell carcinoma. *Cancer Res* 1993;53(13):3092-7.

124. Suzuki Y, Tamura G, Satodate R, Fujioka T. Infrequent mutation of p53 gene in human renal cell carcinoma detected by polymerase chain reaction single-strand conformation polymorphism analysis. *Jpn J Cancer Res* 1992;83(3):233-5.

125. Torigoe S, Shuin T, Kubota Y, Horikoshi T, Danenberg K, Danenberg PV. p53 gene mutation in primary human renal cell carcinoma. *Oncol Res* 1992;4(11-12):467-72.

126. Wurl P, Meye A, Schmidt H, et al. High prognostic significance of Mdm2/p53 co-overexpression in soft tissue sarcomas of the extremities. *Oncogene* 1998;16(9):1183-5.

127. Lu ML, Wikman F, Orntoft TF, et al. Impact of alterations affecting the p53 pathway in bladder cancer on clinical outcome, assessed by conventional and array-based methods. *Clin Cancer Res* 2002;8(1):171-9.

128. Warburton HE, Brady M, Vlatkovic N, Linehan WM, Parsons K, Boyd MT. p53 regulation and function in renal cell carcinoma. *Cancer Res* 2005;65(15):6498-503.

129. Gurova KV, Hill JE, Razorenova OV, Chumakov PM, Gudkov AV. p53 pathway in renal cell carcinoma is repressed by a dominant mechanism. *Cancer Res* 2004;64(6):1951-8.

130. Carroll VA, Ashcroft M. Regulation of angiogenic factors by HDM2 in renal cell carcinoma. *Cancer Res* 2008;68(2):545-52.

131. Yang JY, Zong CS, Xia W, et al. MDM2 promotes cell motility and invasiveness by regulating E-cadherin degradation. *Mol Cell Biol* 2006;26(19):7269-82.

132. Dearth LR, Qian H, Wang T, et al. Inactive full-length p53 mutants lacking dominant wild-type p53 inhibition highlight loss of heterozygosity as an important aspect of p53 status in human cancers. *Carcinogenesis* 2007;28(2):289-98.

133. Anglard P, Trahan E, Liu S, et al. Molecular and cellular characterization of human renal cell carcinoma cell lines. *Cancer Res* 1992;52(2):348-56.

134. Kovacs G, Akhtar M, Beckwith BJ, et al. The Heidelberg classification of renal cell tumours. *J Pathol* 1997;183(2):131-3.

135. Csermely P, Schnaider T, Soti C, Prohaszka Z, Nardai G. The 90-kDa molecular chaperone family: structure, function, and clinical applications. A comprehensive review. *Pharmacol Ther* 1998;79(2):129-68.

136. Hartl FU. Molecular chaperones in cellular protein folding. *Nature* 1996;381(6583):571-9.

137. Picard D. Heat-shock protein 90, a chaperone for folding and regulation. *Cell Mol Life Sci* 2002;59(10):1640-8.

138. Sharp S, Workman P. Inhibitors of the HSP90 molecular chaperone: current status. *Adv Cancer Res* 2006;95:323-48.

139. Sepehrnia B, Paz IB, Dasgupta G, Momand J. Heat shock protein 84 forms a complex with mutant p53 protein predominantly within a cytoplasmic compartment of the cell. *J Biol Chem* 1996;271(25):15084-90.

140. Nagata Y, Anan T, Yoshida T, et al. The stabilization mechanism of mutant-type p53 by impaired ubiquitination: the loss of wild-type p53 function and the hsp90 association. *Oncogene* 1999;18(44):6037-49.

141. Young JC, Moarefi I, Hartl FU. Hsp90: a specialized but essential protein-folding tool. *J Cell Biol* 2001;154(2):267-73.

142. Hohfeld J, Minami Y, Hartl FU. Hip, a novel cochaperone involved in the eukaryotic Hsc70/Hsp40 reaction cycle. *Cell* 1995;83(4):589-98.

143. Isaacs JS, Xu W, Neckers L. Heat shock protein 90 as a molecular target for cancer therapeutics. *Cancer Cell* 2003;3(3):213-7.

144. Zou J, Guo Y, Guettouche T, Smith DF, Voellmy R. Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1. *Cell* 1998;94(4):471-80.

145. Kamal A, Thao L, Sensintaffar J, et al. A high-affinity conformation of Hsp90 confers tumour selectivity on Hsp90 inhibitors. *Nature* 2003;425(6956):407-10.

146. Prodromou C, Roe SM, O'Brien R, Ladbury JE, Piper PW, Pearl LH. Identification and structural characterization of the ATP/ADP-binding site in the Hsp90 molecular chaperone. *Cell* 1997;90(1):65-75.

147. Blagosklonny MV, Toretsky J, Neckers L. Geldanamycin selectively destabilizes and conformationally alters mutated p53. *Oncogene* 1995;11(5):933-9.

148. Blagosklonny MV, Toretsky J, Bohen S, Neckers L. Mutant conformation of p53 translated in vitro or in vivo requires functional HSP90. *Proc Natl Acad Sci U S A* 1996;93(16):8379-83.

149. Whitesell L, Sutphin P, An WG, Schulte T, Blagosklonny MV, Neckers L. Geldanamycin-stimulated destabilization of mutated p53 is mediated by the proteasome in vivo. *Oncogene* 1997;14(23):2809-16.

150. Whitesell L, Sutphin PD, Pulcini EJ, Martinez JD, Cook PH. The physical association of multiple molecular chaperone proteins with mutant p53 is altered by geldanamycin, an hsp90-binding agent. *Mol Cell Biol* 1998;18(3):1517-24.

151. Marcu MG, Schulte TW, Neckers L. Novobiocin and related coumarins and depletion of heat shock protein 90-dependent signaling proteins. *J Natl Cancer Inst* 2000;92(3):242-8.

152. Rudiger S, Freund SM, Veprintsev DB, Fersht AR. CRINEPT-TROSY NMR reveals p53 core domain bound in an unfolded form to the chaperone Hsp90. *Proc Natl Acad Sci U S A* 2002;99(17):11085-90.

153. Muller L, Schaupp A, Walerych D, Wegele H, Buchner J. Hsp90 regulates the activity of wild type p53 under physiological and elevated temperatures. *J Biol Chem* 2004;279(47):48846-54.

154. Walerych D, Kudla G, Gutkowska M, et al. Hsp90 chaperones wild-type p53 tumor suppressor protein. *J Biol Chem* 2004;279(47):48836-45.

155. Wang C, Chen J. Phosphorylation and hsp90 binding mediate heat shock stabilization of p53. *J Biol Chem* 2003;278(3):2066-71.

156. Peng Y, Chen L, Li C, Lu W, Chen J. Inhibition of MDM2 by hsp90 contributes to mutant p53 stabilization. *J Biol Chem* 2001;276(44):40583-90.

157. Burnette WN. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate--polyacrylamide gels to unmodified nitrocellulose and radiographic

detection with antibody and radioiodinated protein A. *Anal Biochem* 1981;112(2):195-203.

158. Rouviere O, Bouvier R, Negrier S, Badet L, Lyonnet D. Nonmetastatic renal-cell carcinoma: is it really possible to define rational guidelines for post-treatment follow-up? *Nat Clin Pract Oncol* 2006;3(4):200-13.

159. Noon AP, Polański R, El-Fert AY, et al. Combined p53 & MDM2 biomarker analysis demonstrates a unique pattern of expression associated with poor prognosis in renal cell carcinoma patients undergoing radical nephrectomy. *BJU Int* 2011 (article in press accepted April 2011).

160. Welman A, Barraclough J, Dive C. Generation of cells expressing improved doxycycline-regulated reverse transcriptional transactivator rtTA2S-M2. *Nat Protoc* 2006;1(2):803-11.

161. Arya AK, El-Fert A, Devling T, et al. Nutlin-3, the small-molecule inhibitor of MDM2, promotes senescence and radiosensitises laryngeal carcinoma cells harbouring wild-type p53. *Br J Cancer*;103(2):186-95.

162. Germain D, Russell A, Thompson A, Hendley J. Ubiquitination of free cyclin D1 is independent of phosphorylation on threonine 286. *J Biol Chem* 2000;275(16):12074-9.

163. Kim JK, Diehl JA. Nuclear cyclin D1: an oncogenic driver in human cancer. *J Cell Physiol* 2009;220(2):292-6.

164. Itahana K, Mao H, Jin A, et al. Targeted inactivation of Mdm2 RING finger E3 ubiquitin ligase activity in the mouse reveals mechanistic insights into p53 regulation. *Cancer Cell* 2007;12(4):355-66.

165. Burch L, Shimizu H, Smith A, Patterson C, Hupp TR. Expansion of protein interaction maps by phage peptide display using MDM2 as a prototypical conformationally flexible target protein. *J Mol Biol* 2004;337(1):129-45.

166. Kubbutat MH, Vousden KH. Proteolytic cleavage of human p53 by calpain: a potential regulator of protein stability. *Mol Cell Biol* 1997;17(1):460-8.

167. Maguire M, Nield PC, Devling T, et al. MDM2 regulates dihydrofolate reductase activity through monoubiquitination. *Cancer Res* 2008;68(9):3232-42.

168. Giovannetti E, Backus HH, Wouters D, Peters GJ. Functional inactivity and mutations of p53 differentially affect sensitivity to 5-fluorouracil and antifolate inhibitors of thymidylate synthase (TS) by altering TS levels in colorectal cancer cells. *Nucleosides Nucleotides Nucleic Acids* 2008;27(6):740-5.

169. Ronnen EA, Kondagunta GV, Ishill N, et al. A phase II trial of 17-(Allylamino)-17-demethoxygeldanamycin in patients with papillary and clear cell renal cell carcinoma. *Invest New Drugs* 2006;24(6):543-6.

170. Eggener SE, Yossepowitch O, Pettus JA, Snyder ME, Motzer RJ, Russo P. Renal cell carcinoma recurrence after nephrectomy for localized disease: predicting survival from time of recurrence. *J Clin Oncol* 2006;24(19):3101-6.

APPENDIX -1 - ADDITIONAL DATA

Detailed in this appendix are a number of additional experiments the results of which have been summarised but not displayed in results chapter 4.

A1.1 Results

A1.1.1 Optimisation of in situ β –gal assay for the 117 cell line

To determine the efficiency of transfection of a variety of measurements or assays are available. A basic and reproducible method is the in-situ β-galactosidase (β-gal) assay. In this method a recombinant DNA plasmid containing the gene for bacterial β-gal, is transfected into the cells. Expression of β-gal can be detected histochemically through hydrolysis of X-Gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside), which yields a blue precipitate. A comparison was made between the stained cells and unstained cells, and transfection efficiency was calculated as a percentage of stained /total cells. The amount of β-gal plasmid transfected and the affect on transfection efficiency was evaluated in the H1299 cell line using an in situ β-gal assay. H1299 cells were used as these can be readily transfected. GeneJuice™ (a liposomal transfection reagent) was used as the transfection reagent again due to prior experience within our laboratory. The experiment was designed to calculate the optimum weight of pβgal DNA plasmid to be used for the in situ β-gal assay. Four conditions were tested; 2.5μg of pβgal, 2.5μg of pβgal and 7.5 μg of the empty backbone plasmid pCEP4, 5μg of pβgal and 5μg of pβgal

and 5µg of pCEP4. Cells were left to incubate for 24 hours at 37°Cand photographed at various powers of magnification, as indicated in Figure A1.1.1. The highest transfection efficiency was seen with 5µg of pβgal and 5 µg pCEP4 estimated at 50%.

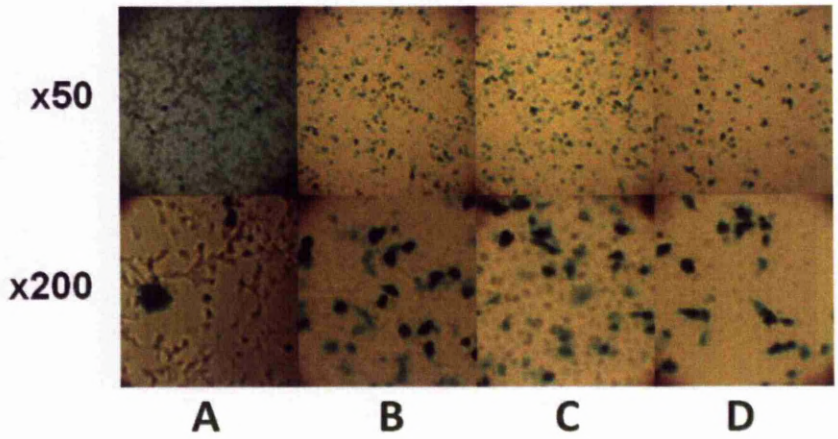


Figure A1.1.1 In situ β-gal assay of H1299 cells transfected with various amounts of plasmid DNA, using GeneJuice™ transfection reagent. Condition A = pβ-Gal (2.5µg), B = pβ-Gal (2.5µg) & pCEP4 (7.5 µg), C = pβ-Gal (5µg), D = pβ-Gal (5µg) & pCEP4 (5µg). Cells were treated for 24 hours before performing the in situ β-gal assay. Photographs were taken at x50 magnification (top row of Figure) and x200 magnification (bottom row of Figure).

A1.1.2 Transfection efficiency of the 117 cells and 117 derived clones using GeneJuice™

Using the optimized β-gal assay, the transfection efficiency in the 117 cell lines (117, pCEP3 and ΔNp53) was assessed, using GeneJuice™ (Novagen). The Cell lines were transfected with pβ-Gal (5ug) and pCEP4 (5ug) using GeneJuice™ transfection reagent. Figure A1.1.2 shows the results of the assay at a low and high power magnification. The 117 parent cell line had a superior transfection efficiency compared to the two derived clones. This may be in part due to the fact that the clones were the results of previous transfections, (see section 1.6). This may render the cells more resistant to further transfection. This hypothesis was not further tested. All cell lines had transfection efficiency less than 10%.

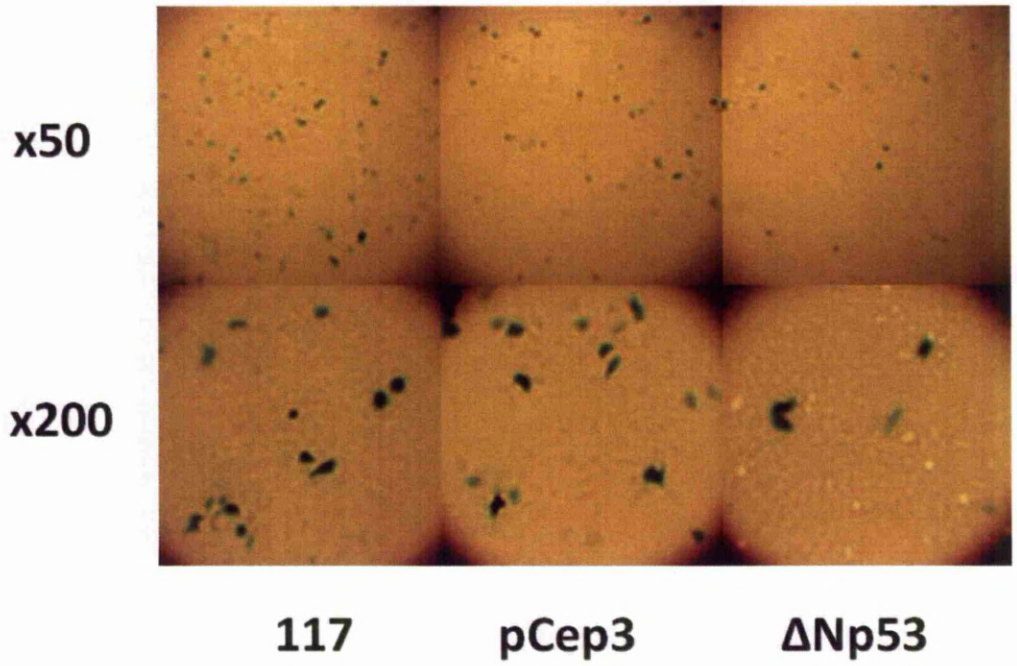


Figure A1.1.2 In situ β-gal assays of 117 cell line and derived clones transfected with pβ-Gal (5μg) and pCEP4 (5μg) using GeneJuice™ transfection reagent. Cells were left for 24 hours before performing the in situ β-gal assay. A photograph was taken at the indicated magnifications.

A1.1.3 Comparison of transfection efficiency in 117 cells using Genejuice™ and FuGENE HD™ transfection reagents

A new non-liposomal transfection reagent, FuGENE HD™ (Roche), had recently become commercially available. Using the previously described amount of DNA plasmids, transfection efficiency using Genejuice™ was compared to FuGENE HD™ in the 117 cell line only. Comparison was made using transfection reagent volume (μl) to DNA weight (μg) ratios of 3:1 and 6:1 to investigate if differing ratios also altered the transfection efficiency. Transfection efficiency was estimated using, in situ β-gal assay, western blotting and β-gal enzyme assay. The β-gal enzyme assay used a non-biological substrate for the enzyme, Ortho-nitrophenyl-ß-galactoside (ONPG). In the presence of ß-gal, ONPG was converted to galactose and Ortho-nitrophenyl (ONP). ONP is colourless at neutral or acid pH, but in an alkaline solution it is bright yellow. The amount of colour change was measured in a spectrophotometer, results were referenced to a non-transfected 117 control. It was hoped that the β-gal enzyme assay would be more quantitative than the in situ β-gal assay. The results are displayed in Figure A1.1.3.

These three experiments clearly show that FuGENE HD™ at a transfection reagent volume (μl) to DNA weight (μg) ratio of 3:1, is the optimum transfection condition. The transfection efficiency was approximately 30 – 40% with FuGENE HD™. This was also reflected in the western blot and the enzyme assay. FuGENE HD™ was so dramatically superior to GeneJuice™ that further optimisation focused on FuGENE HD™. Although quantitative the β-gal enzyme assay did not add sufficiently to the in situ β-gal assay to

be used for further experimental assessment of transfection efficiency.

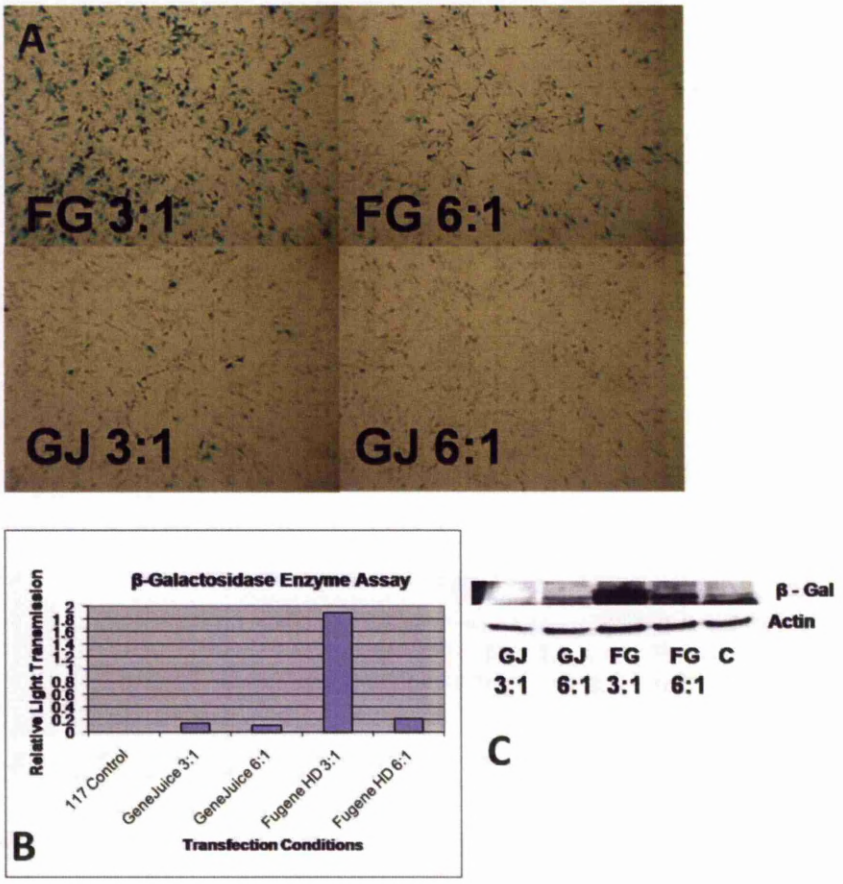


Figure A1.1.3. Comparing transfection of 117 cell line with GeneJuice™ (GJ) and FuGENE HD™ (FG) in differing transfection reagent volume (µl) to DNA weight (µg) ratios. 117 cells were transfected with pβ-gal (5µg) and pCEP4 (5µg), at transfection reagent volume (µl) to DNA weight (µg) ratios indicated in the Figure. 117 Control (C) cells were transfected with pCEP4 (10µg). Cells were incubated for 24 hours at 37°C before performing the in situ β-gal assay. Photographs was taken at 50x magnification (Panel **A**). **B** Western blot, proteins probed for as indicated in figure. C β-gal enzyme assay GJ = Genejuice™. FG = FuGENE HD™.

A1.1.4 Optimisation of FuGENE HD™ volume to DNA weight ratio for transfection in the 117 cell line.

In order to find the optimum transfection volume (µl) to DNA weight (µg) ratio, for FuGENE HD™ in 117 cells, in situ β-gal assays were performed. A range of FuGENE HD™ reagent volume (µl) to DNA weight (µg) ratios were used (2:1 3:1 5:2 & 3:2), this time below the 3:1 ratio used earlier to investigate if lower ratios resulted in higher transfection efficiency. Figure A1.1.4, shows the optimum volume (µl) to DNA weight (µg) ratio to be 2:1, with a transfection efficiency estimated to be around 40 – 45%.

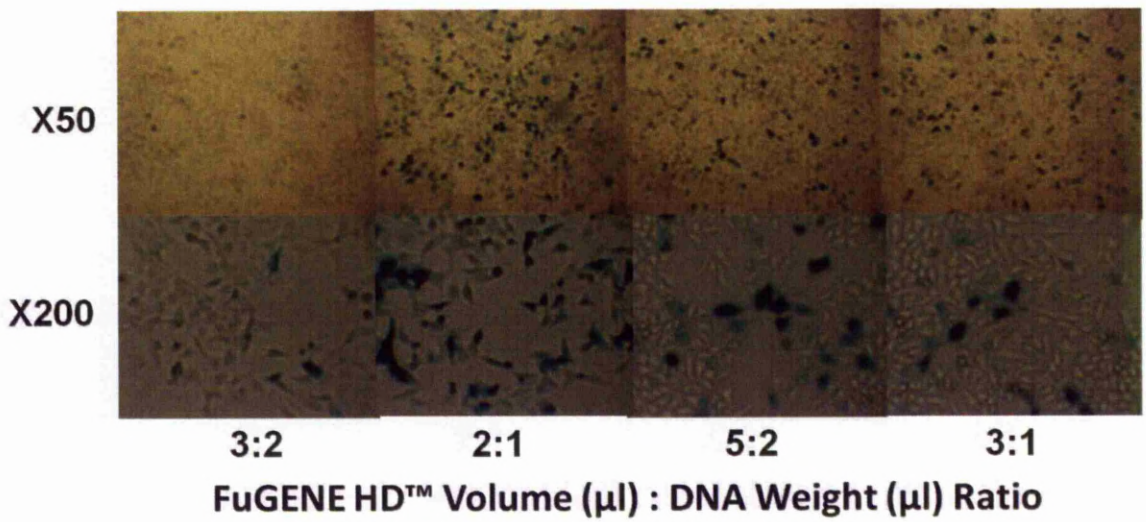


Figure A1.1.4. Optimisation of FuGENE HD volume to DNA weight ratio for transfection of 117. 117 cells were transfected with pβ-gal (5µg) and pCEP4 (5µg) using FuGENE HD™ transfection reagent, at transfection reagent volume (µl) to DNA weight (µg) ratio indicated in the Figure. Cells were incubated for 24 hours at 37°C before performing the in situ β-gal assay. A photograph was taken at 50x and 400x magnification.

A1.1.5 Transfection efficiency of 117 cell line using Lipofectamine 2000™

Another commercially available transfection reagent, Lipofectamine 2000™ (Invitrogen), was evaluated. The 117 cell line was transfected with β-gal (as described previously) with four different ratios of reagent volume (µl) to DNA weight (µg). In situ β-gal assays were performed; the results after 24 hours can be seen in Figure A1.1.5. The optimum reagent volume (µl) to DNA weight (µg) ratio seen was 5:1. The transfection efficiency was estimated at 40%, similar to FuGene HD™. A comparison between FuGENE HD™ and Lipofectamine 2000™ was not carried out as both were considered to be unsuitable methods for high transfection efficiency also transfection with Lipofectamine 2000™ did seem to result in some cell death. This had not been observed with FuGENE HD™ transfection.

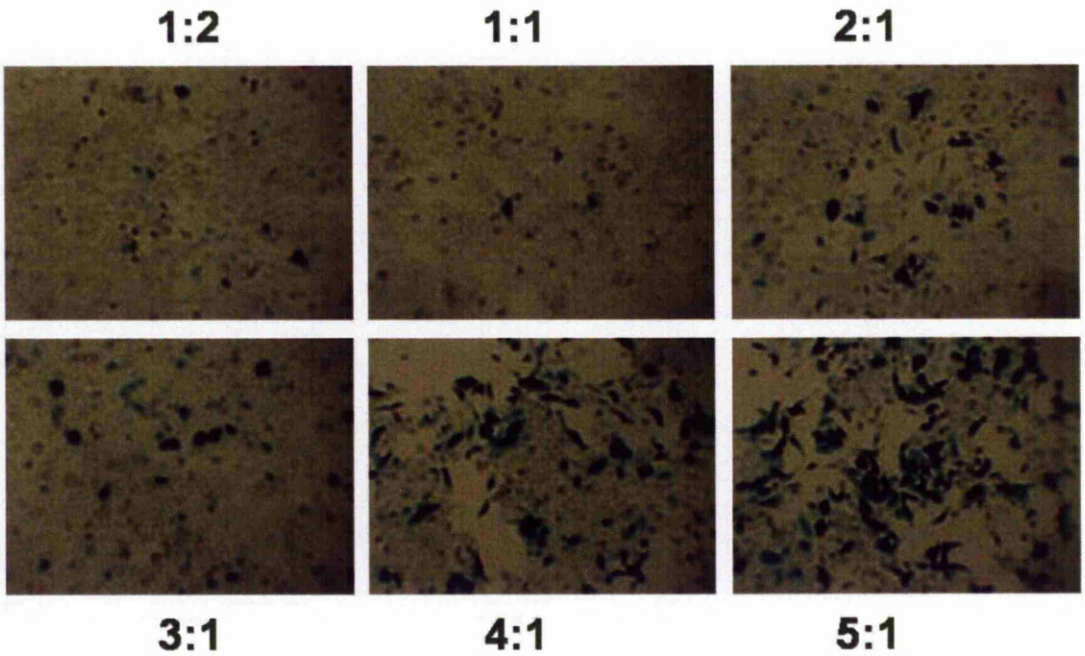


Figure A1.1.5 In situ β-gal assays of four different Lipofectamine 2000 volume (µl) to DNA weight (µg) ratios. 117 cells were transfected with pβ-Gal (5µg) and pCEP4

(5μg) using the Lipofectamine transfection reagent, at transfection reagent volume (μl) to DNA weight (μg) ratios indicated in the Figure. Cells were incubated for 24 hours at 37°C before performing the in situ β-gal assay. A representative photograph was taken at 50x magnifications. The highest transfection efficiency is seen in cells transfected using Lipofectamine 2000™ at a reagent volume (μl) to DNA weight (μg) ratio of 5:1.

A1.1.6 Transfection efficiency of 117 cells using Magnetofection™ - Polymag

Another transient transfection system was evaluated. This method associated DNA with magnetic nanoparticles coated with cationic molecules. The resulting molecular complexes were then transported into cells supported by an appropriate magnetic field. The magnetofection™ system, exploited magnetic forces exerted upon gene plasmid towards, possibly even into, the target cells. In theory, the complete applied plasmid dose gets concentrated onto the cells within a few minutes. This experiment was conducted in six well plates, pβ-Gal (2μg) was transfected. Six different ratios of reagent volume (μl) to DNA weight (μg) ratio were assessed (1:0.5, 1:1, 1:2, 1:3, 1:4, & 1:5) using an in situ β-gal assay. Of the six conditions evaluated, only the 1:1 ratio (Figure A2.2.6), showed any evidence of transfection. This method was therefore discounted.

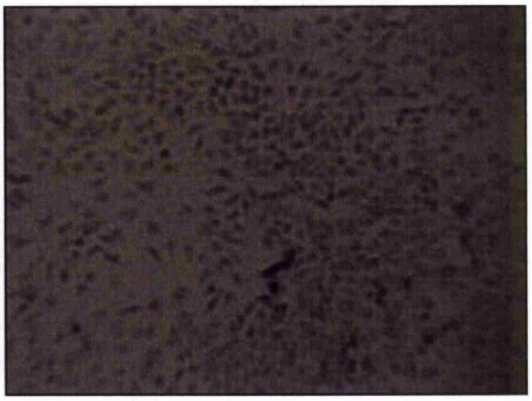


Figure A1.1.6 In situ β-gal assay of 117 transfected with pβ-Gal (2μg) using a Polymag™ reagent volume (μl) to DNA weight (μl) ratio of 1:1. Photograph taken at x50 magnification

A1.1.7 Determination of G418 concentration for 1st round clonal selection of 117 and derivived clones – G418 response assay

G418 is an aminoglycoside antibiotic that selects for mammalian cells expressing neomycin phosphotransferase, from the neo gene (contained within the pN1pβactin-rtTA2S-M2-IRES-EGFP vector). As can be seen from table A1.1.7, 800 μg/ml causes cell death in all three cell lines after 5 days. This dose of G418 would be used for clonal selection.

Cell Line	117					pCep 3					ΔNp53				
Day	0	3	4	5	6	0	3	4	5	6	0	3	4	5	6
Dosage (μg/ml)	15	75	75	75	75	20	75	75	75	75	20	75	75	75	75
0	15	75	75	75	75	20	75	75	75	75	20	75	75	75	75
50	15	75	75	75	75	20	75	75	75	75	20	75	75	75	75
100	15	75	75	75	75	20	75	75	75	75	20	75	75	75	75
200	15	45	45	45	30	20	45	45	45	30	20	45	45	45	30
400	15	45	45	30	D	20	45	45	30	D	20	45	45	30	D
800	15	45	30	D	D	20	45	30	D	D	20	45	30	D	D

Table A1.1.7 G418 response assay. Numbers refer to an estimation of the percentage of the area of the tissue culture dish occupied with viable cells. D = no viable cells visible.

APPENDIX 2 – PRESENTATIONS AND PUBLICATIONS

A2.1 Summary of presentations and publications

A2.1.1 British Association of Urological Surgeons Annual Meeting June 2008

MDM2 and p53 coexpression is associated with poor prognosis in renal cell carcinoma in patients undergoing radical nephrectomy. Podium presentation

Noon AP, Warburton HE, Shawki H, Campbell F, Parsons K & Boyd MT

Abstract published in British Journal of Urology International, June 2008, Vol 101, Supplement 5, page 2.

A2.1.2 National Cancer Research Institute Conference October 2007

Investigating the consequences of high MDM2 expression in renal cell carcinoma using an inducible gene expression system. Poster presentation

Aidan Paul Noon, H Warburton, K Parsons, M T Boyd[1]

A2.1.3 Review Paper

p53 and MDM2 in renal cell carcinoma: biomarkers for disease progression and future therapeutic targets? **Noon AP**, Vlatković N, Polański R, Maguire M, Shawki H, Parsons K, Boyd MT. Cancer. 2010 Feb 15;116(4):780-90.

A2.1.4 Published Paper

Combined p53 & MDM2 biomarker analysis demonstrates a unique pattern of expression associated with poor prognosis in renal cell carcinoma patients undergoing

radical nephrectomy Noon AP, Polański R, El-Fert AY, et al. *BJU Int* 2011 (article in press accepted April 2011).

A2.2 Abstracts and Posters

A2.2.1 British Association of Urological Surgeons Annual Meeting June 2008

MDM2 and p53 coexpression is associated with poor prognosis in renal cell carcinoma patients undergoing radical nephrectomy.
Noon AP, Warburton HE, Shawki H, Campbell F, Parsons K & Boyd MT

Introduction:

Compromise of the p53 tumour suppressor pathway has been shown to be an important event in the progression of a number of human cancers. Studies have shown that renal cell carcinomas (RCC) with high expression of p53 have a poorer prognosis. RCC's that over express p53 retain its normal wild type function. We wanted to investigate the role of the p53 counter-regulatory protein and oncogene MDM2 in patients with RCC.

Materials and Methods:

A recently created tissue microarray of 91 RCC nephrectomy samples was used to detect the presence of p53 and MDM2 expression by immunohistochemical analysis. A scoring system was devised and two consultant histopathologists independently scored the tumour samples for p53 and MDM2 staining.

Results:

Analysis of 91 patient samples revealed that p53 was expressed in 14 (15.4%) and MDM2 was expressed in 24 (26.4%). 11 tumours (12%) expressed both MDM2 and p53 and this association was highly significant $p<0.0005$. Five year analysis of patients with tumours that coexpressed p53 and MDM2 showed a significant decreased disease specific survival ($p<0.05$).

Conclusion:

Our results show an intriguing phenotype whereby two normally counter-regulatory proteins are both over expressed in poor prognosis RCC.

A2.2.2 National Cancer Research Institute Conference 2007

Investigating the consequences of high MDM2 expression in renal cell carcinoma using an inducible gene expression system

AP Noon[1], H Warburton[1], K Parsons[2], M T Boyd[1]

[1]Division of Surgery and Oncology, University of Liverpool, Liverpool, UK; [2]Department of Urology, Royal Liverpool and Broadgreen University Hospital, Liverpool, UK

High level co-expression of MDM2 and p53 occur in renal cell carcinomas (RCC) with the poorest prognosis. To study the consequences of MDM2 expression in renal cells we attempted to generate stable MDM2 expressing RCC cells. RCC cell lines frequently display high levels of MDM2 but our studies suggested that specific cellular events are required to permit increased MDM2 expression. Since a strong link ($P<0.0004$) between p53 expression and MDM2 up-regulation has been observed in RCC, we hypothesised that introducing a dominant negative mutant of p53 (R175H) might render cells susceptible to MDM2 expression. Stable clones that expressed MDM2 were indeed generated, but we also observed that some clones in which only empty vector had been transfected also spontaneously acquired increased p53 and/or MDM2. These observations suggest that events other than alterations in p53 function can permit increased MDM2 expression. We have created sub-clones of the parental cell line that are tolerant to MDM2 expression and we propose to use these to define events that contribute to tolerance to MDM2 expression in renal cells. We have generated cells in which we can inducibly express MDM2, that are then analysed by performing gene expression profiling using DNA microarrays. Our goal is to identify clusters of genes whose expression is altered upon MDM2 expression and which differ in permissive and non-permissive cells. Identifying such genes may provide important information regarding events that contribute to MDM2 oncogenicity and through this to tumour progression in RCC.

Investigating the consequences of high MDM2 expression in renal cell carcinoma using an inducible gene expression system

THE UNIVERSITY of LIVERPOOL

AP Noon[1], H Warburton[1], K Parsons[2] & MT Boyd[1]
[1]Division of Surgery and Oncology, University of Liverpool, Liverpool, UK.
[2]Department of Urology, Royal Liverpool and Broadgreen University Hospital, Liverpool, UK

Hypothesis

High level co-expression of the tumour suppressor p53 and its negative regulator MDM2 has been found in renal cell carcinoma (RCC) patients with poor prognosis. In our recently constructed RCC tissue microarray (TMA) we also found a strong association ($p<0.0005$) of p53 and MDM2 high co-expression. We set out to try and understand the mechanisms leading to this phenotype and its consequences by constructing a RCC poor prognosis progression model.

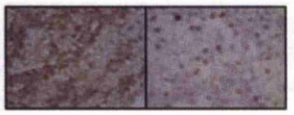


Fig 1.Expression of MDM2 and p53 in RCC from our TMA.
Left: MDM2. Right: p53

Method

We first obtained a panel of RCC cell lines and described their p53 and MDM2 status (Fig 2). We decided to take the 117 cell line (Low wt p53 and low MDM2 levels), and force it to over express MDM2 through clonal selection. This will mimic the progression from good to poor prognosis RCC.

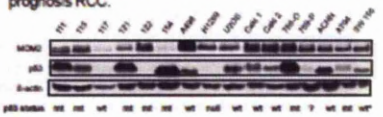
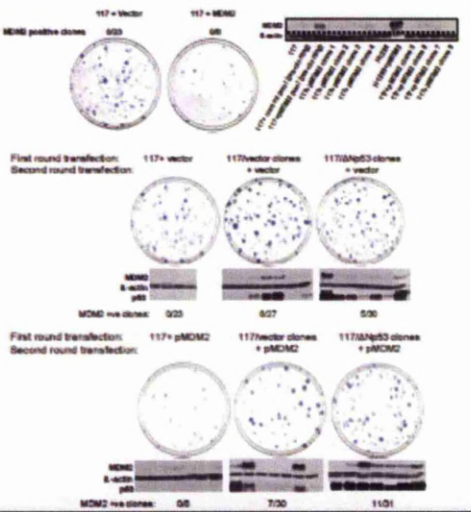


Fig 2. MDM2 and p53 expression in a panel of RCC cell lines. *Western blot analysis of 14 RCC cell lines and U2OS osteosarcoma cell line (high MDM2) and the p53 Null NSCLC cell line H1299.*

Results

Fig 3. Colony formation assays and western blots of generated 117 clones.



We were not able to generate any MDM2 positive 117 clones after transfection with pCMV-MDM2-Neo-Bam (Fig 3 Top). We hypothesised that p53 mutation may render these cells permissible to MDM2 expression. Indeed after transfection with the dominant negative p53 mutant R175H clones were generated that expressed MDM2 (Fig 3 Middle). To our surprise MDM2 expressing clones were also generated from the empty vector (pCEP4) control. This suggests mechanisms other than p53 mutation may be important for MDM2 expression. Clones from the ΔNp53 and empty vector pCEP4 can now be stably transfected with MDM2 (Fig 3 bottom).

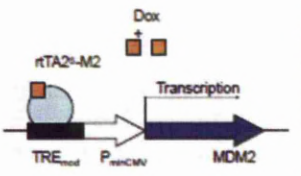


Fig 4. The Tet-On advanced gene expression system. In the presence of doxycycline the rtTA2s-M2 protein binds the TREmod sequence in pTight. This causes expression of the gene of interest, in our case MDM2.

We next wanted to generate inducible MDM2 clones of MDM2 permissive cell lines (pCEP4 and ΔNp53 clones) and the non permissive (117). This would allow us to study the differences that MDM2 expression has in these cells.

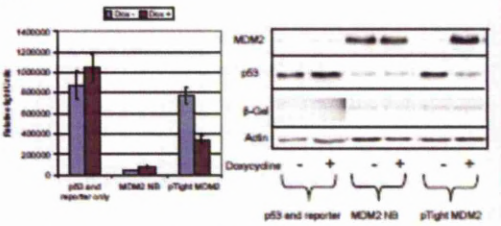


Fig 5. Inducible expression of MDM2 inhibits p53 in RCC cells. 117 RCC cells stably expressing rtTA2s-M2 were transiently transfected with pCEP p53 and pp53-TA-Luciferase reporter construct alone and with either pCMV-MDM2-Neo-Bam (MDM2 NB) or pTight MDM2. After 24 hours media containing 1μg/ml of doxycycline was added, a control dish had its media replaced. The left panel shows the luciferase assay of the cellular lysates. The right panel shows the western blot of the same lysates. β-Galactosidase was used as a control of transfection.

Induction of pTight MDM2 with doxycycline results in decreased p53 activity and destabilisation as expected.

Conclusion

The Tet on inducible gene expression system will allow us to study the effects of MDM2 expression in the 117 RCC cell line, that can not tolerate high MDM2 expression. We intend to perform gene expression analysis using a DNA microarray to look for the consequences of MDM2 expression in non permissive 117 and MDM2 permissive 117 clones (pCEP4 and ΔNp53). We hope this will enable a better understanding of the effects of MDM2 in poor prognosis RCC.

Acknowledgements

This work is being supported by a grant from Mersey Kidney Research.

The pN1pbactin-rtTA2S-M2-IRES-EGFP construct was generously donated by Dr Arkadiusz Welman of the Paterson Institute for Cancer Research, Christie Hospital NHS Trust.

MERSEY KIDNEY RESEARCH

A2.3 Published review paper

Review Article

p53 and MDM2 in Renal Cell Carcinoma

Biomarkers for Disease Progression and Future Therapeutic Targets?

Aidan P. Noon, MB ChB[1]; Nikolina Vlatković, PhD[1]; Radosław Polański, BSc[1]; Maria Maguire, PhD[1]; Howida Shawki, FRCPath[2]; Keith Parsons, FRCS[3]; and Mark T. Boyd, PhD[1]

Renal cell carcinoma (RCC) is the most common type of kidney cancer and follows an unpredictable disease course. To improve prognostication, a better understanding of critical genes associated with disease progression is required. The objective of this review was to focus attention on 2 such genes, p53 and murine double minute 2 (MDM2), and to provide a comprehensive summary and critical analysis of the literature regarding these genes in RCC. Information was compiled by searching the PubMed database for articles that were published or e-published up to April 1, 2009. Search terms included renal cancer, renal cell carcinoma, p53, and MDM2. Full articles and any supplementary data were examined; and, when appropriate, references were checked for additional material. All studies that described assessment of p53 and/or MDM2 in renal cancer were included. The authors concluded that increased p53 expression, but not p53 mutation, is associated with reduced overall survival/more rapid disease progression in RCC. There also was evidence that MDM2 up-regulation is associated with decreased disease-specific survival. Two features of RCC stood out as unusual and will require further investigation. First, increased p53 expression is tightly linked with increased MDM2 expression; and, second, patients who have tumors that display increased p53 and MDM2 expression may have the poorest overall survival. Because there was no evidence to support the conclusion that p53 mutation is associated with poorer survival, it seemed clear that increased p53 expression in RCC occurs independent of mutation. Further investigation of the mechanisms leading to increased p53/MDM2 expression in RCC may lead to improved prognostication and to the identification of novel therapeutic interventions. *Cancer* 2010;00:000–000. © *2010 American Cancer Society.*

KEYWORDS: renal cancer, renal cell carcinoma, p53, murine double minute 2.

The latest available figures from the National Cancer Institute's Surveillance, Epidemiology, and End Results Program predict that there will be 49,096 new cases of kidney cancer (renal cancer and cancer of the renal pelvis) and 11,033 deaths from the disease in the United States in 2009.[1] In the United Kingdom, the latest figures for 2007 indicate that 7380 individuals were diagnosed with the disease and that 3752 died from it.[2] Advances in our understanding of the molecular biology of renal cell carcinoma (RCC) have helped classify this heterogeneous disease and led to the development of several new "von Hippel-Lindau (VHL) pathway"-targeted molecular drug treatments.[3] Nevertheless, patients with metastatic disease still have an extremely short life expectancy.[3] Certainly other molecular pathways must contribute to the poor prognosis observed for those who have advanced RCC. The objective of the current review was to turn the molecular focus back to 1 of the most important genes in cancer biology, p53, and its counterpart, murine double minute 2 (MDM2), and to describe and critically review what is known of their role in RCC.

For the purposes of this review, the 3 most common histologic subtypes of RCC (as set out in the 2004 World Health Organization classification; see Lopez-Beltran et al[4]) are discussed. Clear cell RCC (CCRCC) is the commonest histologic subtype and is believed to account for 75% of all RCCs. Papillary RCC (PRCC) is the second most common and accounts for 10% of RCC; PRCC is subclassified into type I and type II. Chromophobe RCC is the third most common subtype and accounts for 5% of RCCs. Sarcomatoid RCC is a high-grade histologic variation that can arise from all types of RCC, although it is not a separate entity.

Corresponding author: Mark T. Boyd, PhD, Division of Surgery and Oncology, School of Cancer Studies, University of Liverpool, 5th Floor, UCD Building, Daulby Street, Liverpool, L69 3GA, United Kingdom; Fax: (011) 44 151 706 5826; mboyd@liv.ac.uk

[1]Division of Surgery and Oncology, School of Cancer Studies, University of Liverpool, Liverpool, United Kingdom; [2]Department of Pathology, Royal Liverpool University Hospital, Liverpool, United Kingdom; [3]Department of Urology, Royal Liverpool University Hospital, Liverpool, United Kingdom

We apologize to colleagues whose articles we have not cited through lack of space.

DOI: 10.1002/cncr.24841, **Received:** January 6, 2009; **Revised:** June 15, 2009; **Accepted:** June 15, 2009, **Published online** in Wiley InterScience (www.interscience.wiley.com)

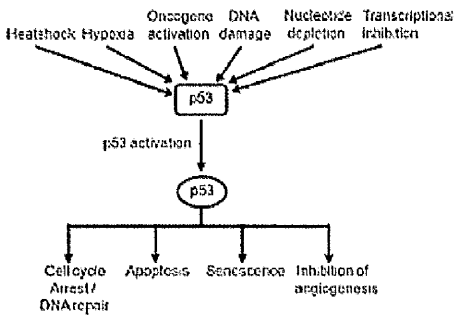


Figure 1. Pathways leading to and consequences of p53 activation are shown.

The p53 and MDM2 Pathway

In 50% of human cancers, p53 is mutated, and it has become 1 of the most studied molecules in science.[5] The p53 protein accumulates at times of cellular or genotoxic stress, when it functions primarily as a transcription factor to promote cell cycle arrest and DNA repair, to initiate and maintain a senescent phenotype, or to promote apoptosis if the normal cellular conditions are not restored (Fig. 1). This latter function helps prevent conditions arising within the cell that can lead to the establishment of mutations with the consequent increased risk of malignant disease. The importance of the tumor suppressor function of p53 also is highlighted by the high frequency of tumors that occur in individuals with a monoallelic germline mutation of p53, as observed in patients with Li-Fraumeni syndrome.[6]

The potentially lethal activities of p53 are regulated by the proto-oncogene Mdm2 (murine double minute 2) or MDM2 in humans. Transgenic mice that are Mdm2 null display early embryonic lethality and die at around Day 5 or 6 of embryogenesis. This lethality can be rescued by concomitant deletion of the p53 gene,[7] thus demonstrating that loss of Mdm2 is lethal because of the lethal effects of unregulated p53. Then, as levels of p53 rise, transcription of MDM2 is induced; thus p53 and MDM2 exist in an autoregulatory feedback loop.[8] Binding of Mdm2 to p53 and can block p53 transcriptional activity by preventing it from interacting with the transcriptional machinery.[9] MDM2 also causes p53 degradation by targeting it for destruction by the 26S proteasome. The latter effect is caused by the ability of MDM2 to act as an E3 ubiquitin ligase with specificity for p53 (among other targets).[10] MDM2 also can target itself for ubiquitylation and, thus, can regulate its own stability, although how these competing activities (ubiquitylation with concomitant degradation of p53 and/or MDM2) are regulated remains unclear.[11] Thus, increased levels of MDM2 can lead to a reduction in p53 levels as a result of this ubiquitin ligase activity, which, in turn, results in decreased p53-dependent MDM2 transcription, restoring the normal cellular status quo.

With respect to cancer, MDM2 over expression has been associated with increased metastasis and advanced disease in several cancers, including breast carcinoma.[12] It is noteworthy that the oncogenic effects of MDM2 are not caused simply by the inhibition of p53 function, because they often are detectable in tumors that harbor p53 mutations,[13] as indicated by further evidence provided from in vivo studies.[14] How MDM2 elicits p53-independent oncogenic effects is unclear, although Yang et al[12] demonstrated that MDM2 expression led to a decrease in E-cadherin levels and a subsequent increase in cell motility in breast carcinoma. Those authors also demonstrated that high expression of MDM2 with low E-cadherin expression was more frequent in metastatic tumor samples.

The balance between MDM2 and p53 is modulated in several ways, depending on the nature of cellular stress. For example, in response to ionizing radiation, p53 is phosphorylated by the ataxia telangiectasia mutated (ATM) kinase, which inhibits MDM2 binding[14]; whereas, after exposure to ultraviolet radiation, p53 is modified by the related ATM and Rad3-related (ATR) kinase.[15] The complex interplay between p53 and MDM2 presumably has evolved to ensure that cells are able to respond rapidly and appropriately to a wide range of genotoxic stresses. One consequence of this sophistication in the regulation of p53 is that it can lead to unreliable conclusions when attempting to determine p53 status from simple assays, such as immunohistochemical (IHC) analyses.[16,17]

In addition to the variable outcomes induced by p53 in response to different types or amounts of stress/damage, there is also a high degree of spatial (ie, tissue) variability in response to genotoxic stress, for example, within an organism (for review, see Slee et al[18]). Studies of transgenic mice exposed to ionizing radiation have revealed broadly 3 classes of p53 response[19]: In the first class of tissues, p53 is up-regulated and elicits a dramatic apoptotic response, as typified by tissues like the small intestine, spleen, and thymus. In the second class of tissues, which includes the kidney, p53 is up-regulated, but little or no apoptotic response is detected. In the third class of tissues,

there is little or no apparent induction of p53. There are also clear differences within tissues. Even when p53 is activated, cellular responses are determined by both tissue-specific and cell-intrinsic factors, which may vary according to the status of the individual cell.[18,20]

Therefore, in the kidney, it appears that there already may be factors at work that compromise the ability of p53 to activate an apoptotic response to genotoxic stress.[19,21] This suggests that p53 tumor suppression may be less effective in the kidney than in some other tissues, and the reason for this remains a major, unanswered question. Moreover, the identification of such a tissue-specific mechanism might provide an opportunity for therapeutic "reactivation" of p53 in these cells. Nevertheless, studies in vitro have questioned this interpretation, demonstrating that p53 is normally functional in renal cancer cells and is regulated by MDM2 in a manner that is typical of cells from other tissues, findings that require further investigation.[22,23]

The majority of studies of p53 and MDM2 in clinical material from renal cancers have been based on IHC of these proteins. This approach is technically simple but is not quantitative and relies on the observation that mutant p53 often is present at higher levels in cells than the wild-type protein.[16,17] The standard explanation for this genotype/phenotype correlation is that mutant p53 lacks the capacity to up-regulate MDM2; thus, an imbalance in p53/MDM2 homeostasis develops and leads to excess p53. In a study of the use of IHC to interrogate p53 status, Nenutil et al[16] observed that combining highly sensitive IHC for p53 (with the ability to detect low levels of wild-type expression and, thus, distinguish this from an absence of expression) with IHC for downstream markers (MDM2 and p21[CDKN1A]) increased the reliability of predicting p53 status. In that study, high levels of p53 almost always were indicative of p53 mutation when MDM2 expression was low, thus according with the standard model for mutant p53 up-regulation. This is not the case in renal cancer, as detailed below.

p53 and MDM2 in Renal Cell Carcinoma

Relatively little is known with certainty about the status and role of p53 or MDM2 in RCC, in striking contrast to some other, albeit more common cancers. To date, at least 31 studies have investigated the expression of p53 in RCC (Table 1).

All of these studies used IHC staining of formalin-fixed, paraffin-embedded tumor samples. When they were available, the percentages of samples from CCRCC, the range of disease stages, and the number of metastatic samples are indicated. All samples contained a mixture of high-grade and low-grade tumors. Several different antibodies were used, although the majority of studies used the p53 DO-7 monoclonal antibody. When available, the criterion for dichotomizing p53 staining has been indicated. According to published data (excluding articles by Klatte et al[24] and Kim et al,[34] in which no details of p53 status were provided), 2519 tumors were stained for p53, and 618 tumors were deemed positive for p53 for p53-positive frequency of 24.5%; however the heterogeneity of the samples must be taken into consideration (see below). Variability between studies may be attributed in part to the lack of a consensus on p53 dichotomization (see Munro et al[17]) compounded by differences in antibody choice and also by processing techniques. One study[26] reported the use of 2 different p53 antibodies, which led to a higher overall p53 detection level/expression rate of 60% (DO-7, 51% positive; p53 antibody 240, 30% positive). This highlights the finding that using different antibodies can result in apparent differences in p53 expression. Interpretation of these results also is hindered by variations in the numbers of different histologic subtypes of RCC tumors, tumor stages, and grades and the variable presence of metastases in the sample populations.

Analysis of p53 expression in primary and metastatic samples has demonstrated an increased frequency of staining of 51.8% in metastatic samples versus 22% in primary samples.[33] This suggests that p53 expression may be a relatively late event in the evolution of RCC and may be associated with metastatic capabilities. If this is correct, then it seems reasonable to expect that p53 expression will be associated with a poorer prognosis regardless of a functional or causal connection. In the rarer histologic subtypes of RCC, considerably more heterogeneity is apparent; and, inevitably, the smaller number of samples analyzed makes interpretation more difficult.

p53 and MDM2 Expression and Prognostic Implications in Renal Cell Carcinoma

The prognostic implications of p53 expression were evaluated, and the results are summarized in Table 1. Of the 27 studies that evaluated p53 expression as a clinical outcome predictor, 18 studies indicated that it predicted a poor outcome, and 10 studies did not. The 10 articles that did not had a smaller study size (mean, 62 patients per study compared with 119 patients per study in the positive articles). The 4 largest studies to date, all of which

Table 1. Studies Investigating the Expression of p53 in Renal Cell Carcinoma

Study	No. of Specimens (% CCRCC)	Antibody	Positive Criteria, %	No. Positive (%)	p53 Prognostic Value	Comments
Kate 2009[34]	178 (100); All stages; M=0	?[a]	Any	?[a]	Yes	Decreased DFS
Perrot 2008[35]	50 (0); All PRCC; 25 type I/25 type II; M=8	DO-7	>20	22 (44); Type I=12/type 2=53	Yes	Decreased OS in type II PRCC
Pruss 2007[36]	119 (100); All stages; M=23	DO-7	>10	64 (54)	Yes	Decreased DSS in all patients
Karkan 2006[37]	117 (96); All stages; M=29	DO-7	>10	15 (12.8)	Yes	Decreased OS in patients with metastases
Kramer 2006[38]	117 (99); All stages; M=21	?[a]	>5	16 (13.6)	No	
Langner 2006[23]	95 (79); stage pT1 only; M=0	DO-7	?[a]	23 (24)	Not evaluated	
Cho 2005[39]	92 (100); All stages; M=7	?[a]	>10	11 (12)	Yes	Decreased DSS
Shvarts 2005[21]	193 (85); All stages; M=0	DO-7	Any; >20	111 (57.5), 14 (7.2)	Yes	20% Cutoff predicted recurrence
Uzzalar 2005[22]	57 (77.1); All stages and grades; M=?	?[a]	>1	20 (35)	Yes	Decreased DSS
Zigeuner 2004[20]	184 (70.7)Metastases, 58 (94.8)[b]	DO-7	>1	42 (22.8)/29 (51.8)	Yes	Decreased metastasis-free survival in CCRCC only
Kim 2004[24]	318; All stages; M=153	DO-7	>15	?[a]	Yes	Decreased DSS
Ueda 2003[25]	112 (73); All stages; M=2	DO-7	>1	15 (13.4)	Yes	Decreased OS
Ohmi 2001[26]	43 (100); All stages; M=14	DO-7/PAB240/both	>10, Either	22 (51)/13 (30)/29 (90)	No	Combined antibody positivity was 90%
Hungerford 2001[27]	99 (74); All stages; M=?	DO-7	>5	17 (19)	Yes	Decreased survival in non-CCRCC
Girgin 2001[28]	50 (82); All stages; M=0	DO-1	>20	18 (36)	Yes	Decreased disease-specific death
Hakl 2000[29]	97 (100); All stages; M=15	DO-1	>5	35 (36)	Yes	Decreased DSS
Raue-Lederecq 2000[40]	66 (?); All stages; M=10	DO-7	>20	11 (17)	Yes	Decreased DSS
Sejima & Miyagawa 1999[41]	53 (?); All stages; M=25	RS53	?[a]	1 (2)	No	
Watanabe 1998[42]	39 (71); T1 and T2 only; M=0	DO-7	>1	0	No	
Sink 1997[43]	39 (100); All stages; M=2	DO-7	>10	7 (17.5)	No	
Papadopoulos 1997[44]	90 (?); T1 and T2; M=14	DO-1	Any positive nuclei	30 (33)	No	
Zhang 1997[45]	70 (?); All stages; M=?	Ab-6	>10	18 (23)	Not evaluated	
Gelb 1997[46]	52 (100); T1 and T2 only; M=0	DO-7	>5	2 (2)	No	
Shiina 1997[47]	72 (?); All stages; M=8	DO-7	>10	29 (40.3)	Yes	Decreased OS
Moch 1997[48]	50 (100); T3 only; M=1	DO-7	?[a]	8 (16)	Yes	Decreased OS
Hohenfellner 1996[49]	51 (?); T1-T3; M=0	DO-7	>1%	5 (10)	No	
Chemets 1995[50]	82 (49)[c]; M=?	DO-1	?[a]	43 (52)	Not evaluated	
Lipponen 1994[51]	123 (?); M=29	CM1	Any positive	41 (33)	Yes	Increased recurrence-free survival
Kamal 1994[52]	56 (?); All stages; M=13	CM1	>1	6 (11)	No	
Bol 1994[53]	100 (74); T1-T3 only; M=0	DO-7	>50	22 (22)	No	
Uhlman 1994[54]	175 (?); All stages; M=45	?[a]	>1	49 (28)	Yes	Decreased DSS

CCRCC indicates clear cell renal cell carcinoma; PRCC, papillary renal cell carcinoma; M, metastatic disease; DFS, disease-free survival; OS, overall survival; DSS, disease-specific survival; pT1, pathologic T1 tumor classification.

[a] Information not provided.

[b] In addition to 182 renal cancers, 58 surgically removed metastatic tissues were analyzed.

[c] Thirty-three CCRCCs and 47 noneosinophilic renal cell carcinomas.

used tumor microarrays, indicated that p53 is a prognostic predictor. Kim et al evaluated 318 patients with CCRCC who had local and metastatic disease (49%; ie, 155 of 318 patients had metastatic disease). Those authors observed that p53 was an independent predictor of decreased disease-specific survival on univariate ($P<.001$) and on multivariate Cox regression analysis ($P = .014$) in which the presence of metastasis was included as a covariate.[34] Klatte et al,[24] in a similar study of 170 patients with CCRCC, also reported that p53 was retained in a multivariate Cox regression analysis for predicting disease-free survival. In the study by Klatte et al, the percentage of tumors that stained for p53 was evaluated, obviating the need to describe tumors as either positive or negative for p53 staining. Zigeuner and colleagues[33] demonstrated that p53 was a predictor of disease progression (metastasis-free survival) on multivariate Cox regression analysis in their study of 130 patients with CCRCC. Those patients were followed for a median of 26 months, and 9 of 16 patients with p53-positive tumors progressed versus 20 of 114 patients with p53-negative tumors ($P = .0005$). In another substantial study, Shvarts et al[31] evaluated p53 staining as a predictor of 5-year recurrence in 193 patients who underwent surgery for localized disease. Those authors also reported that a p53-positive cutoff of 20% expression detected by IHC was a predictor of recurrence (hazard ratio, 3.28; $P = .0108$) on univariate and multivariate Cox regression analysis. When considering such studies of biomarkers of disease outcome, the possibility of publication bias leading to the publication of fewer articles that demonstrate no disease outcome correlation should not be neglected. Nevertheless, the trend appears to be that more recent studies with higher numbers of patients indicate that p53 protein levels are prognostically significant in RCC.

p53 Mutational Analysis in Renal Cell Carcinoma

When examining the significance of p53 involvement in cancer in general and in RCC in particular, it is clear that IHC detection of p53 alone cannot reliably inform us whether the protein is functional or mutated.[16,55] However, p53 mutational status often has been inferred in such studies, because high-level expression of p53 is used as a surrogate indicator of mutation. This interpretation certainly is not correct for renal cancer cells in culture, in which relatively high levels of p53 protein frequently are detected in the absence of p53 mutation.[22] In addition, in some 10% to 20% of cases that harbor p53 mutations, tumors may harbor nonsense (truncating) mutations, which can lead to less stable mutant proteins that are unlikely to be detected by IHC but that would be inferred to possess wild-type p53 according to typical IHC analyses (not all truncated forms of p53 are unstable, although their expression still may be suppressed by nonsense-mediated decay[56]). In any event, these proteins will be expressed at low levels, and such cases would be grouped together with samples that have low-level wild-type p53 expression. Because recent studies in other cancers, such as squamous cell cancer of the head and neck,[57] have indicated that deletion of p53 defines a group of patients with the worst outcome, such a grouping together of wild-type with deletions/nonsense mutations will obscure the significance of p53 in prognostication. To date, at least 14 articles have been published in which the p53 mutational rate was evaluated in RCC (Table 2).

In most of these studies, single-strand conformation polymorphism (SSCP) was used as an initial screen to detect mutations. The majority of studies analyzed the central core domain of the gene (exons 4-8 or 5-8), because this is the most common site of p53 mutation.[71] Approximately 15% of p53 mutations occur outside exons 5 through 8, in exons 4, 9, and 10[72]; therefore, it is likely that there will be some underreporting of p53 mutations in these studies. The frequency of p53 mutations reported is between 0% and 44% (excluding the study by Oda et al,[63] in which sarcomatoid tumors were evaluated). For comparison, in other tumors, the reported incidence of p53 mutations typically has been between 60% and 65% for lung and colon cancers; between 40% and 45% for stomach, esophageal, and bladder cancers; between 25% and 30% for breast, liver, and prostate cancers and lymphomas; and between 10% and 15% for leukemias (further information available at: http://www-p53.iarc.fr/ accessed June 15, 2009).[71] One intriguing observation derives from the study by Chemeris and colleagues, who observed that 0 of 29 RCC samples, all positive for p53 by IHC, had a p53 point mutation.[58] However, in another study, Zhang and colleagues observed that 44% of tumors with p53 staining (n = 16) had a p53 point mutation.[45] It is possible that the contamination of samples with normal tissue might lead to reduced detection of p53 mutations by SSCP; thus, variations in the extent of this may explain the differences between these 2 studies. This conclusion is supported indirectly by the observation that 33% of RCC-derived tumor cell lines harbored p53 mutations.[68] However, this assertion should be tempered by the possibility that the selection of cells to adapt to growth in vitro

Table 2. Studies Evaluating the p53 Mutational Rate in Renal Cell Carcinoma

Study	Sample Type	Technique	p53 Mutation Frequency, %[a]
Gad 2000[59]	46 Chromophobe/19 clear cell/9 papillary	Direct sequencing, exons 2-11	23.9/5.3/11.1
Kawasaki 1999[57]	5	SSCP and direct sequencing, exons 4-8	20
Zhang 1997[45]	16; All p53 positive by IHC[a]	SSCP only, exons 5-8	44[a]
Contractor 1997[60]	30 Clear cell/20 chromophobe	SSCP and direct sequencing or subclone sequencing, exons 5-8	3/30
Dahiya 1993[61]	40	SSCP and direct sequencing, exons 5-9	35
Dijkhuizen 1993[62]	14 Papillary	SSCP only, exons 2-11	0
Oda 1995[63]	14 Sarcomatoid	Subclone sequencing, exons 1-8	79
Chemaris 1995[58]	29; All p53 positive by IHC[a]	SSCP and direct sequencing, exons 4-8	0[a]
Kaczyk 1995[64]	33	SSCP and direct sequencing, exons 5-8	6
Uchida 1994[65]	36	SSCP and direct sequencing, exons 5-8	5
Kikuchi 1994[66]	118	SSCP and subclone sequencing, exons 4-9	2
Imai 1994[67]	50	SSCP and direct sequencing, exons 4-8	9
Reiter 1993[68]	31 RCC cell lines	SSCP and subclone sequencing, exons 5-9	33
Suzuki 1992[69]	23	SSCP and direct sequencing, exons 5-8	4.8
Tanigaw 1992[70]	21	SSCP, exons 2-11	10

SSCP indicates single-strand conformation polymorphism; IHC, immunohistochemistry; RCC, renal cell carcinoma.
[a]Positive IHC results were used to preselect samples for genetic analysis. Note that p53 mutation frequency refers only to the percentage of mutations identified in the samples that were analyzed in this manner.

may have resulted in an increased frequency of p53 mutation. It is noteworthy that data from CCRCC cell lines contrast with data from the study by Dijkhuizen et al[62] in which no p53 mutations were identified in 29 PRCC-derived tumor cell lines; thus, it seems possible that different mechanisms other than direct p53 mutation may inactivate p53 more frequently in PRCC than in RCC and that these also may result in higher expression.[57,63] One other finding of note is the high p53 mutation rate reported in sarcomatoid tumors.[62] Sarcomatoid change is a histologic finding associated with a poor prognosis in renal carcinoma in which a high p53 mutation rate (79%; n = 14)[63] was detected. Notwithstanding these differences between individual studies and RCC subtypes, it seems clear that, in contrast to both other cancers and p53 protein detection in RCC, p53 mutational analysis has yet to demonstrate prognostic utility.

MDM2 Expression and Prognosis in Renal Cell Carcinoma

Four studies have evaluated MDM2 and its prognostic value in RCC. Imai et al[67] screened 53 RCC tumor samples for MDM2 amplifications and identified none. In another study, IHC was used, and MDM2 expression was detected in 2 of 112 tumor samples (2%).[55] When 50 consecutive T3 and T4 tumors were screened by IHC, MDM2 expression was identified in 30%, but it reportedly had no prognostic significance in terms of overall survival.[48] However, it was observed that 7 of 8 patients who expressed p53 also expressed MDM2, raising the possibility that MDM2 expression may be linked to the up-regulation or activation of p53. This idea, that p53 and MDM2 expression may be linked in RCC, was supported by statistical analysis in that study (P = .0006). Another study that analyzed this question from Haitel and colleagues[59] examined 97 CCRCCs of all stages. MDM2 expression was detected in 19% of tumors and was significantly more frequent in high-grade tumors (P = .0149). In addition, MDM2 staining was strongly associated with tumor progression (P = .00113), and p53 expression was detected in 36% of the samples and was correlated with decreased progression-free survival (P = .00291). When different p53 and MDM2 phenotypes were compared (Fig. 2), it was observed that patients who had tumors with both MDM2 expression and p53 expression had the shortest progression-free survival (P = .00179). Perhaps most interesting from a mechanistic perspective and in accordance with the study by Moch and colleagues, the authors observed a highly significant correlation between MDM2 expression and p53 expression (P < .00004). These studies of MDM2 in RCC examined relatively small numbers of patients using different cutoff values for MDM2 expression. Nevertheless, the association of p53 and MDM2 detected by Moch et al and Haitel et al suggests that tumor progression in RCC may present a

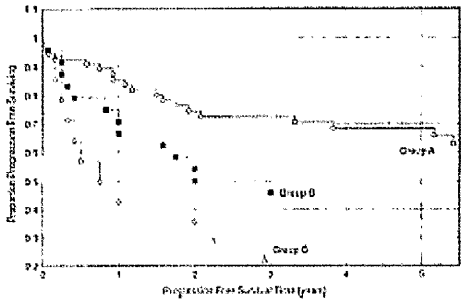


Figure 2. Kaplan-Meier disease-free survival curves are shown for patients with murine double minute 2 (mdm2)-negative/p53-negative tumors (Group A) versus patients with mdm2-negative/p53-positive or mdm2-positive/p53-negative tumors (Group B) versus patients with mdm2-positive/p53-positive tumors (Group C; $P = .00179$). Reproduced with permission from Haltel A, Wiener HG, Baethge U, Marberger M, Susani M. mdm2 Expression as a prognostic indicator in clear cell renal cell carcinoma: comparison with p53 overexpression and clinicopathological parameters. *Clin Cancer Res.* 2000; 6:1840-1844.[29]

tissue-specific pattern that has not been observed in many other cancers. For example, this link has not been observed in soft tissue sarcomas[73] or in bladder cancer,[13] although, in the latter, patients who had tumors that expressed mutant p53 and increased MDM2 had a poorer prognosis similar to that reported for patients with RCC.

Regarding MDM2 in RCC, an additional noteworthy point is the recently described single nucleotide polymorphism at codon 309 (SNP309 thymine/guanine [T/G]). This polymorphism lies in the intronic promoter region of the MDM2 gene and reportedly alters binding of the Sp1 transcription factor, with the G/G variant displaying increased binding and increased transcription of MDM2. It also has been reported that, in RCC, differences in the SNP309 genotype lead to different levels of MDM2 expression, as detected by IHC,[74] and that the G/G SNP309 genotype is an independent predictor of poor prognosis. Clearly, further analysis of this polymorphism is warranted.

In Vitro Studies of p53 and MDM2 Function in Renal Cell Carcinoma

Several groups, including our own, have used in vitro analyses to investigate the functional relation between p53 and MDM2 in RCC cells. We previously investigated a panel of RCC cell lines and concluded that p53 is regulated by MDM2, several of which retain relatively high levels of both wild-type p53 and MDM2.[22] p53 mutations have been detected in approximately 30% of RCC-derived cell lines, as discussed previously.[68] This, together with other studies demonstrating that p53 expression is an independent prognostic indicator, suggests that p53 function (or, rather, its loss or aberration) contributes to tumor evolution in the kidney. It has been suggested, however, that an alternative, novel, dominant mechanism leads to the inactivation of p53 in RCC. Although our own studies and those of others have provided evidence that does not appear to support this conclusion,[22,23] good reasons remain to continue investigating the possibility. Two arguments for this are immediately apparent. First, there is evidence from several studies that p53 is not mutated as frequently in RCC as it is in many other cancers. Second, the strong association between p53 expression and MDM2 expression may suggest a functional link between them, with 1 obvious possibility that MDM2 expression may be driven by wild-type p53. It is intriguing to note that in vitro studies have connected MDM2 with 2 critical phenotypes: the ability to promote both motility and invasiveness[12] and the regulation of angiogenic factors, such as hypoxia-inducible factor 1 (HIF-1).[22,25] Either or both of these phenotypic connections (MDM2 expression with metastasis and/or angiogenesis) may have important consequences.

Manipulating the p53 and MDM2 Pathway

Given the link between p53 and MDM2 expression with poor outcome, a key question becomes whether this is an association or a causal relation. In the event of the former, screening for p53 and/or MDM2 becomes justified for prognostic purposes and, in the future, might be used to stratify therapy according to an individual patient's risk. In the event of the latter, therapies that target the p53/MDM2 axis also become desirable. The best characterized drug that targets this pathway at the present time is nutlin-3,[75] which acts by competing with p53 for binding to the hydrophobic cleft in MDM2 and, thus, up-regulates wild-type p53. Other conceptually similar compounds that prevent MDM2-p53 interactions have been described recently that up-regulate wild-type p53.[76] Alternatively, drugs like the HLI98 family of compounds, which inhibit the E3 ligase activity of MDM2, may play a role in RCC, because high levels of MDM2 are linked to tumor progression. However, the associated co-up-regulation of MDM2 with p53 raises questions regarding the activity of MDM2 in these cells[77] with implications for

the utility of MDM2 enzymatic inhibitors. Therefore, it will be important to determine whether the role of MDM2 in disease progression in RCC depends on its enzymatic activity as an E3 ligase or whether this relates to some other function of MDM2. An alternative strategy would be to focus on the wild-type p53 present in these cells, which, clearly, is not fully active; otherwise, the cells would undergo a classic antiproliferative response. Moreover, we have demonstrated that the introduction of wild-type p53 into RCC cells does elicit a response, at least with respect to a limited set of outputs.[22] Thus, a potentially potent therapeutic option would be to reactivate the already high levels of p53 in these cells. RCC is notoriously insensitive to traditional chemotherapeutic and radiotherapeutic regimens that normally might activate p53,[78] possibly indicating that signaling to p53 is defective in these cells. However, p53 coexists with high levels of MDM2, suggesting that there is a breakdown in the interaction of these proteins. For future identification of effective therapy, it would be advantageous to identify the actual mechanism that prevents p53 activity in these cells. Notwithstanding this, several compounds aimed at p53 have demonstrated effectiveness in vitro in activating p53 in renal cancer cells. Examples include RITA (reactivation of p53 and induction of tumor cell apoptosis) and derivatives of 9-aminoacridine (including quinacrine).[79] It has been reported that RITA induces predominantly growth arrest rather than an apoptotic response in A498 RCC cells in vitro and that 9-aminoacridine and its derivatives, including quinacrine, indirectly inhibit MDM2 in RCC cells in vitro.[79,80] It remains unclear whether these compounds specifically target the pathway(s) that regulates p53/MDM2 co-up-regulation in RCC cells, and, to date, none of these compounds or their derivatives have been evaluated in clinical trials for RCC.

In conclusion, for many years, the role of the p53 pathway in renal cancer has been the subject of seemingly conflicting results. However, recent studies appear to be generating a consensus, which suggests a clear link between p53 expression and disease progression, particularly in CCRCC. It is noteworthy that this p53 positivity and link with prognosis is not corroborated by studies of p53 mutation; indeed, there are data suggesting that mutation of p53 may not be linked to outcome or progression in renal cancer and, thus, that the defect in p53 leading to its up-regulation lies elsewhere in the pathway. To our knowledge, only 2 studies of p53 in RCC have examined both p53 protein expression and p53 gene mutations,[45,50] and those results were conflicting. Given the disparity between the results from studies of p53 mutation and the more frequent observations of p53 up-regulation, it appears likely that p53 up-regulation is not caused by mutation in most cases of RCC. If this finding is correct, then it has important implications for understanding the nature of the defects in this pathway in RCC. For example, the up-regulation of wild-type p53 is linked to the up-regulation of MDM2, and it appears probable that this is because of p53 transcriptional activation of MDM2. Although it has been studied less than p53, MDM2 expression appears to be linked with disease progression. It remains unclear why this should be so when, clearly, it occurs in the context of high levels of p53 (ie, MDM2 is not performing its normal function to degrade p53).

The expression of p53 identifies a population of patients with RCC who are more likely to perform poorly. However, pathologists do not routinely monitor p53 in patients with RCC despite an increasing body of evidence suggesting that positive p53 status can predict recurrence and decreased survival. The ability to predict which patients will follow a poor disease course clearly would benefit clinicians, because more rigorous follow-up maybe indicated. Because small tumors (<4 cm; T1a) can present with metastases, more prognostic information clearly is required.[81] The limitations of current follow-up practice and the potential for molecular markers to improve this situation are reviewed excellently by Rouviere et al.[82] Moreover, 2 studies that included p53 status in their predictive nomograms demonstrated that it contributed to better prediction of survival.[24,34] Although the tumor microenvironment and other host factors will have an impact on outcome, intrinsic tumor mutations predominantly determine tumor growth, invasion, and metastasis. Following the existing paradigm (which led to the discovery of the role of VHL mutations; the consequences of these for HIF-1α and HIF-2α expression and for oncogenic mediators, such as vascular endothelial growth factor; and recent developments in therapeutic targeting of such tumor determinants) should lead to the identification of these personalized indicators. Loss of p53 function is a key event in carcinogenesis; and the evidence suggests that p53 and MDM2 not only may provide 2 components of such improved prognostication but, at the same time, also may provide for novel, potentially tissue-specific therapeutic target(s) to improve treatment.

CONFLICT OF INTEREST DISCLOSURES

Supported by Mersey Kidney Research, Cancer Research UK, and the North West Cancer Research Fund.

REFERENCES

1. Horner MJ, Ries LAG, Krapcho M, et al, eds. SEER Cancer Statistics Review, 1975-2006. Bethesda, Md: National Cancer Institute. Based on the November 2008 SEER data submission; posted to the SEER website, 2009. Available at: http://info.cancerresearch.uk.org/cancerstats/types/kidney/incidence/. Accessed on June 15, 2009.
2. Cancer Research UK. UK Kidney Cancer Incidence Statistics, 2009. London, United Kingdom: Cancer Research UK; 2009.
3. Lane BR, Rini BI, Novick AC, Campbell SC. Targeted molecular therapy for renal cell carcinoma. *Urology*. 2007; 69:3-10.
4. Lopez-Beltran A, Scarpelli M, Montironi R, Kirkali Z. 2004 WHO classification of the renal tumors of the adults. *Eur Urol*. 2006;49:798-805.
5. Hollstein M, Rice K, Greenblatt MS, et al. Database of p53 gene somatic mutations in human tumors and cell lines. *Nucleic Acids Res*. 1994;22:3551-3555.
6. Malkin D, Li FP, Strong LC, et al. Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. *Science*. 1990;250:1233-1238.
7. Montes de Oca Luna R, Wagner DS, Lozano G. Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. *Nature*. 1995;378:203-206.
8. Wu X, Bayle JH, Olson D, Levine AJ. The p53-mdm-2 autoregulatory feedback loop. *Genes Dev*. 1993;7:1126-1132.
9. Thut CJ, Goodrich JA, Tjian R. Repression of p53-mediated transcription by MDM2: a dual mechanism. *Genes Dev*. 1997;11:1974-1986.
10. Kubbutat MH, Jones SN, Vousden KH. Regulation of p53 stability by Mdm2. *Nature*. 1997;387:299-303.
11. Fang S, Jensen JP, Ludwig RL, Vousden KH, Weissman AM. Mdm2 is a RING finger-dependent ubiquitin protein ligase for itself and p53. *J Biol Chem*. 2000;275: 8945-8951.
12. Yang JY, Zong CS, Xia W, et al. MDM2 promotes cell motility and invasiveness by regulating E-cadherin degradation. *Mol Cell Biol*. 2006;26:7269-7282.
13. Lu ML, Wikman F, Orntoft TF, et al. Impact of alterations affecting the p53 pathway in bladder cancer on clinical outcome, assessed by conventional and array-based methods. *Clin Cancer Res*. 2002;8:171-179.
14. Canman CE, Lim DS, Cimprich KA, et al. Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. *Science*. 1998;281:1677-1679.
15. Bode AM, Dong Z. Post-translational modification of p53 in tumorigenesis. *Nat Rev Cancer*. 2004;4:793-805.
16. Nenutil R, Smardova J, Pavlova S, et al. Discriminating functional and non-functional p53 in human tumours by p53 and MDM2 immunohistochemistry. *J Pathol*. 2005; 207:251-259.
17. Munro AJ, Lain S, Lane DP. P53 abnormalities and outcomes in colorectal cancer: a systematic review. *Br J Cancer*. 2005;92:434-444.
18. Slee EA, O'Connor DJ, Lu X. To die or not to die: how does p53 decide? *Oncogene*. 2004;23:2809-2818.
19. MacCallum DE, Hupp TR, Midgley CA, et al. The p53 response to ionising radiation in adult and developing murine tissues. *Oncogene*. 1996;13:2575-2587.
20. Lahav G, Rosenfeld N, Sigal A, et al. Dynamics of the p53-Mdm2 feedback loop in individual cells. *Nat Genet*. 2004;36:147-150.
21. Gurova KV, Hill JE, Razorenova OV, Chumakov PM, Gudkov AV. p53 pathway in renal cell carcinoma is repressed by a dominant mechanism. *Cancer Res*. 2004; 64:1951-1958.
22. Warburton HE, Brady M, Vlatkovic N, Linehan WM, Parsons K, Boyd MT. p53 regulation and function in renal cell carcinoma. *Cancer Res*. 2005;65:6498-6503.
23. Carroll VA, Ashcroft M. Regulation of angiogenic factors by HDM2 in renal cell carcinoma. *Cancer Res*. 2008; 68:545-552.
24. Klatte T, Seligson DB, LaRochelle J, et al. Molecular signatures of localized clear cell renal cell carcinoma to predict disease-free survival after nephrectomy. *Cancer Epidemiol Biomarkers Prev*. 2009;18:894-900.
25. Perret AG, Clemencon A, Li G, Toutain J, Peoc'h M. Differential expression of prognostic markers in histological subtypes of papillary renal cell carcinoma. *BJU Int*. 2008;102:183-187.
26. Phinse NB, Ehara H, Gotoh T, et al. Immunohistochemical analysis with multiple antibodies in search of prognostic markers for clear cell renal cell carcinoma. *Urology*. 2007; 69:843-848.
27. Kankuri M, Soderstrom KO, Pelliniemi TT, Vahlberg T, Pyrhonen S, Salminen E. The association of immunoreactive p53 and Ki-67 with T-stage, grade, occurrence of metastases and survival in renal cell carcinoma. *Anticancer Res*. 2006;26(5B):3825-3833.
28. Kramer BA, Gao X, Davis M, Hall M, Holzbeierlein J, Tawfik O. Prognostic significance of ploidy, MIB-1 proliferation marker, and p53 in renal cell carcinoma. *J Am Coll Surg*. 2005;201:565-570.
29. Langner C, Ratschek M, Rehak P, Tsybrovskyy O, Zigeuner R. The pT1a and pT1b category subdivision in renal cell carcinoma: is it reflected by differences in tumour biology? *BJU Int*. 2005;95:310-314.
30. Cho DS, Joo HJ, Oh DK, et al. Cyclooxygenase-2 and p53 expression as prognostic indicators in conventional renal cell carcinoma. *Yonsei Med J*. 2005;46:133-140.
31. Shvarts O, Seligson D, Lam J, et al. p53 is an independent predictor of tumor recurrence and progression after nephrectomy in patients with localized renal cell carcinoma. *J Urol*. 2005;173:725-728.
32. Uzunlar AK, Sahin H, Yilmaz F, Ozekinci S. Expression of p53 oncoprotein and bcl-2 in renal cell carcinoma. *Saudi Med J*. 2005;26:37-41.
33. Zigeuner R, Ratschek M, Rehak P, Schips L, Langner C. Value of p53 as a prognostic marker in histologic subtypes of renal cell carcinoma: a systematic analysis of primary and metastatic tumor tissue. *Urology*. 2004;63: 651-655.
34. Kim HL, Seligson D, Liu X, et al. Using protein expression to predict survival in clear cell renal carcinoma. *Clin Cancer Res*. 2004;10:5464-5471.
35. Uchida T, Gao JP, Wang C, et al. Clinical significance of p53, mdm2, and bcl-2 proteins in renal cell carcinoma. *Urology*. 2002;59:615-620.
36. Olumi AF, Weidner N, Presti JC. p53 immunoreactivity correlates with Ki-67 and bcl-2 expression in renal cell carcinoma. *Urol Oncol*. 2001;6:63-67.
37. Hungerg B, Bozoky B, Kovacs G, et al. p53 expression in correlation to clinical outcome in patients with renal cell carcinoma. *Scand J Urol Nephrol*. 2001;35:15-20.
38. Girgin C, Tarhan H, Hekimgil M, Sezer A, Gurel G. P53 mutations and other prognostic factors of renal cell carcinoma. *Urol Int*. 2001;66:78-83.

39. Haitel A, Wiener HG, Baethge U, Marberger M, Susani M. mdm2 expression as a prognostic indicator in clear cell renal cell carcinoma: comparison with p53 overexpression and clinicopathological parameters. *Clin Cancer Res.* 2000; 6:1810-1814.
40. Rioux-Leclercq N, Turlin B, Bansard J, et al. Value of immunohistochemical Ki-67 and p53 determinations as predictive factors of outcome in renal cell carcinoma. *Urology.* 2000;55:501-505.
41. Sejima T, Miyagawa I. Expression of bcl-2, p53 oncoprotein, and proliferating cell nuclear antigen in renal cell carcinoma. *Eur Urol.* 1999;35:242-248.
42. Vasavada SP, Novick AC, Williams BR. P53, bcl-2, and Bax expression in renal cell carcinoma. *Urology.* 1998; 51: 1057-1061.
43. Sinik Z, Alkibay T, Atasoglu O, et al. Nuclear p53 overexpression in bladder, prostate, and renal carcinomas. *Int J Urol.* 1997;4:546-551.
44. Papadopoulos I, Rudolph P, Weichert-Jacobsen K. Value of p53 expression, cellular proliferation, and DNA content as prognostic indicators in renal cell carcinoma. *Eur Urol.* 1997;32:110-117.
45. Zhang XH, Takenaka I, Sato C, Sakamoto H. p53 and HER-2 alterations in renal cell carcinoma. *Urology.* 1997; 50:636-642.
46. Gelb AB, Suddlowsky D, Wu CD, Weiss LM, Medeiros LJ. Appraisal of intratumoral microvessel density, MIB-1 score, DNA content, and p53 protein expression as prognostic indicators in patients with locally confined renal cell carcinoma. *Cancer.* 1997;80:1768-1775.
47. Shiina H, Igawa M, Urakami S, Shirakawa H, Ishibe T, Kawanishi M. Clinical significance of immunohistochemically detectable p53 protein in renal cell carcinoma. *Eur Urol.* 1997;31:73-80.
48. Moch H, Sauter G, Gasser TC, et al. p53 protein expression but not mdm-2 protein expression is associated with rapid tumor cell proliferation and prognosis in renal cell carcinoma. *Urol Res.* 1997;25(suppl 1):S25-S30.
49. Hofmockel G, Wittmann A, Dammrich J, Bassukas ID. Expression of p53 and bcl-2 in primary locally confined renal cell carcinomas: no evidence for prognostic significance. *Anticancer Res.* 1996;16(6B):3807-3811.
50. Chemeris G, Loktinov A, Rempel A, Schwarz M, Bannasch P. Elevated content of p53 protein in the absence of p53 gene mutations as a possible prognostic marker for human renal cell tumors. *Virchows Arch.* 1995;426:563-569.
51. Lipponen P, Eskelinen M, Hietala K, Syrjanen K, Gambetta RA. Expression of proliferating cell nuclear antigen (PC10), p53 protein and c-erbB-2 in renal adenocarcinoma. *Int J Cancer.* 1994;57:275-280.
52. Kamel D, Turpeenniemi-Hujanen T, Vahakangas K, Paakko P, Soini Y. Proliferating cell nuclear antigen but not p53 or human papillomavirus DNA correlates with advanced clinical stage in renal cell carcinoma. *Histopathology.* 1994;25:339-347.
53. Bot FJ, Godschalk JC, Krishnadath KK, van der Kwast TH, Bosman FT. Prognostic factors in renal-cell carcinoma: immunohistochemical detection of p53 protein versus clinicopathological parameters. *Int J Cancer.* 1994; 57:634-637.
54. Uhlman DL, Nguyen PL, Manivel JC, et al. Association of immunohistochemical staining for p53 with metastatic progression and poor survival in patients with renal cell carcinoma. *J Natl Cancer Inst.* 1994;86:1470-1475.
55. Boyd MT, Vlatkovic N. p53: a molecular marker for the detection of cancer. *Expert Opin Med Diagn.* 2008;2:1013-1024.
56. Anensikow O, Ware MD, Bulsson M, et al. Does the nonsense-mediated mRNA decay mechanism prevent the synthesis of truncated BRCA1, CHK2, and p53 proteins? *Hum Mutat.* 2008;29:65-73.
57. Poeta ML, Manola J, Goldwasser MA, et al. TP53 mutations and survival in squamous-cell carcinoma of the head and neck. *N Engl J Med.* 2007;357:2552-2561.
58. Gad S, Lefevre SH, Khoo SK, et al. Mutations in BHD and TP53 genes, but not in HNF1beta gene, in a large series of sporadic chromophobe renal cell carcinoma. *Br J Cancer.* 2007;96:336-340.
59. Kawazaki T, Bilim V, Takahashi K, Tomita Y. Infrequent alteration of p53 pathway in metastatic renal cell carcinoma. *Oncol Rep.* 1999;6:329-333.
60. Contractor H, Zariwala M, Bugert P, Zeider J, Kovacs G. Mutation of the p53 tumour suppressor gene occurs preferentially in the chromophobe type of renal cell tumour. *J Pathol.* 1997;181:136-139.
61. Dahiya R, Deng G, Selph C, Carroll P, Presti J Jr. A novel p53 mutation hotspot at codon 132 (AAG→AGG) in human renal cancer. *Biochem Mol Biol Int.* 1998;44:107-115.
62. Dijkhuizen T, Van den Berg E, Van den Berg A, et al. Chromosomal findings and p53-mutation analysis in chromophilic renal-cell carcinomas. *Int J Cancer.* 1995;63:47-50.
63. Oda H, Nakatsuru Y, Ishikawa T. Mutations of the p53 gene and p53 protein overexpression are associated with sarcomatoid transformation in renal cell carcinomas. *Cancer Res.* 1995;55:658-662.
64. Kuczyk MA, Serth J, Bokemeyer C, et al. Detection of p53 gene alteration in renal-cell cancer by micropreparation techniques of tumor specimens. *Int J Cancer.* 1995;64:399-406.
65. Uchida T, Wada C, Wang C, Egawa S, Ohtani H, Koshiba K. Genomic instability of microsatellite repeats and mutations of H-, K-, and N-ras, and p53 genes in renal cell carcinoma. *Cancer Res.* 1994;54:3682-3685.
66. Kikuchi Y, Kishi T, Suzuki M, Furusato M, Aizawa S. Polymerase chain reaction-single strand conformation polymorphism analysis of the p53 gene in paraffin-embedded surgical material from human renal cell carcinomas. *Virchows Arch.* 1994;424:229-233.
67. Imai Y, Strohmeyer TG, Fleischhacker M, Slamon DJ, Koeffler HP. p53 mutations and MDM-2 amplification in renal cell cancers. *Mod Pathol.* 1994;7:765-770.
68. Reiter RE, Anglard P, Liu S, Gnarra JR, Linehan WM. Chromosome 17p deletions and p53 mutations in renal cell carcinoma. *Cancer Res.* 1993;53:3092-3097.
69. Suzuki Y, Tamura G, Satodate R, Fujiola T. Infrequent mutation of p53 gene in human renal cell carcinoma detected by polymerase chain reaction single-strand conformation polymorphism analysis. *Jpn J Cancer Res.* 1992;83:233-235.
70. Torigoe S, Shuin T, Kubota Y, Horikoshi T, Danenberg K, Danenberg PV. p53 gene mutation in primary human renal cell carcinoma. *Oncol Res.* 1992;4:467-472.
71. Pethjean A, Mathe E, Kato S, et al. Impact of mutant p53 functional properties on TP53 mutation patterns and tumor phenotype: lessons from recent developments in the IARC TP53 database. *Hum Mutat.* 2007;28:622-629.
72. Soussi T, Beroud C. Assessing TP53 status in human tumours to evaluate clinical outcome. *Nat Rev Cancer.* 2001;1:233-240.

73. Cordon-Cardo C, Latres E, Drobnjak M, et al. Molecular abnormalities of mdm2 and p53 genes in adult soft tissue sarcomas. *Cancer Res.* 1994;54:794-799.
74. Hirata H, Hinoda Y, Kikuno N, et al. MDM2 SNP309 polymorphism as risk factor for susceptibility and poor prognosis in renal cell carcinoma. *Clin Cancer Res.* 2007;13:4123-4129.
75. Vassilev LT, Vu BT, Graves B, et al. In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science.* 2004;303:844-848.
76. Shangary S, Qin D, McEachern D, et al. Temporal activation of p53 by a specific MDM2 inhibitor is selectively toxic to tumors and leads to complete tumor growth inhibition. *Proc Natl Acad Sci USA.* 2008;105:3933-3938.
77. Yang Y, Ludwig RL, Jensen JP, et al. Small molecule inhibitors of HDM2 ubiquitin ligase activity stabilize and activate p53 in cells. *Cancer Cell.* 2005;7:547-559.
78. Motzer RJ, Bander NH, Nanus DM. Renal-cell carcinoma. *N Engl J Med.* 1996;335:865-875.
79. Guo C, Gasparian AV, Zhuang Z, et al. 9-Aminoacridine-based anticancer drugs target the PI3K/AKT/mTOR, NF-kappaB and p53 pathways. *Oncogene.* 2009;28:1151-1161.
80. Enge M, Bao W, Hedström E, Jackson SP, Moumen A, Selivanova G. MDM2-dependent downregulation of p21 and hnRNP K provides a switch between apoptosis and growth arrest induced by pharmacologically activated p53. *Cancer Cell.* 2009;15:171-183.
81. Nguyen MM, Gill IS. Effect of renal cancer size on the prevalence of metastasis at diagnosis and mortality. *J Urol.* 2009;181:1020-1027; discussion 1027.
82. Rouviere O, Bouvier R, Negrier S, Badet L, Lyonnet D. Nonmetastatic renal-cell carcinoma: is it really possible to define rational guidelines for post-treatment follow-up? *Nat Clin Pract Oncol.* 2006;3:209-213.

A2.4 Published paper (accepted for publication in the BJUi – April 2011)

BJU International

BJUI

BJU INTERNATIONAL

Combined p53 & MDM2 biomarker analysis demonstrates a unique pattern of expression associated with poor prognosis in renal cell carcinoma patients undergoing radical nephrectomy

Journal:	*BJU International*
Manuscript ID:	BJU-2011-0132.R1
Manuscript Type:	Original Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Noon, Aidan; University of Liverpool, Cancer Studies Polanski, Radoslaw; University of Liverpool, Cancer Studies El-Fert, Ashraf; University of Liverpool, Cancer Studies Kalirai, Helen; University of Liverpool, Cancer Studies Shawki, Howida; Royal Liverpool University Hospital, Pathology Campbell, Fiona; Royal Liverpool University Hospital, Pathology Dodson, Andy; Royal Liverpool University Hospital, Pathology Eccles, Richard; University of Liverpool, Cancer Studies Lloyd, Bryony; University of Liverpool, Cancer Studies Sibson, David; University of Liverpool, Cancer Studies Coupland, Sarah; University of Liverpool, Cancer Studies Lake, Sarah; University of Liverpool, Cancer Studies Parsons, Keith; Royal Liverpool University Hospital, Urology Vlatkovic, Nikolina; University of Liverpool, Cancer Studies Boyd, Mark; University of Liverpool, Surgery and Oncology
keywords:	p53, mdm2, renal cancer, prognosis, survival
Abstract:	Objectives: The role of p53 in RCC has been much debated. To resolve issues surrounding p53 function, expression and mutation we have performed the first study to simultaneously determine p53/MDM2 expression, TP53 mutational status (in p53-positive patients) and outcomes in RCC. Patients and Methods: 90 specimens from patients with RCC, treated by radical nephrectomy, were analysed by immunohistochemistry for p53 and MDM2 on a tissue microarray and p53 was functionally and genetically analysed in p53 positive samples. Outcomes analysis

was by Kaplan-Meier method and univariate analysis was used to identify variables for subsequent multivariate analysis of correlations between clinical parameters and biomarker expression.

Results:

Up-regulation of p53 in RCC is strongly linked with MDM2 up-regulation (P=0.000013). Increased co-expression of p53 and MDM2 identifies patients with significantly reduced DSS by univariate (P=0.036) and Cox multiple regression analysis (P=0.027, RR=3.20). Functional (FASAY) and genetic analysis of tumours with increased p53 expression demonstrates that the majority (86%) retain wild-type p53.

Conclusions:

Co-expression of p53/MDM2 identifies a sub-set of patients with poor prognosis despite all having organ confined disease. Up-regulated p53 is typically wild-type and thus provides a mechanistic explanation for the association between p53 and MDM2 expression. Our results suggest that the p53 pathway is altered in a tissue/disease-specific manner and that up-regulated wild-type p53 likely promotes the observed MDM2 co-expression. Therapeutic strategies targeting this pathway should be investigated to determine whether the tumour suppressive function of p53 can be rescued in RCC.

Running title: p53 and MDM2 association in renal cancer

Combined p53 & MDM2 biomarker analysis demonstrates a unique pattern of expression associated with poor prognosis in renal cell carcinoma patients undergoing radical nephrectomy

Aidan P. Noon[†], Ashraf Y. El-Fert[†], Radosław Polański[†], Helen Kalirai[#], Howida Shawki[+], Fiona Campbell[+], Andy Dodson[+], Keith Parsons[%], Nikolina Vlatković[†] and Mark T. Boyd[†]

From the [†]p53/MDM2 Research Group, and the [#]Division of Pathology, Department of Molecular and Clinical Cancer Medicine, University of Liverpool, L69 3GA, UK, and from the [+]Departments of Pathology, and [%]Urology, Royal Liverpool University Hospital, Liverpool, L7 8XP, UK

Correspondence to M. Boyd:

Division of Surgery and Oncology, Department of Molecular and Clinical Cancer Medicine, University of Liverpool, 5th Fl. UCD Building, Daulby St, Liverpool L69 3GA

Tel 0151 706 4185,

Fax 0151 706 5826

mboyd@liv.ac.uk

Word count: 3,713 (not incl. figures, tables, references and abstract)

Abstract (240 words)

Objectives:

The role of p53 in RCC has been much debated. To resolve issues surrounding p53 function, expression and mutation we have performed the first study to simultaneously determine p53/MDM2 expression, TP53 mutational status (in p53-positive patients) and outcomes in RCC.

Patients and Methods:

90 specimens from patients with RCC, treated by radical nephrectomy, were analysed by immunohistochemistry for p53 and MDM2 on a tissue microarray and p53 was functionally and genetically analysed in p53 positive samples. Outcomes analysis was by Kaplan-Meier method and univariate analysis was used to identify variables for subsequent multivariate analysis of correlations between clinical parameters and biomarker expression.

Results:

Up-regulation of p53 in RCC is strongly linked with MDM2 up-regulation (P=0.000013). Increased co-expression of p53 and MDM2 identifies patients with significantly reduced DSS by univariate (P=0.036) and Cox multiple regression analysis (P=0.027, RR=3.20). Functional (FASAY) and genetic analysis of tumours with increased p53 expression demonstrates that the majority (86%) retain wild-type p53.

Conclusions:

Co-expression of p53/MDM2 identifies a sub-set of patients with poor prognosis despite all having organ confined disease. Up-regulated p53 is typically wild-type and thus

provides a mechanistic explanation for the association between p53 and MDM2 expression. Our results suggest that the p53 pathway is altered in a tissue/disease-specific manner and that up-regulated wild-type p53 likely promotes the observed MDM2 co-expression. Therapeutic strategies targeting this pathway should be investigated to determine whether the tumour suppressive function of p53 can be rescued in RCC.

Keywords: p53, MDM2, renal cell carcinoma, clear cell renal cell carcinoma, prognosis, survival,

Introduction

The incidence of cancer of the kidney is increasing rapidly in the developed world, for example in the USA, the age-adjusted incidence has risen from 8 to approximately 14/100,000 in 30 years [1]. It has been estimated that in the USA in 2010, 58,240 people will have been diagnosed with kidney cancers and that 13,040 people will have died from the disease [2]. The present study focuses primarily on the most common histological sub-type of kidney cancer; clear cell renal cell carcinoma (RCC; 2004, WHO classification system) [3] which typically accounts for 75% of all kidney cancers [4].

Despite advances in our understanding of the molecular biology of renal cell carcinoma which have led to the development of a number of new targeted molecular drug treatments (targeting consequences of VHL mutations (increased VEGF), or mTOR dependence), patients with metastatic disease still have an extremely short life expectancy [5]. Identifying other pathways, that determine for example tumour metastatic potential, is a high priority not only for prognostication, but also for defining novel therapeutic targets.

The role of the p53 pathway in RCC remains unclear and in part this stems from the literature on p53 and the p53 pathway in RCC which appears to be ambiguous. However, in a recent review of his literature, we found that that information on the status of the p53/MDM2 pathway may nevertheless provide significant prognostic information, though this requires careful interpretation due to peculiarities of this pathway in kidney [6]. The present study is therefore aimed at an examination of p53 expression as a biomarker and combining this information with data on MDM2, one of

the essential negative regulators of p53. Our objective being to determine whether the combined use of these potential biomarkers can provide further insights into the functional status of the p53 pathway in clinical samples, as has previously been proposed for other solid tumours [7]. Since the reason for analysing the combination of p53 and MDM2 in other cancers has been to provide a surrogate indication of p53 mutational status, in cancers where p53 mutations are rare, such as RCC, this kind of analysis would be expected to be either uninformative, or informative only in rare cases where p53 is mutatated. However, in settings where p53 mutations are rare, as occurs in RCC for example, patterns of expression of p53 and MDM2 would be expected either to be uninformative, or at least to only to be so in rare cases since the reason for analysing MDM2 and p53 in other cancers has been to provide a surrogate indication of the likelihood of p53 mutations to be inferred [7]. From this it follows that in cancers with rare p53 mutations, if p53/MDM2 expression patterns were found to be associated with disease progression/outcomes then this would require new biological explanations for the defects in this pathway since the association is not due to mutations in p53. That the kidney behaves in an atypical way wrt p53 function is not without precedent and there is evidence biologically of unique characteristics regarding p53 and p53-function in the kidney [6, 8, 9], as well as evidence of other distinct mechanisms involved in renal carcinogenesis such as loss of VHL function. Thus, unlike many other cancers where p53 and/or MDM2 expression or genetic analysis is well understood, the role of these in RCC remains enigmatic [6].

Accordingly, we set out to investigate the expression of p53 and MDM2 in a cohort of 90 RCC patients treated by radical nephrectomy at the Royal Liverpool University

Hospital and to determine *TP53* mutational status both functionally using the yeast functional assay (FASAY) [10] and by genotyping for all p53 positive/expressing samples to determine whether these potential biomarkers correlate with patient outcomes.

Patients and methods

Patient samples and Tissue Micro-Array (TMA) construction

Between 1992 and 2007 the Liverpool Tissue Bank (LTB) collected specimens from 97 patients undergoing radical nephrectomy for RCC and for this study data was obtained from 90 cases of pathologically confirmed renal carcinoma (haematoxylin and eosin (H&E) stained slides and formalin fixed paraffin embedded (FFPE) blocks) which were retrieved from the archives of the LTB. These patients had consented for the storage and use of their tissue for research between 1993 and 2007 and study-specific ethical approval was obtained from the Liverpool Adult Research Ethics Committee. Data on the stage and grade of the tumours were collected prospectively by the LTB. The cases analysed were of the following sub-types: clear cell type (n=87), papillary cell type (n=2) and chromophobe cell type (n=1). All H&E-stained slides from the FFPE material were evaluated by a Pathologist for the presence of tissue regions optimally representative of the renal cell carcinoma. An appropriate slide was selected and representative regions of tumour were circled from each case. This was also performed for a slide containing adjacent non involved renal tissue from each case. From each corresponding FFPE tumour block, at least duplicate cores (0.6mm in diameter) were taken from the marked area and mounted into a recipient paraffin block using the Manual Arrayer (Beecher Instruments Inc). Duplicate tumour cores were not placed next to each other in the

recipient paraffin block to eliminate both scoring and staining biases. A single core of non-involved renal tissue was also mounted into the recipient block. Cores of normal colon, liver and testis were also included for orientation and as controls during immunohistochemistry (IHC). Serial 5μm sections were cut from the tissue microarray (TMA) and collected onto X-tra™ adhesive slides (Surgipath).

Antibodies

Murine monoclonal antibodies for MDM2 (clone SMP14, used at 1/100) and for p53 (clone DO-7) used at 1/200 for IHC were obtained from Santa Cruz Biotechnology and DAKO respectively. For immunofluorescence, rabbit polyclonal anti-p53 antiserum (CM1, Leica Microsystems) and mouse Mab anti-MDM2 (sc-965, Santa Cruz Biotechnology) were used.

Immunohistochemistry

Samples prepared as described above were de-waxed, re-hydrated and endogenous peroxidase was blocked with H_2O_2 in methanol prior to high temp antigen retrieval (pressure cooker with 10mM EDTA pH7.0), and all were stained on a Dako Autostainer using labelled polymer detection ADVANCE™ (Dako). Sections were counterstained with Mayer's haematoxylin. Slides of stained specimens were reviewed by two consultant histopathologists (blinded to clinical outcome) and a scoring system was determined empirically as follows. The intensity of staining was graded 0 (no staining) through 1 (weakly stained) and 2 (moderately stained) to 3 (highly stained). The percentage of cells showing positive staining was graded as follows 1 = 0 - 2%, 2 = 3 -

10%, 3 = 11 - 50% and 4 > 50%. The TMA sections were then independently scored and results analysed. In the event of any discrepancy these specimens were reviewed by the histopathologists together and a score agreed upon. For each tumour there were two cores on the TMA thus 180 cores were scored. For assessment and statistical analyses of p53 and MDM2 staining there were a number of specimens which were found to clearly stain more profoundly than the remainder (intensity score 3 and percentage score 4) and thus these parameters were used to dichotomize p53 and/or MDM2 staining into positive and negative groups.

Dual Fluorescence Staining

For dual staining and detection by immunofluorescence, serially sectioned 4μm paraffin sections were de-waxed in xylene and re-hydrated in graded ethanol to distilled water. Heat mediated antigen retrieval was performed in a pressure cooker filled with 10mM EDTA (pH 7.0) in which sections were treated at full pressure for 3 minutes. Following this, manual staining was undertaken in a flat-bed incubation tray. Primary antibodies were applied as required to serial sections. Negative controls were performed using antibody diluent in place of antibody. Primary antibodies diluted in commercial diluent solution (Dako) were applied for 60 minutes following which sections were washed in TBS and incubated for 60 minutes with a mixture of two fluorescently labeled secondary antibodies: FITC labeled horse anti-mouse IgG (Vector Laboratories), and TRITC labeled swine anti-rabbit Ig's (Dako). Sections were washed in TBS and mounted in aqueous mounting medium containing DAPI (Vector Laboratories).

FASAY and genotyping

FASAY was performed essentially as described [10]. pRDI-22 was a kind gift of Richard Iggo. For genetic analysis of FASAY products PCR was performed on at least three independent red (i.e. mutant) clones. For some samples PCR amplified p53 exons 1 to 10 (plus the coding part of exon 11) including the exon – intron junctions of the *TP53* gene were subject to direct DNA sequence analysis. PCR primers are summarised in supplementary data Table 1. DNA sequencing reactions were performed using DYEnamic ET Dye Terminators (GE Healthcare) and capillary electrophoresis (Megabace 1000– GE Healthcare). Sequence variants were scored if present in both the sense and anti-sense strand of all three triplicates.

Statistical analysis

All data was entered into a database using Microsoft Office Excel 2007. Statistical analysis was performed using SPSS version 16. The association between p53 and MDM2 expression with patient and tumour factors was analysed using a two-sided Fisher's exact test. Disease-free and overall survival curves of the nephrectomy patients were estimated according to the Kaplan-Meier method. Statistical analyses of the differences between curves were performed using the log-rank test. Variables that significantly influenced survival ($P < 0.05$) in the univariate analysis were entered into a multivariate Cox regression model. In all of the analyses, the significance level was set at 0.05.

Results

Only two studies have previously examined p53 and MDM2 expression in RCC and neither of these determined the genetic status of the expressed *TP53* [12, 13]. We therefore examined p53 and MDM2 expression and the status of *TP53* in the p53 positive/expressing samples from a cohort of 90 renal cell carcinomas. Figure 1 shows examples of typical staining observed for p53 and MDM2 and also Figure 1C shows that in an individual p53/MDM2 co-expressing sample, the same cells express both p53 and MDM2 protein at relatively high levels. A proportion of the tumours (14/90) displayed high intensity staining for p53 (scored as grade =3) of at least 50% of the specimen. Since this staining pattern was distinct from the remainder of the samples it was therefore used to dichotomise the samples into p53 positive and negative groups. As for p53, the same criteria when applied to MDM2 also identified a clearly distinct sub-set of the RCC specimens (24/90) and therefore this was used to dichotomise samples as MDM2 positive and negative.. Table 1 summarises the clinical parameters of the cohort used for this study and the results of immunohistochemical analyses for p53 and MDM2. As has been observed previously [12, 13], and as illustrated in Table 2.i there is a very strong association in renal cell carcinomas between p53 and MDM2 expression (P=0.000013).

To investigate the expressed p53 further, we performed a yeast functional assay (FASAY) for p53 and genetic analysis of the *TP53* gene in samples displaying p53 expression to determine whether p53 was wild type or mutant. The results of these analyses are shown in Table 3 which demonstrates that 12/14 (86%) of p53 expressing RCCs retained wild-type *TP53*. We next investigated whether p53 or MDM2 expression was linked with disease specific survival (DSS) and generated Kaplan-Meier plots which indicate that increased co-expression of p53 with MDM2 is associated (P=0.027)

with reduced DSS during the full follow-up period (median follow up 4.99 years, range 0.79 - 13.27 years) for this cohort of patients as shown in Figure 2. Note that only the combination of dual positivity is significant; plots dichotomizing for p53 or MDM2 individually were not significantly associated with DSS (not shown). In addition, as Table 2.ii shows, co-expression of p53 and MDM2 is significantly linked with reduced DSS in early disease.

On univariate analysis only tumour stage (pT1-2 vs pT3, 2002 TNM classification) and the combination of p53 positive co-expressing MDM2 were significant predictors of outcome as Table 2.iii illustrates. Finally, Table 2.iv shows the results of Cox multiple regression analysis which demonstrates that the combination of p53 and concomitant MDM2 expression was significantly linked in both univariate (P=0.036) and multivariate (P=0.027) analyses. It is noteworthy that the relative risk of death from disease was comparable for concomitant p53/MDM2 positivity (RR 3.203 95% CI 1.143 - 8.98) to that observed when comparing pT1-2 tumours with pT3 (RR 2.897 95% CI 1.155 - 7.27). In addition, whilst pT1-2 and pT3 were significant indicators of outcome in univariate analyses, Fuhrman grade was not and therefore grade was not included as a parameter in multiple regression analyses. This may partly be due to the fact that this cohort is predominantly of low stage disease, all N=0 and M=0 at diagnosis/pre-operatively and with predominantly T1/2 disease (stage T1 (n=44), stage T2 (n=25)) and with only 4 cases of Fuhrman grade 4 (see Table 1). Note that none of the patients from whom samples were collected had evidence of metastatic disease at the time of nephrectomy and thus neither metastasis nor nodal involvement were analysed as parameters in this cohort.

Discussion

The p53 pathway, with its associated regulators is one of the most studied of all biological pathways that have been examined in human cancers. However, there are tissue-specific issues in the kidney which appear to complicate our understanding of the several analyses performed to date of p53 in RCC (and to a lesser extent of MDM2) [6]. In addition, since p53 mutations are rare in RCC [6] and as we show here, p53 is rarely mutated in the p53 expressing RCC samples, it has been necessary to devise an empirical approach to scoring p53 and MDM2 for RCCs.

Nevertheless, from the analysis presented here we draw three potentially important conclusions which have significant implications for our understanding of underlying molecular events in RCC: firstly, p53 and MDM2 co-expression is very strongly linked in RCC (P=0.000013). Secondly, that this co-expression defines a sub-set of patients with significantly reduced DSS (P=0.027, RR=3.20 95% CI 1.143 – 8.98) and this is particularly apparent for early (stage 1) disease as Table 2.ii shows. Thirdly, our analysis indicates that when p53 is expressed in RCC it is typically wild-type (12/14 cases) which immediately suggests a mechanism to explain the co-expression observed in RCC in which wild-type p53 promotes up-regulation of MDM2. This accords with our previous *in vitro* studies suggesting that wild-type p53 is at least partially functional in RCC cells [14, 15] and has implications for potential therapeutic targeting of the p53/MDM2 pathway in RCC (discussed below).

Our cohort was selected based on records from a larger archive of recorded RCCs stored at the Liverpool Tissue Bank and is biased towards the most common sub-type of RCC; clear cell RCC (ccRCC). There are a number of possible reasons for this bias. Firstly in such a small number of samples there may arise a sampling bias due to the nature of samples archived and the quality of these. In addition, samples were selected for inclusion on the TMA based upon histological condition and pathological criteria as determined by two histopathologists and this has also contributed to the observed bias in the TMA towards ccRCC. Whilst several of our observations are significant, there is clearly a need for a follow-up study which includes more samples and particularly of a cohort that has more samples from later disease stages, including node positive and metastatic cancers, to evaluate whether analysis of p53 and MDM2 expression is informative for other RCC cohorts. It would be interesting in future studies to examine whether patients with RCC staining positive for p53 and MDM2 show a different response to kinase inhibitors targeting consequences of VHL mutations. Five studies of larger cohorts (n>170) appear to have resolved the long-standing lack of clarity regarding p53 expression in RCC with all of them finding that up-regulation of p53 is associated with more advanced or aggressive disease (the studies determined variously: decreased disease–specific survival/disease recurrence/decreased metastasis-fee survival/ decreased disease free survival) [16-20]. However, to date, MDM2 expression and the mutational status of the expressed p53 have not been analysed in a large cohort study.

Regarding p53/MDM2 co-expression: The low frequency of p53 mutation in RCCs suggests that the strong association between p53 positivity and MDM2 positivity reported here and elsewhere in renal cancers [12, 13], is likely a consequence of p53-mediated up-regulation of MDM2 and this conclusion is supported by our functional and genetic analysis of p53 which demonstrates that the majority of cases with p53 up-regulation retain wild-type p53 (86%, 12/14 cases). In normal cells p53 is maintained at very low levels through the action of MDM2, an essential negative regulator of p53 [21]. MDM2 negatively regulates p53 in several ways by binding to it [22] and by acting as an E3 ubiquitin ligase promoting nuclear export and degradation of p53 [23]. MDM2 is also a transcriptional target of p53 and thus an auto-regulatory feedback loop exists between p53 and MDM2 [15]. The notion that MDM2 is transcriptionally up-regulated by p53 in this sub-set of RCCs accords with our earlier studies in which we showed that wild-type p53 can up-regulate MDM2 in RCC cells [14], and with data from others [24, 25]. In addition, we have recently shown that p53 and MDM2 can become spontaneously up-regulated in RCC cells *in vitro* and the up-regulation of MDM2 is a direct consequence of p53 up-regulation [26]. We have also found that p53 retains some specific transcriptional activity in RCC cells and it appears that this enables the selective activation of a sub-set of p53 target genes, for example we have found that p21 (CDKN1A) and MDM2 are up-regulated by p53 in RCC cells *in vitro* whereas Bax is less so. Taking the literature for p53 in RCC as a whole and including the data presented here, we propose a model (see Figure 3) for the p53 pathway in RCC cells in which p53 up-regulation results from a partial break in the p53/MDM2 loop where p53 retains the ability to up-regulate some targets including MDM2, but that up-regulated MDM2 appears to be unable to fully complete the loop and promote degradation of p53 effectively enough to return it to normal levels.

Why the up-regulated MDM2 fails to promote effective degradation of p53 in this setting and in so doing, return expression of both p53 and MDM2 to their normal low levels, remains unclear. Regardless, since the observed strong association between p53 and MDM2 co-expression in RCC has not been observed in most other cancers, this indicates that there must exist kidney-specific (perhaps unique) aspects to the function/regulation of the p53/MDM2 axis, and hence to renal carcinogenesis which future investigations must take into account.

Regarding the association between co-expression and outcome: up-regulation of mutant p53 has frequently been linked with poorer outcome measures in many solid tumours which is not surprising since p53 is a tumour suppressor implicated in a wide range of cancers [27]. Mutations in the p53 gene (*TP53*) occur in approximately 50% of all human cancers and are the most commonly observed genetic lesion detected to date [28].

However, in RCC p53 mutation is rare so the problem becomes how can up-regulation of normal (wild-type) p53 promote cancer? By itself, this seems incongruous, so it appears more likely that since up-regulation of wild-type p53 leads to increased expression (and reduced expression in some cases) of a sub-set of p53 target genes, then it seems probable that the target genes themselves must be responsible for promoting disease progression with the MDM2 proto-oncogene being a front runner for consideration in the first instance.

Although best understood for its critical role as a negative regulator of p53 [21], MDM2 can also promote cancer through as yet unidentified p53-independent mechanisms [29], that may be associated with invasion and/or metastasis [30, 31]. Two studies of MDM2 and p53 in renal cancer have been performed previously and in both a correlation was observed between p53 and MDM2 expression [12, 13]. There is good evidence that relatively minor changes in the balance between p53 and MDM2 may have pathological consequences. For example, a roughly two-fold increase in MDM2 levels associated with the G/G haplotype at SNP309 can result in an increased risk of cancer development [32]. In addition our own recent studies have shown that MDM2 can promote increased motility and invasion in RCC cells [26] and this might provide an explanation for the observed association between MDM2 expression and reduced DSS.

Our findings add further support for kidney-specific events in carcinogenesis since the combination of linked co-expression of p53 and MDM2 in more aggressive cases has not been observed in other cancers. With respect to p53 in kidney, there is additional evidence supporting this conclusion, since p53 is up-regulated in response to DNA damage in this tissue, as in many others, but unusually up-regulation of p53 does not result in apoptosis [8]. Notwithstanding these tissue-specific issues, there is also good evidence that loss of p53 function can contribute to renal carcinogenesis. For example, loss of p53 function through mutation accelerates renal carcinogenesis in a murine model of cancer [9].

A key question that arises is the interaction of the p53/MDM2 pathway with other critical pathways in the kidney such as VHL/HIF [33]. Mutations in VHL are the most frequent genetic event reported in renal cancer with a reported incidence in sporadic clear cell RCC of around 70% [34]. Although VHL mutations also occur in sporadic haemangioblastomas, it is clear that loss of VHL function is an event that is of most significance in the kidney and clearly further investigations of the p53 pathway should incorporate analyses of VHL and the pathways regulated by it.

In conclusion, one of the most exciting prospects to arise from these studies is the potential that exists for therapeutic targeting of the up-regulated wild-type p53 and/or MDM2 in a sub-set of patients with reduced DSS. This opportunity appears likely to be particularly applicable to RCC as a consequence of the unusual association we have detected between co-expression and outcome. Thus these studies may lead, not only to the identification of biomarker-defined sub-sets of patients displaying altered survival, but also to the development of personalised therapeutic strategies, including the use of MDM2 inhibitors currently being developed (such as Nutlin-3 and the MI- series of compounds [35, 36]) for selected patients with RCC.

Acknowledgements

This work was funded by grants from Mersey Kidney Research and Cancer Research UK and to DRS from Clatterbridge Cancer Research (genotyping).

References

[1] Surveillance Epidemiology and End Results (SEER) Program: Age adjusted SEER Incidence Rates By Cancer Site All Ages, All Races, Both Sexes 1975-2007 (SEER 9) [cited 2011 January 17]. Available from: http://seer.cancer.gov/faststats/ [cited; Available from:

[2] National Cancer Institute, Cancer topics: Kidney Cancer: Estimated new cases and deaths from kidney (renal cell and renal pelvis) cancer in the United States in 2010. [cited 2011 January 15]. Available from: http://www.cancer.gov/cancertopics/types/kidney [cited; Available from:

[3] Lopez-Beltran A, Scarpelli M, Montironi R, Kirkali Z. 2004 WHO classification of the renal tumors of the adults. Eur Urol. 2006 May: **49**:798-805

[4] Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer statistics, 2007. CA: a cancer journal for clinicians. 2007 Jan-Feb: **57**:43-66

[5] Lane BR, Rini BI, Novick AC, Campbell SC. Targeted molecular therapy for renal cell carcinoma. Urology. 2007 Jan: **69**:3-10

[6] Noon AP, Vlatkovic N, Polanski R, et al. p53 and MDM2 in renal cell carcinoma: biomarkers for disease progression and future therapeutic targets? Cancer. 2010 Jan 5: **116**:780-90

[7] Nenutil R, Smardova J, Pavlova S, et al. Discriminating functional and non-functional p53 in human tumours by p53 and MDM2 immunohistochemistry. The Journal of pathology. 2005 Nov: **207**:251-9

[8] MacCallum DE, Hupp TR, Midgley CA, et al. The p53 response to ionising radiation in adult and developing murine tissues. Oncogene. 1996 Dec 19: **13**:2575-87

[9] Sansom OJ, Griffiths DF, Reed KR, Winton DJ, Clarke AR. Apc deficiency predisposes to renal carcinoma in the mouse. Oncogene. 2005 Dec 8: **24**:8205-10

[10] Ishioka C, Frebourg T, Yan YX, et al. Screening patients for heterozygous p53 mutations using a functional assay in yeast. Nat Genet. 1993 Oct: **5**:124-9

[11] Shangary S, Wang S. Small-molecule inhibitors of the MDM2-p53 protein-protein interaction to reactivate p53 function: a novel approach for cancer therapy. Annu Rev Pharmacol Toxicol. 2009: **49**:223-41

[12] Haitel A, Wiener HG, Baethge U, Marberger M, Susani M. mdm2 expression as a prognostic indicator in clear cell renal cell carcinoma: comparison with p53 overexpression and clinicopathological parameters. Clin Cancer Res. 2000 May: **6**:1840-4

[13] Moch H, Sauter G, Gasser TC, et al. p53 protein expression but not mdm-2 protein expression is associated with rapid tumor cell proliferation and prognosis in renal cell carcinoma. Urol Res. 1997: **25 Suppl 1**:S25-30

[14] Warburton HE, Brady M, Vlatkovic N, Linehan WM, Parsons K, Boyd MT. p53 regulation and function in renal cell carcinoma. Cancer Res. 2005 Aug 1: **65**:6498-503

[15] Wu X, Bayle JH, Olson D, Levine AJ. The p53-mdm-2 autoregulatory feedback loop. Genes Dev. 1993 Jul: **7**:1126-32

[16] Kim HL, Seligson D, Liu X, et al. Using protein expressions to predict survival in clear cell renal carcinoma. Clin Cancer Res. 2004 Aug 15: **10**:5464-71

[17] Klatte T, Seligson DB, LaRochelle J, et al. Molecular signatures of localized clear cell renal cell carcinoma to predict disease-free survival after nephrectomy. Cancer Epidemiol Biomarkers Prev. 2009 Mar: **18**:894-900

[18] Shvarts O, Seligson D, Lam J, et al. p53 is an independent predictor of tumor recurrence and progression after nephrectomy in patients with localized renal cell carcinoma. The Journal of urology. 2005 Mar: **173**:725-8

[19] Uhlman DL, Nguyen PL, Manivel JC, et al. Association of immunohistochemical staining for p53 with metastatic progression and poor survival in patients with renal cell carcinoma. J Natl Cancer Inst. 1994 Oct 5: **86**:1470-5

[20] Zigeuner R, Ratschek M, Rehak P, Schips L, Langner C. Value of p53 as a prognostic marker in histologic subtypes of renal cell carcinoma: a systematic analysis of primary and metastatic tumor tissue. Urology. 2004 Apr: **63**:651-5

[21] Iwakuma T, Lozano G. MDM2, an introduction. Mol Cancer Res. 2003 Dec: **1**:993-1000

[22] Oliner JD, Pietenpol JA, Thiagalingam S, Gyuris J, Kinzler KW, Vogelstein B. Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. Nature. 1993 Apr 29: **362**:857-60

[23] Toledo F, Wahl GM. Regulating the p53 pathway: in vitro hypotheses, in vivo veritas. Nat Rev Cancer. 2006 Dec: **6**:909-23

[24] Carroll VA, Ashcroft M. Regulation of angiogenic factors by HDM2 in renal cell carcinoma. Cancer Res. 2008 Jan 15: **68**:545-52

[25] Roberts AM, Watson IR, Evans AJ, Foster DA, Irwin MS, Ohh M. Suppression of hypoxia-inducible factor 2alpha restores p53 activity via Hdm2 and reverses chemoresistance of renal carcinoma cells. Cancer Res. 2009 Dec 1: **69**:9056-64

[26] Polanski R, Warburton HE, Ray-Sinha A, et al. MDM2 promotes cell motility and invasiveness through a RING-finger independent mechanism. FEBS Lett. 2010 Oct 29: **584**:4695-702

[27] Hainaut P, Hollstein M. p53 and human cancer: the first ten thousand mutations. Adv Cancer Res. 2000: **77**:81-137

[28] Petitjean A, Mathe E, Kato S, et al. Impact of mutant p53 functional properties on TP53 mutation patterns and tumor phenotype: lessons from recent developments in the IARC TP53 database. Hum Mutat. 2007 Jun: **28**:622-9

[29] Ganguli G, Wasylyk B. p53-independent functions of MDM2. Mol Cancer Res. 2003 Dec: **1**:1027-35

[30] Wang SP, Wang WL, Chang YL, et al. p53 controls cancer cell invasion by inducing the MDM2-mediated degradation of Slug. Nat Cell Biol. 2009 Jun: **11**:694-704

[31] Yang JY, Zong CS, Xia W, et al. MDM2 promotes cell motility and invasiveness by regulating E-cadherin degradation. Mol Cell Biol. 2006 Oct: **26**:7269-82

[32] Bond GL, Hu W, Bond EE, et al. A single nucleotide polymorphism in the MDM2 promoter attenuates the p53 tumor suppressor pathway and accelerates tumor formation in humans. Cell. 2004 Nov 24: **119**:591-602

[33] Kaelin WG, Jr. The von Hippel-Lindau tumour suppressor protein: O2 sensing and cancer. Nat Rev Cancer. 2008 Nov: **8**:865-73

[34] Eichelberg C, Junker K, Ljungberg B, Moch H. Diagnostic and Prognostic Molecular Markers for Renal Cell Carcinoma: A Critical Appraisal of the Current State of Research and Clinical Applicability. Eur Urol. 2009 Jan 13:

[35] Ding K, Lu Y, Nikolovska-Coleska Z, et al. Structure-based design of spiro-oxindoles as potent, specific small-molecule inhibitors of the MDM2-p53 interaction. J Med Chem. 2006 Jun 15: **49**:3432-5

[36] Vassilev LT, Vu BT, Graves B, et al. In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. Science. 2004 Feb 6: **303**:844-8

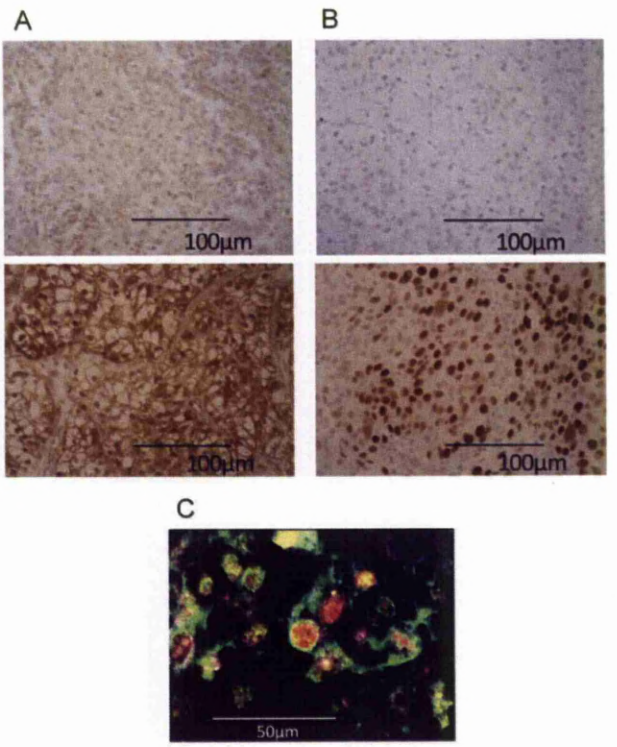
Figure legends

Figure 1. p53 and MDM2 expression in RCC cells from samples on the TMA. A. and B. Typical examples of negative and positive immunohistochemical samples of A. MDM2 and B. p53 are shown on the upper and lower panels respectively. C. Dual immunofluorescence detection of p53 (red) and MDM2 (green) demonstrating that individual RCC tumour cells express both proteins.

Figure 2. p53 and MDM2 up-regulation is linked with reduced disease specific survival. A and B. Kaplan-Meier plots for A. stage pT1-2 versus stage pT3. and B. for disease specific survival for patients dichotomized according to p53/MDM2 dual positive cancers versus p53 and/or MDM2 negative cancers (i.e. p53/MDM2 (+/+ vs +/-, -/+ or -/-)). Statistical analysis by log-rank test.

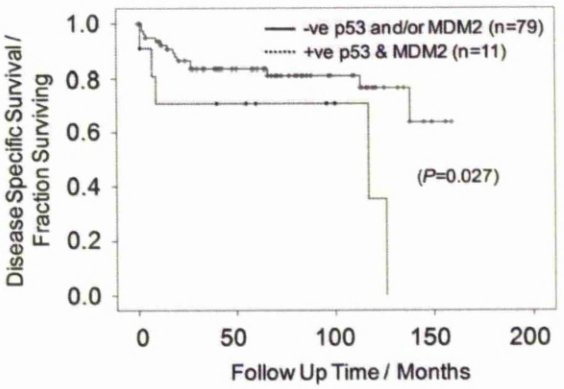
Figure 3. A model of p53/MDM2 homeostasis in different settings and for RCC based upon the literature and on the present study. In normal cells p53 and MDM2 co-exist at relatively low levels and this is regulated by MDM2 produced constitutively (from the p53-independent P1 promoter) promoting degradation of p53. Following stress, MDM2 no longer interacts with p53 leading to a rapid increase in p53 levels and subsequently p53 target genes are induced. MDM2 is also up-regulated from the p53 responsive P2 promoter and thus p53/MDM2 homeostasis is regulated by an auto-regulatory feedback loop. If the stress signal is removed, in surviving cells, the up-regulated MDM2 binds to p53 returning the cell to the normal steady state of low levels

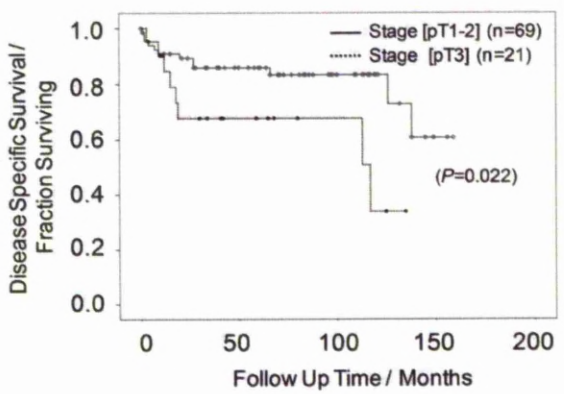
of p53 and MDM2. In most cancer cells that harbour mutant p53 (***p53****), p53 exists in excess due an imbalance in p53/MDM2 homeostasis which arises because of failure of mutant p53 to induce MDM2 expression. In normal kidney p53 is up-regulated in response to stress e.g. DNA damage but this fails to elicit an apoptotic response due to unknown mechanisms. There is clearly a block to MDM2-mediated degradation of p53 in RCC, but the nature of this remains to be elucidated. Text in bold indicates increased protein abundance.



Noon et al Figure 1

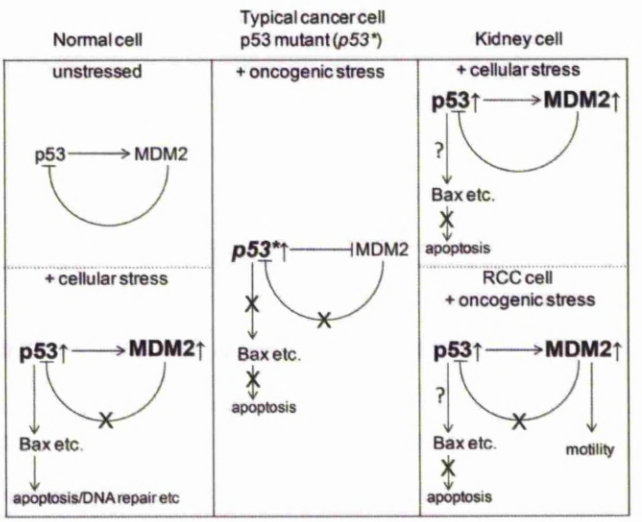
232x309mm (300 x 300 DPI)





Noon et al Figure 2

164x266mm (300 x 300 DPI)



Noon et al Figure 3

218x278mm (300 x 300 DPI)

Table 1. Patient cohort summary and IHC data summary

	Number of patients in each category by IHC phenotype (percentage)[1]					
	All	p53 +	p53 -	MDM2 +	MDM2 -	p53 +/ MDM2 +
No. of Tumours	90	14 (15.6)	76 (84.4)	24 (26.7)	66 (73.3)	11 (12.2)
Male	60 (66.7)	8	52	12	48	6
Female	30 (33.3)	6	24	12	18	5
Mean Age (24 – 82)	60.6	55.2	61.5	58.2	61.4	59.8
Histological subtype						
Clear cell	87 (96.7)	14 (100)	73 (96.1)	24 (100)	63 (95.5)	11 (100)
Papillary	2 (2.2)	0	2 (2.6)	0	2 (3)	0
Chromophobe	1 (1.1)	0	1(1.3)	0	1 (1.5)	0
Fuhrman Grade						
1	20 (22.2)	3	17	7	13	2
2	37 (41.1)	3	34	10	27	3
3	29 (32.2)	7	22	6	23	5
4	4 (4.4)	1	3	1	3	1
Tumour Stage[2]						
1	44 (48.4)	8	36	15	29	6
1a	6	0	6	1	5	0
1b	38	8	30	14	24	6
2	25 (29.6)	5	20	6	19	4
3	21 (22)	1	20	3	18	1
3a	17	1	16	3	14	1
3b	4	0	4	0	4	0
3c	0	0	0	0	0	0
4	0 (0)	0	0	0	0	0

[1] No patients had evidence of metastatic disease at the time of nephrectomy

Noon et al Table 1

Table 3. Analysis of selected tumour samples

Specimen ID	p53 Positive by IHC	MDM2 Positive by IHC	p53 status by FASAY/DNA seq. analysis	Died of RCC	Died
1	YES	YES	Wild-type[1]	NO	NO
2	YES	YES	Wild-type[1]	NO	NO
3	YES	YES	Wild-type[1]	NO	NO
4	YES	YES	Wild-type[1]	NO	NO
5	YES	YES	Mutant/G245C[1]	YES	YES
6	YES	YES	Wild-type[1]	NO	NO
7	YES	YES	Wild-type[1]	YES	YES
8	YES	YES	Wild-type[1]	YES	YES
9	YES	YES	Mutant/Q136E[1]	NO	NO
10	YES	YES	Wild-type[1]	YES	YES
11	YES	YES	Wild-type[1]	YES	YES
12	NO	YES	ND	NO	NO
13	NO	YES	ND	NO	NO
14	NO	YES	ND	YES	YES
15	NO	YES	ND	YES	YES
16	NO	YES	ND	NO	NO
17	NO	YES	Wild-type[1]	NO	NO
18	NO	YES	ND	NO	NO
19	NO	YES	ND	NO	YES
20	NO	YES	ND	NO	YES
21	NO	YES	Wild-type[1]	NO	YES
22	NO	YES	ND	NO	NO
23	NO	YES	Wild-type[1]	NO	NO
24	NO	YES	ND	NO	NO
25	YES	NO	Wild-type[1]	NO	NO
26	YES	NO	Wild-type[1]	NO	NO
27	YES	NO	Wild-type[1]	NO	NO
28	NO	NO	Wild-type[1]	NO	NO

IHC, immunohistochemistry, FASAY, functional assay of separated alleles in yeast, ND= not done.

[1] Sequence confirmed for at least three clones or by direct sequencing of PCR products. Sequences were compared to the *Homo sapiens* chromosome 17 contig NT_010718.15, positions 7189581-7169068 bp, using Sequencher v 4.9 software (Gene Codes Corporation).

Noon et al Table 3