

**A STUDY OF THE MOLECULAR GENETICS OF RENAL CELL
CARCINOMA IN MAN**

by

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Abstract

The aim was to measure the expression levels of hypoxia inducible factor 1- α (HIF-1 α), glucose transporter one (GLUT-1), and vascular endothelial growth factor (VEGF) isoforms 165 and 189 mRNA in patients with renal cell carcinoma (RCC). Patients with RCC underwent radical nephrectomy at Derriford Hospital, Plymouth. Tumour as well as matched normal tissue from the kidney was harvested, snap frozen and stored in liquid nitrogen, and used to quantitate VEGF165, VEGF189, GLUT-1 and HIF-1 α mRNA expression using ribonuclease protection assays, and quantified using a Phosphor-imager system.

The VEGF 165 isoform was increased in the tumour tissue in comparison with the adjacent normal tissue (3.05 vs. 1.56 $P=0.00002$) as was the VEGF 189 isoform (2.41 vs. 1.43 $P=0.0002$). Forty four patients were analysed for the expression of HIF-1 α and GLUT-1 with statically significant differences seen between the tumour tissues with respect to the normal tissue for both HIF-1 α (1.34 vs. 1.10 $P=0.01$) and GLUT-1 (1.99 vs. 1.63 $P=0.003$).

Hypoxia inducible factor 1 (HIF-1) is a key regulator of genes involved in the hypoxic response. HIF consists of alpha and beta subunits, with the alpha subunit being degraded under normoxic conditions and stabilised under hypoxia. Polymorphisms in exon 12 of the HIF gene have been recently been identified consisting of nucleotide changes (C+1772T and G+1790A) resulting in an amino acid substitution from Proline 582 to Serine, and Alanine 588 to Threonine respectively. These polymorphisms are found within the oxygen-dependent degradation domain (ODD) of the HIF-1 α protein when transcribed which is important in the oxygen regulation of the protein via hydroxylation of the proline residue at position 564 (P564) by HIF- α prolyl-hydroxylase (HIF-PH). The regulation of HIF-1 α by this method is a novel way of regulating the levels of the protein, and polymorphisms in the ODD of HIF-1 α may affect the ability of VHL to direct the alpha subunit for destruction.

We have genotyped 146 patients and 288 controls for the G+1790A, and 160 patients and 162 controls for the C+1772T polymorphisms respectively. We found an increase in both the GA and CC ($P<0.00001$ and $P=0.00002$) genotypes in our patients with renal cell carcinoma, and a decrease in GG and CT ($P<0.00001$ and $P=0.00002$) genotypes respectively. Haplotype analysis revealed there to be an increase in the T-A and C-A haplotypes ($P=0.00008$, and $P=0.02$) and a decrease in the T-G haplotype $P=0.01$. No statistical difference was found for the other haplotypes. We have shown that these HIF-1 α polymorphisms are present in RCC with increased frequency and may play an important role in the disease process, leading to increased angiogenesis in the tumour.

Vascular endothelial growth factor (VEGF) is a highly specific mitogen that is able to stimulate the proliferation of endothelial cells. There have been a number of findings linking the expression of VEGF with RCC, with it also being used to assess the prognosis of the disease. Polymorphisms in the VEGF gene have been recently identified. A possible link between promoter polymorphisms and expression of mRNA isoforms has been found in a variety of cytokines. Certain polymorphisms in renal cell carcinoma patients can lead to an up regulation of the expression of the mRNA, and may be a factor in the highly vascularized nature of the tumours studied. The aim was to investigate the frequency of polymorphisms within the VEGF gene (C-2578A) in 173 patients with RCC and 142 normal controls. No differences were seen between the patients and control populations, and the polymorphism did not correlate with Robson stage, Fuhrman grade or age and gender. Although a trend was seen between the C-2578A polymorphism and expression of VEGF mRNA species in RCC patients.

For Mary

Always loved never forgotten

CONTENTS

TITLE PAGE	i
ABSTRACT	ii
DEDICATION	iv
CONTENTS	v
LIST OF TABLES	x
LIST OF FIGURES	xiv
ACKNOWLEDGEMENTS	xv
AUTHOR'S DECLARATION	xvi
PUBLICATIONS AND CONFERENCES	xvii
ABBREVIATIONS	xviii
CHAPTER 1: INTRODUCTION	
1.1 Cancer	23
1.1.2 Oncogenes	25
1.1.3 Tumour suppressor genes	28
1.2 The History of Renal Cell Carcinoma	30
1.2.1 Classification of RCC	30
1.2.3 Clinical and environmental factors in the etiology of RCC	31
1.2.4 Genetics of RCC	33
1.2.5 Treatment for RCC	36
1.2.5.1 Surgery	36
1.2.5.2 Immunotherapy	39
1.2.5.3 Vaccines to modulate immunity	41

1.3 History of Angiogenesis	44
1.3.1 Angiogenesis inhibitors	45
1.4 Von Hippel-Lindau gene	50
1.4.1 Variety of neoplasms	50
1.4.2 Identification of the VHL gene	52
1.4.3 Mutational analysis of the VHL gene	53
1.4.4 Localization of pVHL	54
1.4.5 VHL associated proteins	55
1.4.5.1 Elongins B and C	55
1.4.5.2 Cullin-2	56
1.4.6 Downstream targets of pVHL	58
1.5 Vascular Endothelial Growth Factor	61
1.5.1 Isolation and purification of VEGF	61
1.5.2 Alternative splicing of the VEGF gene	61
1.5.3 Differential Isoform expression	62
1.5.4 Role of VEGF in the regulation of blood vessel growth	64
1.5.5 Biological activities of VEGF	64
1.5.6 Regulation of VEGF	65
1.5.7 Other VEGF family members	66
1.5.7.1 VEGF B	66
1.5.7.2 VEGF C	68
1.5.7.3 VEGF D	69
1.5.7.4 PlGF	69
1.5.8 VEGF in RCC	72
1.5.9 VEGF polymorphisms	75
1.5.10 VEGF receptors	78

1.5.10.1 VEGFR signalling	80
1.6 Hypoxia Inducible Factor (HIF)	84
1.6.1 Hypoxic response of HIF	86
1.6.2 Transcriptional activation of HIF	87
1.6.3 Prolyl hydroxylation of HIF-1α	90
1.6.4 Factor inhibiting HIF-1 (FIH)	92
1.6.5 HIF-2α	95
1.6.6 HIF-3α	97
1.6.7 Antisense HIF	100
1.7 HYPOTHESIS	101
CHAPTER 2: MATERIALS AND METHODS	104
2.0 Materials	104
2.1 General reagents and stock solutions	104
2.1.1 Water	104
2.1.2 Diethyl pyrocarbonate (DEPC) treated water	104
2.2 Proprietary solutions, and reagents	104
2.2.1 Stock solutions	104
2.3 Autoclaving	105
2.4 Patients	105
2.5 Ethics	105
2.6 Controls	106
Introduction to Methods	106
2.7 Polymerase chain reaction (PCR)	106
2.7.1 PCR for the polymorphisms in the VEGF gene	109
2.7.1.1 C-2578A in the VEGF gene promoter	109
2.7.2 Polymorphisms in the hypoxia-inducible factor-1α	110
2.7.2.1 C+1772T in the HIF-1α exon 12.	110

2.7.2.2 G+1790A in the HIF-1 α exon 12.	110
2.8 Extraction of high molecular weight DNA from whole blood using standard kits	111
2.8.1 Extraction of high molecular weight DNA from kidney tumour samples using standard kits	113
2.8.2 Extraction of high molecular weight DNA from paraffin embedded sections of kidney from patients with renal cell carcinoma	113
2.8.3 Extraction of high molecular weight DNA from fresh frozen tumour and normal kidney tissue from patients with renal cell carcinoma	114
2.9 Isolation and Culturing of PBMCs from whole blood	115
2.10 Extraction of total and messenger RNA	115
2.11 Preparation of first strand cDNA	116
2.12 Ribonuclease protection assay (RPA)	118
2.12.1 Preparation of antisense RNA probes	118
2.12.2 Hybridisation of RPA probe to RNA sample	119
2.13 Statistical analysis	125
CHAPTER 3: RESULTS	
3.0 Results	126
3.1 Patients for ribonuclease protection assay (RPA)	126
3.1.1 RPA for VEGF 165 & 189, HIF-1 α and Glut-1	126
3.2 VEGF promoter polymorphism	154
3.2.1 VEGF C-2578A polymorphism	156
3.3 HIF-1 α polymorphisms	171
3.3.1 C+1772T in the HIF-1 α exon 12	172
3.3.2 G+1790A in the HIF-1 α exon 12	172

CHAPTER 4: DISCUSSION

4.1 Discussion RPA	199
4.2 Discussion of VEGF polymorphisms	204
4.3 Discussion of HIF-1α polymorphisms	207
4.4 Conclusions	211
4.5 Future work	212

REFERENCES	214
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LIST OF TABLES

1.1	Role of proto-oncogenes in the cell	25
1.2	Pro-angiogenic oncogenes	26
1.3	Tumour suppressor genes	28
1.4	Classification of renal cell carcinomas	31
1.5	The Fuhrman system of nuclear grading for renal cell carcinoma	33
1.6	Tumour, Node, Metastasis, (TNM) staging system for renal cell	35
1.7	Direct angiogenesis inhibitors	47
1.8	Indirect angiogenesis inhibitors	48
1.9	Characteristics of VHL disease	50
1.10	Polymorphisms located in the VEGF gene any associations with disease	76
1.11	Genes regulated by HIF-1	87
2.1	RPA primers for various RNA species	122
2.2	RPA probe sizes and isoform detection	123
2.3	PCR conditions for the RPA probes	124
3.1.1	Characteristics of the patients for ribonuclease protection assays	128
3.1.2	Expression of VEGF isoforms, HIF-1α, and GLUT-1 in patients with renal cell carcinoma	130
3.1.3	VEGF expression vs. histology in RCC patients	132
3.1.4	VEGF expression vs. Fuhrman grade in RCC patients	134
3.1.6	VEGF expression vs. Robson stage in RCC patients	137
3.1.7	Expression of VEGF isoforms 165 and 189 vs. Gender	140
3.1.8	Distribution of Robson stage with gender for RCC patients	143
3.1.9	Distribution of Fuhrman grade with gender for RCC patients	146
3.1.10	Expression of VEGF isoforms with age distribution	149
3.1.11	Distribution of Robson stage with age for RCC patients	152
3.1.12	HIF-1α and GLUT-1 expression vs. histology in RCC patients	155

3.1.13 HIF-1α and GLUT-1 expression vs. Fuhrman grade in RCC patients	157
3.1.14 HIF-1α and GLUT-1 expression vs. Robson stage in RCC patients	160
3.1.15 Expression of HIF-1α and GLUT-1 vs. Gender	163
3.1.16 Expression of HIF-1α and GLUT-1 with age distribution	166
3.1.17 Distribution of Robson stage with sex for RCC patients	169
3.1.18 Distribution of Fuhrman grade with sex for RCC patients	172
3.1.19 Distribution of Robson stage with age for RCC patients	175
3.2.1 Characteristics of patients with RCC and healthy controls for the 18 nucleotide insertion C-2578A polymorphism in the VEGF gene	183
3.2.2 VEGF 18 nucleotide insert C-2578A polymorphism genotype and allele frequencies	185
3.2.3 Distribution of the C-2578A alleles according to Fuhrman grade	187
3.2.4 Distribution of the C-2578A alleles according to Robson stage	188
3.2.5 Distribution of C-2578A alleles with age at operation	190
3.2.6 Expression of VEGF isoforms in comparison with the C-2578A polymorphism	192
3.2.7 Characteristics of patients with clear cell renal cell carcinoma and healthy controls for the 18 nucleotide insertion C-2578A polymorphism in the VEGF gene	194
3.2.8 VEGF 18 nucleotide insert C-2578A polymorphism genotype and allele frequencies for clear cell RRC patients	196
3.2.9 Distribution of the C-2578A alleles for clear cell RCC patients according to Fuhrman grade	198
3.2.10 Distribution of the C-2578A alleles for clear cell RCC patients according to Robson stage	199
3.2.11 Distribution of C-2578A alleles with age at operation	201

3.2.12 Expression of VEGF isoforms in comparison with the C-2578A polymorphism for clear cell RCC patients	203
3.2.13 Characteristics of patients with renal cell carcinoma and healthy controls for HIF-1α polymorphisms	205
3.2.14 Frequency of HIF-1α polymorphisms in patients and controls	209
3.2.15 Haplotype analysis of HIF-1α polymorphisms	211
3.2.16 Combined genotypes for HIF-1α polymorphisms C1772T and G1790A	213
3.2.17 Distribution of the HIF-1α polymorphism C1772T alleles according to Robson stage	215
3.2.18 Distribution of the HIF-1α G1790A alleles according to Robson stage	216
3.2.19 Distribution of the HIF-1α C1772T alleles according to Fuhrman grade	218
3.2.20 Distribution of the HIF-1α G1790A alleles according to Fuhrman grade	219
3.2.21 Distribution of C1772T alleles with age at operation	221
3.2.22 Distribution of HIF-1α polymorphism G1790A alleles with age at operation	222
3.2.23 Characteristics of patients with clear cell renal cell carcinoma and healthy controls for HIF-1α polymorphisms	225
3.2.24 Frequency of HIF-1α polymorphisms in clear cell RCC patients and controls	227
3.2.25 Haplotype analysis of HIF-1α polymorphisms	229
3.2.26 Combined genotypes for HIF-1α polymorphisms C1772T and G1790A	231
3.2.27 Distribution of the C1772T alleles according to Robson stage	233
3.2.28 Distribution of the G1790A alleles according to Robson stage	234
3.2.29 Distribution of the C1772T alleles according to Fuhrman grade	236
3.2.30 Distribution of the G1790A alleles according to Fuhrman grade	237
3.2.31 Distribution of HIF-1α C1772T alleles with age at operation	239
3.2.32 Distribution of G1790A alleles with age at operation	240

LIST OF FIGURES

1.1	Renal cell carcinoma Fuhrman grades 1, 2, and 3	35
1.2	Direct and indirect angiogenesis inhibitors	46
1.3	The von Hippel-Lindau protein (pVHL) and accessory proteins	56
1.4	Regulation of HIF-1 α by pVHL under normoxic and hypoxic conditions	58
1.5	Human VEGF gene	62
1.6	Dual role of PlGF in the regulation of VEGF-induced angiogenesis	72
1.7	The various VEGFs and their receptors.	78
1.8	Signal transduction pathways for VEGFR	81
1.9	Structure of the HIF-1.	84
1.10	Oxygen-dependent regulation of the HIF-1 α transcription factor	90
1.11	Hypothetical model of the complex formation by the FIH-1 dimer, VHL and HIF-1 α .	92
1.12	Structural similarities between the three HIF- α species	98
2.1	Polymerase chain reaction	106
2.2	HIF-1 α exon 12	110
2.3	Preparation of first strand cDNA	117
2.4	Ribonuclease protection assay	119
3.1	Expression of VEGF 165 and VEGF 189 using an RPA assay	127
3.2	RPA assay for VEGF 165 and VEGF 189	128
3.3	RPA assay for VEGF 165 and VEGF 189	129
3.4	Regression analysis for HIF-1 α in relation to GLUT-1	158
3.5	Regression analysis for HIF-1 α in relation to VEGF 165 and VEGF 189	159
3.6	Polymerase chain reaction for the 18-nucleotide insertion at -2578 into the VEGF promoter and a C to A single nucleotide polymorphism	162
3.7	Expression of VEGF isoforms in relation to VEGF polymorphism C-2578	172
3.8	Expression of VEGF isoforms in CCRCC patients in relation to the VEGF polymorphism C-2578A	183
3.9	HIF-1 α C1772T polymorphism	188
3.10	HIF-1 α G1790A polymorphism	189

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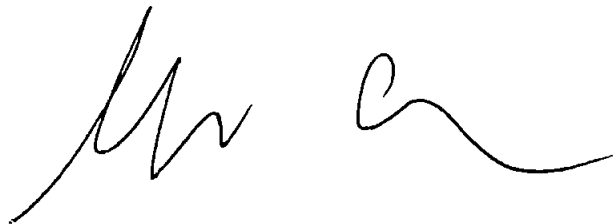
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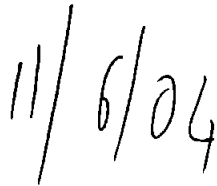
Author's declaration

All of the studies presented in this thesis were performed by the author. At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

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K. L. Søndergaard, M. Penny, M. Ollerenshaw, D. A. Hilton, A G. Demaine. **Expression of Hypoxia Inducible Factor-1 α in Tumours of patients with Glioblastoma.** Neuropathol Appl Neurobiol. 2002. 28 (3) : 210-217

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A.D. Hodgkinson, M. Ollerenshaw, B.A. Millward, A.G. Demaine. **Polymorphisms in the hypoxia-inducible factor-1 gene and diabetic complications.** In submission.

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LIST OF ABBREVIATIONS

$\alpha^{32}\text{P}$ dUTP	alpha phosphate-32 deoxyuridine
ACHN	Renal cell carcinoma cell line
aFGF	Acidic Fibroblast Growth Factor
aHIF	anti-sense Hypoxia inducible factor
APC	Adenomatous polyposis of the Colon
$\alpha\text{V}\beta\text{3}$	Integrin $\alpha\text{V}\beta\text{3}$
B	Elongin B
BCL2	B-cell Lymphomas
bFGF	Basic Fibroblast growth Factor
bp	Base Pairs
BRCA1	breast and ovarian cancer locus
BSA	Bovine serum albumin
C	Elongin C
CBP	CREB-binding protein
CD105	Cluster of differentiation 105
CD44	Cluster of differentiation 44
cDNA	Complementary DNA
CoCl_2	Cobalt Chloride
CREB	cAMP-response element-binding protein
CsCl	Caesium chloride
C-TAD	Carboxy-terminal transactivation domain
Cul-2	Cullin 2
DNA	Deoxyribonucleic acid
dNTPs	2' Deoxyribonucleotide 5'-triphosphates
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra-acetic acid

EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EGLN	EGL-Nine homologs
EG-VEGF	Endocrine-gland-derived vascular endothelial growth factor
EMSA	Electrophoretic mobility shift assay
ER	Endoplasmic reticulum
ES	Embryonic stem cell
Flt-1	Fms-like tyrosine kinase-1
Flk-1	Fetal liver kinase-1
$\gamma^{32}\text{P}$ dATP	gamma phosphate-32 deoxyadenosine
Grb2	Growth Factor Receptor-bound Protein 2
HIF-1	Hypoxia-inducible factor 1
HIF-1 α	Hypoxia-inducible factor 1 alpha
HIF-2 α	Hypoxia inducible factor 2 alpha
HIF-3 α	Hypoxia inducible factor 3 alpha
HIF-1 β / ARNT	Hypoxia inducible factor 1 beta/Aryl hydrocarbon nuclear translocator
HLA	Human leucocyte antigen
HPV-16	human papillomavirus type 16
H-RAS	Harvey strain- Rat Sarcoma
ICAM	Intercellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
kB	Kilo-base
kDa	Kilo-dalton
KDR	Kinase domain region

KRAS	Kirsten strain- Rat Sarcoma
MAPK	Mitogen activated protein kinase
MEK	Mitogen activated kinase kinase
MHC	Major histocompatibility complex
mmHg	Millimetres of mercury
MMP	Matrix metalloproteinase
MOS	Myeloproliferative Sarcoma virus
mRNA	Messenger ribonucleic acid
MVD	Microvessel density
MYB	Myeloblastosis
MYC	Myelocytomatosis
n	Number of subjects
NFκB	Nuclear factor kappa B
NK	Natural killer cell
NRP-1	Neuropillin-1
N/S	Non significant
N-TAD	Amino-terminal transactivation domain
OD	Optical density
ODC	Ornithine Decarboxylase
ODD	Oxygen-dependent degradation domain
P53	Protein 53 tumour suppressor protein
PAI-1	Plasminogen activator inhibitor-1
PDGF	Platelet derived Growth Factor
PDGF-R	Platelet derived growth Factor Receptor
PMBC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PCR-SSCP	PCR-single-stranded conformational polymorphism

PDGF	Platelet derived growth factor
PDK	Phosphoinositide-dependent kinase
PI3K	Phosphatidylinositol-3-kinase
PIP3	Phosphatidylinositol 3, 4, 5-triphosphate
PIGF	Placental growth factor
PKC	Protein Kinase C
PNK	Polynucleotide kinase
PTTG1	Pituitary Tumour-Transforming Gene 1
pVHL	Protein of the von Hippel-Lindau gene
PyMT	Polyoma middle T
RB1	Retinoblastoma
Rbx1	Ring box like protein 1
RCC	Renal cell carcinoma
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RPA	Ribonuclease protection assay
rRNA	Ribosomal ribonucleic acid
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription-polymerase chain reaction
SDS	Sodium dodecyl sulphate
sFlt	Soluble Fms-like tyrosine kinase-1
SHP-1	Ship-1
SHP-2	Ship-2
SOS	Son of Sevenless
SSP	Sequence specific primers
TAD	Transactivation domain
TAM	Tumour associated macrophage

TBE	Tris-borate acid-ethylene diamine tetra-acetic acid
TEMED	N,N,N',N'-Tetramethylethylenediamine
TGF- α	Transforming Growth Factor- α
TGF- β	Transforming Growth Factor- β
TIMP	Tissue inhibitor of metalloproteinase
TNF	Tumour necrosis factor
TSC1	Tuberous sclerosis type 1
TSG	Tumour suppressor gene
UV	Ultraviolet
VEC	VE-cadherin
VEGF	Vascular endothelial growth factor
VEGFb	VEGF b inhibitory isoforms
VEGF-R1	VEGF Receptor-1
VHL	Von Hippel-Lindau

1.1 Cancer

Cancer is the most common human genetic disease. A disease that is caused by changes in DNA resulting in continued and uncontrolled growth of the cell. One in three individuals in Europe and North American will develop one of the approximately 200 different types of cancer, and it is the cause of death for one in five.

Tumour progression is a multi-step process, preceded by multiple alterations from a normal cell to a localized tumour, and then finally to a tumour that acquires the ability to invade and metastasise. Tumours are characterised by many mutations in the form of base substitutions, deletions, chromosomal translocations, and gene amplifications. For many cancers a number of factors have been identified which are of prognostic importance.

These include patient performance status, tumour grade and stage, and response to cytotoxic therapy. Even among patients with a similar tumour there is huge heterogeneity in tumour response, suggesting that the biological properties of the tumour cells themselves may be of prognostic importance. These properties include the expression of drug or radiation resistance genes, specific and non-specific chromosomal abnormalities, and alteration in the expression of a range of oncogenes.

1.1.1 Multistage nature of cancer development

Beginning in 1949, Bereblum and Shublit stated from their experiments that the recognition that carcinogenesis is at least a two stage process should be invariably borne in mind [Bereblum & Shublit 1949]. Armitage and Doll published age/incidence curves for 17 common types of cancer and concluded that carcinogenesis was at least a six or seven stage process [Armitage & Doll 1957]. Being able to define each of these steps to malignancy has been the goal ever since. This step wise progression in which tumours

acquire more aggressive characteristics was determined by the activation, mutation or loss of specific genes [Foulds 1957].

The majority of cancers arise from a single cell that has acquired some heritable form of growth advantage [Nowell 1976]. This initiation step is believed to be caused by some form of genotoxic agent such as radiation or a chemical carcinogen. Cells at this stage, although altered at the DNA level, are still phenotypically normal. It requires further mutational events involving genes responsible for control of cell growth. Supply of blood vessels is of paramount importance to the rapidly growing cells and without recruitment of a new vascular supply they will be unable to survive. The ability to increase expression of angiogenic or decrease the expression of anti-angiogenic peptides will confer a more aggressive phenotype to the tumour mass. The ability of cells to breakaway from the initial clone of cells and travel through the body initiating a new site of malignancy is the final step in conferring a malignant phenotype, by providing the cell a mechanism to escape the basement membrane and circulate through the body.

Each of these sequential changes may result from a variety of different mechanisms. One such mechanism is a mutation in a normal cellular gene controlling cell growth and proliferation, which confers on the cell some potential for malignant transformation

1.1.2 Oncogenes

Oncogenes are mutated forms of proto-oncogenes, whose functions are to promote the normal growth and division of cells. When the gene is either mutated or expressed at abnormally high levels it contributes to converting a normal cell into a cancer cell.

Expressed oncogenes are generally considered to be 'dominantly acting' within a cell because the presence of the mutation, even a single copy, appear to be sufficient to drive the cell towards malignancy. The various types of oncogene can be seen in table 1.1.

Cells are capable of producing and responding to their own growth factors. One group of proto-oncogenes are those, which encode growth factors. The SIS oncogene encodes a protein homologous to PDGF B chain. Another group encode either the growth factor receptors themselves or their functional homologs. Thus the product of the ERBB gene [Sergeant *et al* 1982] is homologous to the epidermal growth factor receptor (EGFR) [Akiyama *et al* 1984] and the FMS product is homologous to the receptor for colony-stimulating factor-1 (CSF1R). A third group are the signal transducers. The signals for stimulation of growth from growth factors and their receptors must ultimately act, via a number of second messengers, at the level of gene expression. Second messengers are essentially signal transducers and transmitters that function by activating a protein cascade. This is controlled at the level of protein phosphorylation by protein kinases. Products of several of the membrane associated proto-oncogenes such as SRC and ABL have tyrosine kinase activity, as do several of those which act in the cytoplasm, such as products of RAF1 and MOS. Several transmembrane growth factor receptors have protein kinase activity associated with the cytoplasmic domain. The final group of proto-oncogenes are those associated with the control of gene expression by their action on DNA itself. This is the final site of action for messages sent from growth factors and is the level at which

control of growth and proliferation ultimately operates. Several proto-oncogene proteins (MYC, FOS, and JUN) have been shown to bind to and control the transcription of genes.

Role	Oncogene	Viral / Homolog
Growth factors	SIS	PDGF
Receptor tyrosine kinases	ERBB / FMS	EGFR / CSF1R
Membrane associated non-receptor kinases	SRC	v-src
G-protein coupled receptor	Mas	v-mas
Membrane associated G-proteins	Ras	v-ras
Serine / Threonine kinases	Raf	v-raf
Nuclear DNA-binding transcription factors	Myc	v-myc

Table1.1 Role of proto-oncogenes in the cell

Proto-oncogenes can be categorized into growth factors, receptor tyrosine kinases, G-protein coupled receptors, serine / threonine kinases or nuclear factors.

Oncogene	Implicated pro-angiogenic activity	Ref
KRAS, HRAS	VEGF upregulation, TSP1 downregulation	Rak <i>et al</i> 1995, Grugel <i>et al</i> 1995
SRC	VEGF upregulation, TSP1 downregulation	Rak <i>et al</i> 2000a
c-MYB	TSP2 downregulation	Bein <i>et al</i> 1998
n-MYC	Angiogenic properties in neuroblastoma	Meitar <i>et al</i> 1995
c-MYC	Angiogenic properties in epidermis	Pelengaris <i>et al</i> 1999
ERBB2	VEGF upregulation	Petit <i>et al</i> 1997
EGFR	VEGF, bFGF, IL-8 upregulation	Perrotte <i>et al</i> 1999
PyMT	TP1 upregulation	Sheibani & Frazier 1996
FOS	VEGF expression	Saez <i>et al</i> 1995
TrkB	VEGF downregulation	McGregor <i>et al</i> 1999
HPV-16	Secretion of VEGF and IFN- α	Le Buanec <i>et al</i> 1999
v-p3k	VEGF production and angiogenesis	Jiang <i>et al</i> 2000
ODC	Novel angiogenic factor	Auvinen <i>et al</i> 1997
PTTG1	VEGF and bFGF upregulation	Heaney <i>et al</i> 1999
E2a-Pbx1	Induction of mouse angiogenin-3	Fu <i>et al</i> 1999
BCL2	VEGF upregulation	Fernandez <i>et al</i> 2001

Table 1.2 Pro-angiogenic oncogenes

This table shows a variety of pro-angiogenic oncogenes.

bFGF, basic fibroblast growth factor; EGFR, epidermal growth factor receptor; IFN- α , interferon- α ; IL-8, interleukin-8; TSP, thrombospondin; VEGF, vascular endothelial growth factor. Adapted from Kerbal & Folkman 2002.

1.1.3 Tumour suppressor genes

A second group of genes, which play a crucial role in tumorigenesis, are the tumour suppressor genes. The normal function of tumour suppressor genes is to inhibit cell growth and the cell cycle. Mutations in tumour suppressor genes cause cells to ignore one or more of the components of the network of inhibitory signals, removing the brakes from the cell cycle and resulting in a higher rate of uncontrolled growth. Tumour suppressor genes are defined by the impact of their absence and thus tend to be recessive, both normal alleles must mutate before cancerous growth begins. The loss or inactivation of a normal tumour suppressor gene may be acquired somatically in a single clone of cells or be constitutionally present throughout the body, including the germ line. Therefore it was hypothesised that it required two separate mutational events. One of these events may occur in the germline and be inherited, the second then occurs somatically. Alternatively, the two mutational events may occur only in the somatic cell of an individual. The 'two hit' model of mutation was proposed by Knudson in 1971 after studying cases of retinoblastoma [Knudson 1971].

Tumour suppressor gene	Description	Cancer type
APC	Adenomatous polyposis of the colon gene	Involved with familial adenomatous polyposis of the colon
BRCA1	Familial breast/ovarian cancer gene 1	Hereditary breast/ovarian cancer
RB1	Retinoblastoma	Familial RB, bone, bladder, small cell lung
P53	Tumour suppressor p53 gene	Wide variety of tumours
TSC1	Tuberous sclerosis type 1 gene	Tuberous sclerosis, some hamartomas and RCC
VHL	von Hippel-Lindau syndrome gene	VHL syndrome, RCC, haemangiomas and pheochromocytomas

Table 1.3 Tumour suppressor genes

This table shows the wide variety of tumour suppressor genes.

1.2 The History of Renal Cell Carcinoma

Renal cell carcinoma (RCC) makes up 2% to 3% of all cancers, and 2% of all cancer deaths. The worldwide mortality was expected to exceed 100,000 in 2000 [Pisani *et al*, 1985]. There is a wide variation in incidence between countries, with Scandinavians and North Americans having the highest rates [Parkin *et al*, 1993]. RCC is twice as common in males as in females although this is beginning to narrow [Landis *et al*, 1998] occurring most commonly in the 5th to 7th decades of life.

1.2.1 Classification of RCC

Since the first misclassification of RCC by Grawitz as a hypernephroma which was thought to originate in the adrenal gland in 1883 [Grawitz, 1883] there have been various attempts to reclassify these tumours according to morphological and genetic patterns. Over the last 10 years there have been advances in the understanding of genetic changes in RCC. The Union Internationale Control le Cancer (UICC) and the American Joint Committee on Cancer (AJCC) classification (1997) identifies five groups of RCC, namely clear cell, papillary, chromophobe, collecting duct and unclassified carcinomas [Storkel *et al*, 1997] see table 1.4. RCC can be graded according to the appearance of the nuclei as described in the Fuhrman grading system (see table 1.5, fig 1.1). The spread of the tumour can also be staged and takes into account the size and any metastasis that has taken place (see table 1.6).

Renal cell carcinomas originate within the renal cortex and are believed to arise from cells of the proximal tubule, and account for 80% to 85% of all primary renal neoplasms [Landis *et al* 1998]. The next most common is transitional carcinomas, which originate in the renal pelvis and account for 7% to 8% of primary neoplasms. The other tumours include, collecting duct tumours, oncocytomas, chromophobic, and chromophilic (previously

classified as papillary). The 5-year survival rate of patients diagnosed with kidney cancer has improved from 52% to 60% for those diagnosed between 1974 and 1976, and those diagnosed between 1986 and 1993 [Landis *et al*, 1998].

1.2.3 Clinical and environmental factors in the etiology of RCC

A number of clinical and environmental factors have been implicated in the etiology of RCC. These include use of tobacco, asbestos, and exposure to cadmium, urbanization, and exposure to petroleum by-products [McLaughlin *et al* 1985, La Vecchia *et al* 1990, Wolk *et al* 1996, Mandel *et al* 1996]. Cigarette smoking has been shown to double the likelihood of RCC and may contribute to as many as one third of cases [McLaughlin *et al* 1984, Yu *et al* 1986, La Vecchia *et al* 1990]. Obesity is also a risk factor, with a linear relationship between increasing body weight and risk of developing RCC, particularly in women [Yu *et al* 1986]. Clinical factors associated with the development of RCC include, hypertension [Yu *et al* 1986], unopposed estrogen therapy [Lindblad *et al* 1995], analgesic abuse [McCredie *et al* 1995], and acquired polycystic disease of the kidney (with a 30 times increased risk than the general population) [Brennan *et al* 1991].

Carcinoma Type	Growth Pattern	Cell of Origin	Cytogenetic characteristics		Incidence (%)
Clear-cell	Acinar or sarcomatoid	Proximal tubule	Major 3p-	Minor +5, +7, +12, - 6q, -8p, -9, - 14q, -Y	75-85
Chromophilic	Papillary or sarcomatoid	Proximal tubule	+7, +17, -Y	12+, +16, +20, -14	12-14
Chromophobic	Solid, tubular, or sarcomatoid	Intercalated cell of cortical collecting duct	Hypodiploidy	-	4-6
Oncocytic	Typified by tumour nests	Intercalated cell of cortical collecting duct	Undetermined	-	2-4
Collecting-duct	Papillary or sarcomatoid	Medullary collecting duct	Undetermined	-	1

Table 1.4 Classification of renal cell carcinomas

Pathological classification of renal cell carcinomas. Adapted from Storkel & van den Berg 1995

1.2.4 Genetics of RCC

RCC can occur in inherited and sporadic forms. The former is usually found in patients suffering from von Hippel-Lindau (VHL) disease, which is associated with a mutation of the VHL gene. This classical tumour suppresser gene is situated on the short arm of chromosome 3 (3p.25). The sporadic form of RCC is associated with somatic mutations in the VHL gene in at least 80% of cases [Foster *et al* 1994b]. Mutation of the VHL gene occurs early in the disease, and as it progresses changes in other chromosomes occur including 3p14, 3p21, 5q, 6q, and 10q [Morita *et al* 1991a, Thrash-Bingham *et al* 1995, Morita *et al* 1991b, Foster *et al* 1994, Kenck *et al* 1997].

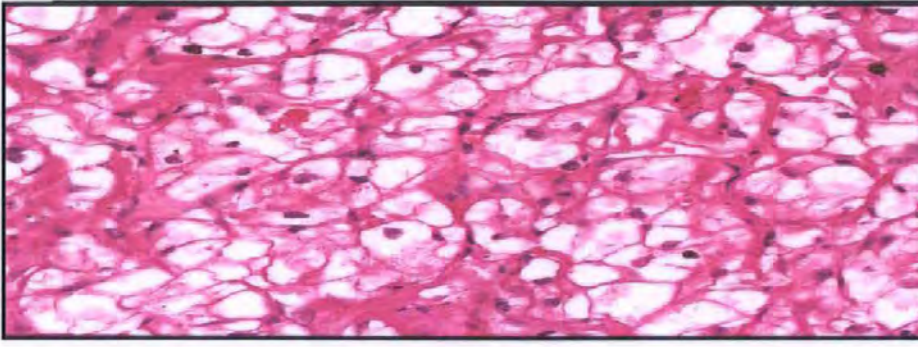
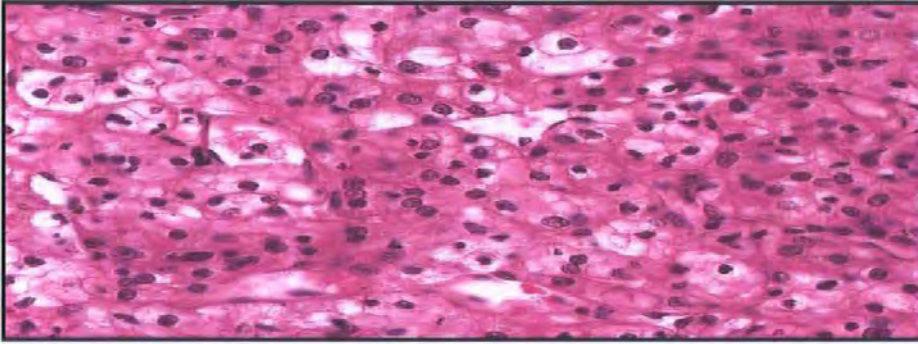
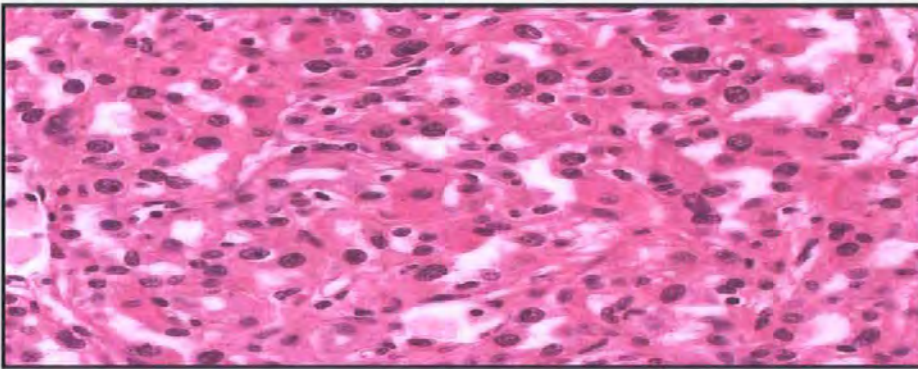
RCC is characteristically associated with an abundant neovasculature. This feature makes RCC an ideal cancer to study in relation to tumour-associated angiogenesis and cellular responses to hypoxic stress [Brown *et al* 1993, Sato *et al* 1994, Krieg *et al* 2000, Hemmerlein *et al* 2001, Le Jan *et al* 2003].

Both forms (sporadic & hereditary) of RCC are associated with a translocation on the short arm of chromosome 3 [Seizinger *et al* 1988]. Southern blot analysis of this region in sporadic cases demonstrated loss of DNA on chromosome 3 in a high percentage of tumours examined [Presti *et al* 1991]. It appears that defects distal on the short arm of chromosome 3 are fundamental to the development of RCC. There is a high frequency of chromosomal breakage at 3p13-14 and 3p21.3 [Yamakawa *et al* 1991]. Other less common abnormalities include trisomy for the region 5q22-qter and the deletion of 14q22-qter [Kovacs & Frisch 1989, Nagao *et al* 2002]. More recently advances in analysis of the breakpoints and translocations, has lead to a more in-depth picture of complex structural rearrangements. Using spectral karyotyping it has been possible to detect these rearrangements which conventional banding analysis was unable to detect [Pavlovich *et al* 2003].

Grade	Criteria
Grade 1	Round uniform nuclei approximately 10µm in diameter with minute or absent nucleoli
Grade 2	Slightly irregular nuclear contours and diameters of approximately 15µm with nucleoli visible at 400X
Grade 3	Moderately to markedly irregular nuclear contours and diameters of approximately 20µm with large nucleoli visible at 100X
Grade 4	Nuclei similar to those of grade 3 but also multi-lobular or multiple nuclei or bizarre nuclei and heavy clumps of chromatin

Table 1.5 Fuhrman system of nuclear grading for renal cell carcinoma

This table shows the Fuhrman grading system for renal cell carcinomas, based on a method of scoring the nucleus of the cells to determine the aggressiveness of the cancer [Fuhrman *et al* 1982].

**A****B****C****Fig 1.1 Renal cell carcinoma Fuhrman grades 1, 2 and 3**

A, RCC Fuhrman grade 1. Grade 1 tumours have round, uniform nuclei with inconspicuous or absent nucleoli. **B**, Fuhrman grade 2, nuclear contours are more irregular than Grade 1; nuclei are about 15 microns in diameter. Nucleoli may be visible at high magnification. **C**, Fuhrman grade 3, nuclear contours are even more irregular. Nuclear diameters can approach 20 microns. Nucleoli are readily seen. Figures adapted from Webpathology.com

Familial renal cancers are characterised by their early age of onset compared to sporadic cases, frequent bilaterality and multicentricity. The mean age of diagnosis in familial cases is about 45 years compared with 60 years for sporadic cases [Cheville *et al* 2003].

1.2.5 Treatment for RCC

There are few treatments for RCC due to the fact that it is highly resistant to traditional therapies like chemotherapy, radiotherapy, hormonal therapy, and immunotherapy [Motzer *et al* 2000]. The currently used strategies to treat RCC are described below.

1.2.5.1 Surgery

Depending on the stage of the tumour the 5-year survival ranges from 30 to 60%. The standard procedure for treatment of localised renal carcinoma is radical nephrectomy. Radical nephrectomy includes complete removal of Gerota's fascia and its contents, including the kidney and the adrenal gland, and provides a better surgical margin than simple removal of the kidney [Robson 1963]. Current opinion on the removal of the adrenal gland is that it only needs to be removed if it is involved in the tumour or contains metastasis, or the tumour is in the upper pole of the kidney [Autorino *et al* 2003]. Nephron sparing surgery (NSS) is emerging as a successful treatment for patients with localised RCC when the need exists to preserve functioning renal parenchyma [Moll *et al* 1993]. NSS has recently been shown to have similar long-term survival statistics in comparison with radical nephrectomy for low stage patients with RCC [Lundstam *et al* 2003].

Partial resection is indicated in cases of a single kidney, bilateral tumours and possibly for tumours smaller than 4 cm in diameter. Analysis of the cause of death in patients with RCC shows that the tumour is not always responsible. A 40-year-old man's lifetime risk of renal carcinoma is 1.27%, and the risk of death (from RCC) is 0.51% [Motzer *et al* 1996].

Kidney tumours are detected more frequently by the newer radiological techniques, and at lower stages, when they can be resected for a cure. Using computed tomography (CT) scan and ultrasound, an increasing number of incidental RCCs have been identified. The Robson stage can be described as an amalgamation of the T, N, M, system to provide a readily usable system (see table 1.6).

Stage			
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T1	N1	M0
	T2	N1	M0
	T3a	N0	M0
	T3a	N1	M0
	T3b	N0	M0
	T3b	N1	M0
	T3c	N0	M0
	T3c	N1	M0
Stage IV	T4	N0	M0
	T4	N1	M0
	Any T	N2	M0
	Any T	Any N	M1

Table 1.6 Tumour, Node, Metastasis, (TNM) staging system for renal cell carcinoma

This table shows the Robson classification with regard to the T, N, M staging of the tumour.

Primary tumour (T)

- Tx Primary tumour cannot be assessed
- T0 No evidence of primary tumour
- T1 Tumour 7cm or less in greatest dimension limited to kidney
- T2 Tumour more than 7cm in greatest dimension limited to kidney
- T3 Tumour extends into major veins or invades the adrenal gland or perinephric tissues, but not beyond Gerota's fascia
- T3a Tumour invades the adrenal gland or perinephric tissues, but not beyond Gerota's fascia
- T3b Tumour grossly extends into the renal vein or vena cava below the diaphragm
- T3c Tumour grossly extends into the renal vein or vena cava above the diaphragm
- T4 Tumour invades beyond Gerota's fascia

Regional lymph nodes (N)

- Nx Regional lymph nodes cannot be assessed
- N0 No regional lymph node metastasis
- N1 Metastasis to single regional lymph node
- N2 Metastasis to more than one regional lymph node

Distant metastasis

- Mx Distant metastasis cannot be assessed
- M0 No distant metastasis
- M1 Distant metastasis

1.2.5.2 Immunotherapy

Although no curative treatment other than surgery is available, new treatment modalities, including immunotherapy (IL-2, Rosenberg *et al* 1985) and anti-angiogenesis therapies are currently being investigated. Anti-angiogenesis therapies include anti-VEGF antibodies [Asano *et al* 1995], and anti-VEGF receptor antibodies that block the VEGF receptors and their signal transduction functions [Zhu *et al* 2003], and thalidomide [D'Amato *et al* 1994, Li *et al* 2003].

The two principle cytokines available as agents for RCC therapy are interferon- α (IFN- α) and interleukin-2 (IL-2). Dosage is measured as millions of units (mIU). IFN- α is usually administered via subcutaneous injection, while IL-2 is administered either by the subcutaneous route or intravenously, usually in the in-patient setting with the higher doses. Prominent cytokine side effects can interfere with delivery of planned drug doses and necessitate hospitalisation and ICU admission for supportive care.

The food and drug administration (FDA) has approved IL-2 for the treatment of metastatic RCC at a dose of 600,000 IU/Kg (approximately 40 to 50 mIU) per dose, administered in a slow intravenous infusion every 8 hours as tolerated to a maximum of 14 doses per cycle (see www.proleukin.com/index.html) and for the FDA approval www.fda.gov/cber/products/aldechi010998.htm).

IFN- α has been approved for use in Europe for the treatment of RCC [Pyrhonen *et al* 1999, Kankuri *et al* 2001], for review see Fossa 2000. Side effects are also dose limiting, the broad range of toxicities can overlap those of IL-2 or other partner drugs. These associated side effects include a flu like syndrome (fatigue, fever, chills, anorexia, myalgia, headache), dry skin and mucus membranes, mental status changes and depression.

Although the exact mechanism of action of cytokines is complex and unknown in an immunotherapy model, the effect of the drugs on the cancer cells is indirect. The cytokines

bind to their specific receptors and initiate intracellular and intercellular signalling cascades. IL-2 is a potent stimulator of T cell proliferation, and if anti-tumour T cells are present, they could be stimulated. The consequence of this action is that tumour specific cytotoxic T-lymphocytes (CTLs), natural killer (NK) cells and tumour-infiltrating lymphocytes (TILs), are activated and these lymphocytes then kill the cancer cells.

Pegylated (40Kd) interferon- α 2a is a modified form of recombinant human IFN- α 2a with sustained absorption and prolonged half-life after subcutaneous injection. Pegylated interferon (PEG-intron) has been used in phase I and II trials in-patients with advanced RCC to assess the efficacy and toxicity advantages over conventional IFN- α therapy. The efficacy and tolerability profile was qualitatively similar to standard IFN- α , and the adverse effects were mostly mild to moderate in nature [Motzer *et al* 2001, Motzer *et al* 2002]. PEG modified IL-2 has also been tested in a phase I trial in-patients with RCC (combined with standard IL-2) [Yang *et al* 1995], and a liposomal IL-2 has also been developed [Kedar *et al* 2000].

Other cytokines are known to be involved in immune signalling. Several have been manufactured in clinical quantities and tested in RCC, including IFN- γ and IL-6. In vitro, IL-2 and IFN- γ act synergistically to generate lymphokine-activated killer cells. Phase II evaluation for RCC therapy in IL-2 combination had 10% to 11% objective response rates [Schimidinger *et al* 2000, Schmidinger *et al* 2001]. A phase I trial using IL-6 and GM-CSF were safe, but further development for RCC application was not recommended [Tate *et al* 2001].

1.2.5.3 Vaccines to modulate immunity

While cytokine therapy activates the immune system, vaccine therapy seeks to direct the immune system specifically to the tumour cell targets. The first step in the clinical development of a vaccine is demonstrating the acquisition or amplification of CTLs with anti-tumour specificity. The process of antigen presentation to lymphocytes is a key step in development of lymphocytes with specificity for a particular tumour antigen. The next crucial step is to get the CTLs to act on the tumour. The presence of the tumour at a particular point in time is evidence that homeostasis has broken down. This may reflect a general immune suppression, or may be a tumour-directed, pathologic, immune-tolerant, anergic state of tumour-specific CTL clones.

At present there are no licensed vaccines available for the treatment of RCC. Several approaches to this problem are currently in the trials phase of development. Vaccination with the peptide corresponding to a particular VHL mutation is one. Individuals whose tumour has a VHL mutation are immunized with the particular mutation (which is an antigen unique to the tumour), with a keyhole limpet hemocyanin (KLH) adjuvant (see www.cancer.gov/search/clinical_trials/). The single peptide vaccination approach is in contrast to the whole protein or cell approaches. The antigenic peptides of cells are associated with the heat shock protein HSPP96. The Oncophage product (Antigenesis Inc) uses this autologous tumour-specific set of peptides and is being tested in two placebo-controlled, randomised phase III trials, one for stage III and one for stage IV disease [Caudill & Li 2001] see also www.antigenic.com/trials/.

Transforming growth factors (TGFs) α and β are two tumour produced regulatory growth factors that may be related to the development of RCC. Using northern blotting it has been shown that enhanced expression of TGF- α and TGF- β 1 mRNA in solid tumour may be related to the development of RCC [Gomella *et al* 1989, Uhlman *et al* 1995, Hise *et al* 1996, Gunaratnam *et al* 2003].

Epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein, which binds the mitogenic polypeptide hormone epidermal growth factor (EGF) as well as the EGF related TGF- α . Increases in the EGFR gene expression and protein production have been implicated in the phenotype of various cancers [Sargent *et al* 1989, Bolufer *et al* 1990, Laderoute *et al* 1992, Di Lorenzo *et al* 2002, Tsutsui *et al* 2003]. The EGFR gene was shown to be constitutively expressed in 90% of normal kidney samples. Tumours expressed an increased amount of EGFR mRNA coupled with the corresponding normal tissue, suggesting that over expression of EGFR mRNA may be associated with malignant transformation in RCC [Sargent *et al* 1989]. Since TGF- α is known to bind to the EGFR, the finding of an increased expression of both TGF- α and EGFR mRNA in kidney tumour tissue suggests that interaction between TGF- α and EGFR may play a role in promoting transformation or production of kidney neoplasm [Mydlo *et al* 1989]. Using two RCC cell lines (UOK-39 and SKRC-7) Gomella *et al* [1989] showed that TGF- β inhibits the proliferation of RCC in vitro and this may be mediated by binding of exogenous TGF- β to functional TGF- β receptors on the cell surface.

By using immunohistochemistry there was no significant correlation between TGF- α staining intensity and tumour size, stage or grade. When the tumour expressed either EGFR or TGF- α , average patient survival was 38 months, whilst the average survival of patients whose tumour expressed both EGFR and TGF- α was 61 months. EGFR and TGF-

α are likely to play a role in the progression of RCC, and their co-expression may have favourable prognostic implications [Lager *et al* 1994]. RCC cell lines SKRC-4 and SKRC-29 were growth stimulated by greater than 35% when cultured in the presence of TGF- α or EGF, and inhibited by 29-46% if cultured with anti-EGF monoclonal antibody 255. This suggests that proliferation of human RCC may be regulated by endogenously produced TGF- α and this pathway can be interrupted using an antibody to its receptor [Atlas *et al* 1992]. Papillary tumours were shown to express relatively lower levels of EGFR in comparison with non-papillary tumours [Uhlman *et al* 1995].

Increased levels of latent TGF- β 1 are common in the plasma of RCC patients. The TGF- β 1 plasma level in RCC was found to be significantly higher than in cases of inflammation, thus the TGF- β 1 is a possible tumour prognostic marker in RCC [Wunderlich *et al* 1998]. Elevated latent TGF- β 1 is common in RCC, and is at least partially produced by the tumour and might be a cause for local immunosuppressive effects within the tumour [Junker *et al* 1996]. Loss of expression of TGF- β R could result in escape from the growth inhibitory effect of TGF- β in TGF- β secreting cancer. Loss of TGF- β -R-II was associated with clinical stage suggesting that loss of TGF- β -R-II expression in the primary tumour is a significant prognostic factor in patients with RCC [Miyajima *et al* 2003]. Using immunohistochemistry VEGF and TGF- β 1 can be a useful prognostic factor in RCC [Yagasaki *et al* 2003]. In contrast, it has recently been shown by ELISA that TGF- β 1 is not a suitable marker for the diagnosis of localized RCC, but may be useful in early detection of RCC recurrence or to control the success of immunochemo-therapy [Hegele *et al* 2002]. TGF- α and FGF are functionally involved in the progression of clear cell RCC, directly stimulating proliferation by autocrine and/or paracrine actions. In contrast TGF- α and FGF did not directly stimulate the proliferation of papillary RCCs, thereby suggesting functional defects or a blockade in the corresponding signalling cascades. This differential

functionality might contribute to the more aggressive behaviour of clear cell RCCs [Ramp *et al* 2000].

TGF- α has been shown to support RCC cell growth through an autocrine loop. VHL has been shown to substantially decrease TGF- α mRNA and protein by shortening TGF- α mRNA half-life. In contrast VHL has no effect on the TGF- α receptor, EGFR, either at the mRNA or protein level [Knebelmann *et al* 1998].

1.3 History of Angiogenesis

Dating back to 1787 surgeon Dr John Hunter first used the term angiogenesis to describe blood vessels growing in the reindeer antler [Hunter 1787]. The term angiogenesis means new blood vessel growth and has become an important process to study in relation to disease over the last 200 years since it was coined. Development of the vascular endothelium, and endothelial cell signal transduction mechanisms involved in angiogenesis are a fundamental requirement for organ development and differentiation in multicellular organisms [Hamilton *et al* 1962]. The relationship between angiogenesis and disease has become a highly studied process over the last few years. Increased knowledge of angiogenesis, coupled with a greater understanding of the diseases that may either have a pro or anti-angiogenic stand point has lead to strategies to inhibit or stimulate angiogenesis. Currently there are over 200 drugs related to angiogenesis in clinical trials and many more that are being developed in laboratories around the world [for review see Kerbal & Folkman 2002 and www.angio.org/ for angiogenesis inhibitors and drugs under current investigation]. The application of pharmacogenomics to drug design will increase our capacity to fight RCC.

The paper that began the current interest in angiogenesis and subsequent therapy to prevent angiogenesis was by surgeon Dr Judah Folkman, published in the New England Journal of Medicine [Folkman, 1971], hypothesizing that tumour growth is dependent on

angiogenesis. This theory was regarded as heresy by leading physicians and scientists at the time. A few years later the first angiogenesis inhibitor was discovered by Henry Brem and Judah Folkman in cartilage and called angiostatin [Brem & Folkman 1975].

Tumour growth depends on angiogenesis, without it the rapidly growing tumour will be unable to survive [Hanahan & Weinberg 2000, Folkman 2001]. Cancer cells have to promote angiogenesis early in the formation of the tumour. This 'angiogenic switch' is characterized by oncogene-driven tumour expression of pro-angiogenic proteins [Rak *et al* 2000b], including bFGF, IL-8, PLGF, VEGF, TGF- β , and PD-EGF [Relf *et al* 1997]. The angiogenic switch also involves the down regulation of angiogenesis suppressor proteins, such as Thrombospondin [Dameron *et al* 1994].

1.3.1 Angiogenesis inhibitors

Angiogenesis inhibitors are a new class of drugs, which do not necessarily follow the general rules of conventional therapies such as chemotherapy and radiotherapy. It is possible to target the genetically stable cells that support tumour growth, and therefore less likely to accumulate mutations that allow them to develop drug resistance. They fall into one of two classes of inhibitor; direct or indirect [see fig 1.2 and tables 1.7 and 1.8]. Direct angiogenesis inhibitors target vascular endothelial cells and prevent them from proliferating, migrating, and avoiding cell death in response to a pro-angiogenic response by proteins including VEGF, bFGF, IL-8, PDGF, PD-EGF (see direct angiogenesis inhibitor table 1.7). It is thought that these direct angiogenesis inhibitors are the least likely to induce acquired drug resistance, as they target genetically stable endothelial cells and not unstable mutating tumour cells [Kerbel 1991]. In comparison indirect angiogenesis inhibitors prevent or block the expression of tumour proteins that activate angiogenesis (see fig 1.2 and table 1.8).

The first use of anti-angiogenic therapy in a human being was in 1988 using a daily low dose of IFN- α (3 million unit/m²) to treat pulmonary haemangiomas in a 12-year-old boy [White *et al* 1989, Folkman 1989]. Tumour angiogenesis was mediated mainly by bFGF in this tumour, and IFN- α has been shown to down-regulate bFGF expression in human cancer cells [Singh *et al* 1995].

Transtuzumab which is an antibody that blocks ERBB2 receptor tyrosine kinase signalling, suppresses cancer-cell production of angiogenic factor such as TGF- β , angiopoietin-1, and plasminogen activated inhibitor-1 [Izumi *et al* 2002] and may also possibly suppress VEGF [Petit *et al* 1997].

In comparison with the traditional methods of testing cytotoxic chemotherapeutic drugs in cancer patients, it has been revealed that many angiogenesis inhibitors are most effective when the dose and schedule maintains a constant concentration of the inhibitor in the circulation, instead of a once daily bolus dose [Kisker *et al* 2001].

Use of anti-cancer drug TNP-470 in RCC patients caused regression of the tumour volume [Stadler *et al* 1999]. TNP-470 is a synthetic analogy of Fumagillin [Ingber *et al* 1990], and inhibits methionine aminopeptidase-2, blocks the activity of cyclin-dependent kinase 2 (CDK2), and inhibits phosphorylation of the RB protein [Griffith *et al* 1997, Sin *et al* 1997].

As well as differences in dosing schedules, the clinical end points applied to anti-angiogenesis therapy are also different. When a cytotoxic drug stabilizes the disease it might be considered a failure because the tumour will eventually become resistant to the drug and begin growing again. Stabilization of disease using an angiogenesis inhibitor on the other hand may arrest the tumour growth for long periods of time because of the genetically stable cells that are targeted.

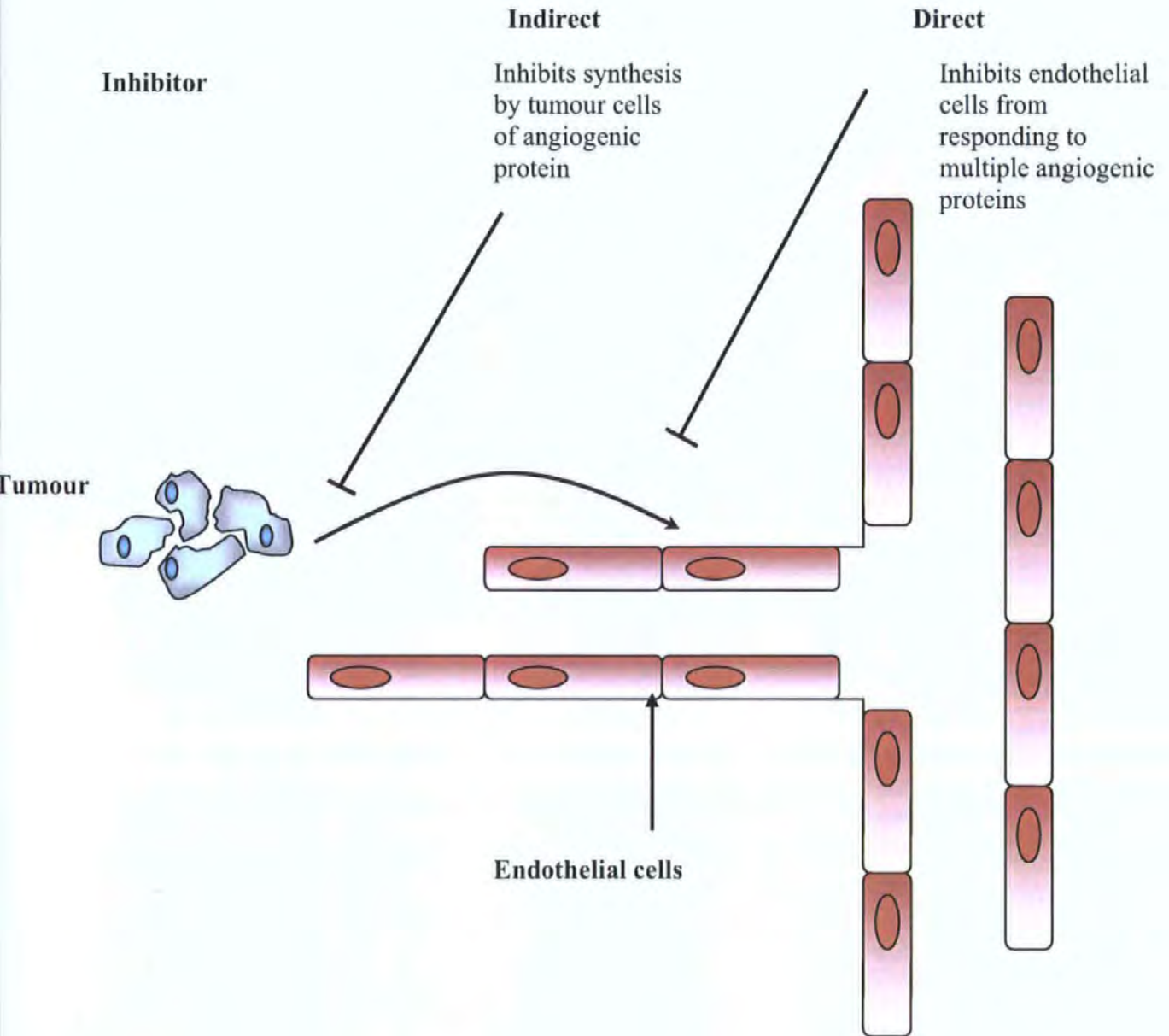


Fig 1.2 Direct and indirect angiogenesis inhibitors

Direct angiogenesis inhibitors target the vascular endothelial cells, which are recruited to the tumour bed and prevent them from responding to multiple angiogenic proteins. Indirect angiogenesis inhibitors target proteins or receptors on the endothelium that are expressed by tumour cells. Adapted from Kerbal & Folkman 2002.

Drug	Cell Target	Clinical trail stage	Authors
Angiostatin	Binds to ATP synthase, angiomin, and annexin II on endothelial cells. Inhibits endothelial cell proliferation and migration	Phase I	O'Reilly <i>et al</i> 1994; Moser <i>et al</i> 1999; Troyanovsky <i>et al</i> 2001
Bevacizumab (Avastin)	Humanized recombinant monoclonal antibody against VEGF	Phase II and III	
Arrestin	Binds to integrin- $\alpha_1\beta_1$ to inhibit endothelial cell proliferation, migration, tube formation, and neovascularization	No	Colorado <i>et al</i> 2000
Canstatin	Binds to integrin- $\alpha_1\beta_1$ to inhibit endothelial cell proliferation, migration, tube formation	Phase I	Kamphaus <i>et al</i> 2000
Combretastatin	Microtubules: Induces reorganization of the of the actin cytoskeleton and early membrane blebbing in human endothelial cells	Completed phase I	Kanthou and Tozer 2002
Endostatin	Targets integrin- $\alpha_1\beta_1$ to inhibit endothelial cell proliferation and migration, and induce apoptosis	Phase I and II	Dixelius <i>et al</i> 2000; O'Reilly <i>et al</i> 1997
NM-3	An isocoumarin small-molecule inhibitor of VEGF	Phase I	Reimer <i>et al</i> 2002
Thrombospondin	Blocks endothelial cell migration and neovascularization in the cornea	No	Dameron <i>et al</i> 1994
Tumstatin	Binds to integrin- $\alpha_1\beta_1$ on endothelial cells, inhibits endothelial cell proliferation and neovascularization	No	Maeshima <i>et al</i> 2001; Maeshima <i>et al</i> 2002
2-methoxyestradiol	Inhibits microtubule function in proliferating endothelial cells, resulting in endothelial cell apoptosis	Phase I and II	D'Amato <i>et al</i> 1994
Vitaxin	A humanized monoclonal antibody against integrin- $\alpha_v\beta_3$	Phase I and II	Gutheil <i>et al</i> 2002

Table 1.7 Direct angiogenesis inhibitors.

This table shows the cell target of various direct angiogenesis inhibitors. Adapted from Kerbal and Folkman 2002.

Cancer-cell target	Pro-angiogenic proteins	Drug	Reference
EGF receptor tyrosine kinase	VEGF, bFGF, TGF- α	ZD1839 (Iressa); ZD6474; OS1774 (Tarceva); CI1033; PKI1666; IMC225 (Erbix)	Giardiello <i>et al</i> 2001
VEGF receptor	VEGF receptor on endothelium	PTK787; SU11248	Tille <i>et al</i> 2001; Hoekman 2001
PDGF receptor	PDGF receptor	PTK787	Tille <i>et al</i> 2001
ERBB-2 (HER-2/neu receptor tyrosine kinase)	VEGF, angiopoietin-1, TGF- β , PAI1, upregulates thrombospondin-1	Herceptin	Kerbal <i>et al</i> 1998; Izumi <i>et al</i> 2002
Interferon (IFN)- α receptor	Inhibits expression of bFGF by cancer cells	IFN- α	Singh <i>et al</i> 1995

Table 1.8 Indirect angiogenesis inhibitors.

This table shows the cancer cell target of indirect angiogenesis inhibitors. Adapted from Kerbal and Folkman 2002.

1.4 von Hippel-Lindau gene

Eugene von Hippel (1867-1939) is credited with providing the first full description of a retinal vascular abnormality [von Hippel 1904], although cases dating back to the 1800s have been identified [Collins 1894]. A Swedish pathologist called Arvind Lindau described patients who were at high risk of developing blood vessel tumour of the brain and spinal cord [Lindau 1927].

von Hippel-Lindau disease is a familial cancer syndrome that is dominantly inherited and predisposes affected individuals to a wide variety of highly vascularized tumours [Maher & Kaelin 1997]. The most frequent tumours are haemangioblastomas of the central nervous system and retina, renal cell carcinoma (RCC), and pheochromocytoma [Maher & Kaelin 1997].

VHL disease affects 1 in 36,000 of the population. Renal carcinomas and central nervous system (CNS) haemangioblastomas remain the major causes of morbidity and mortality in VHL patients [Crossey *et al* 1994, Clifford *et al* 2001]. At the molecular level, VHL disease is autosomal recessive as tumours arise only after the remaining wild-type VHL allele is somatically mutated or silenced [Maher & Kaelin, 1997]. VHL disease can be categorised into four types, termed type 1, 2A, 2B, and 2C (see table 1.9 for descriptions).

1.4.1 Variety of neoplasms

Von Hippel-Lindau disease is a familial multiple cancer syndrome (see table 1.9) in which there is a predisposition to a variety of neoplasm's, including RCC (with clear cell histology), renal cysts, retinal haemangiomas, haemangioblastomas of the cerebellum, spinal cord, pheochromocytomas, pancreatic carcinomas and cysts. RCC develops in

nearly 40% of patients with VHL disease and is a major cause of death among these patients.

Type of VHL disease	VHL mutation type	Molecular defect	Clinical manifestation
Type 1	Loss of <i>VHL</i> or a mutation that effects protein folding	Upregulation of HIF- α and HIF target genes	Haemangioblastomas Diminished risk of pheochromocytoma Renal cell carcinoma
Type 2A	<i>VHL</i> missense mutation	Upregulation of HIF- α and HIF target genes	Haemangioblastomas Pheochromocytoma Low risk of RCC
Type 2B	<i>VHL</i> missense mutation	Upregulation of HIF- α and HIF target genes	Haemangioblastomas Pheochromocytoma High risk of RCC
Type 2C	<i>VHL</i> missense mutation	PVHL retains ability to degrade HIF- α ; decreased binding to fibronectin, fibronectin matrix assembly defect	Pheochromocytoma only

Table 1.9 Characteristics of VHL disease.

This table shows the four types of VHL disease according to the mutations and functional differences that occur. Adapted from Kaelin 2002.

1.4.2 Identification of the VHL gene

Seizinger *et al* mapped the VHL tumour suppressor gene locus to the short arm of chromosome 3 in 1988. The gene was located in 1993 by Latif *et al* using positional cloning, with the promoter being identified by Kuzmin *et al* in 1995. This method identified the gene using cDNA libraries that were scanned using a cosmid (cos11) that mapped to a commonly deleted region. Two apparently unrelated clones were identified and denoted g6 and g7. It was concluded that g7 proved to be a strong candidate for the VHL gene. The expression pattern of g7 was first studied using northern blotting, and a transcript was observed in all human tissues tested, including brain and kidney, tissues which are frequently affected in VHL disease [Latif *et al*, 1993]. The positional cloning strategies delineated the VHL gene to the region on the human chromosome 3p25-p26. The expression of the VHL transcript is not restricted to organs affected by VHL disease [Ohh & Kaelin, 1999]. The VHL coding sequence is contained within 3 exons, which can be alternatively spliced to produce two mRNAs that reflect the presence (isoform I) or absence (isoform II) of exon two.

The VHL gene encodes a 4.7 Kb mRNA that is widely expressed in both foetal and adult tissues [Iliopoulos *et al*, 1995, Latif *et al*, 1993]. The VHL mRNA encodes two protein products (pVHL) with molecular weights of ~30 and ~19 kDa [Iliopoulos *et al* 1995], the former being 213 amino acids long and the latter containing 160 amino acids as the result of internal translation from the second methionine residue at codon 54 within the VHL open reading frame [Iliopoulos *et al*, 1998]. The smaller form of the protein appears to predominate in many tissues. It is not currently known why cells make two forms of pVHL, and the two isoforms behave similarly in biochemical and functional assay performed on them.

1.4.3 Mutational analysis of the VHL gene

A comprehensive database of mutations in the VHL gene called the Universal VHL-mutation database can be found at www.umd.necker.fr:2005/ and includes 747 mutations of the gene from 62 references. Identification of the VHL gene has allowed the detection of germline mutation in approximately 75% of VHL families. A number of mutations have been identified in the VHL gene of patients with RCC, spanning across the whole gene [Gnarra *et al*, 1994].

A mutational hot spot was identified at a CpG island in codon 167 where 12% of the mutations were found. The codon 167 mutation was associated with a phenotype characterised by minimal or absence of renal carcinoma and frequent pheochromocytomas [Maher *et al* 1996].

Reintroduction of the VHL gene into renal carcinoma cell lines lacking normal expression of the gene restored the expression of the protein and led to a dramatic suppression of growth [Chen *et al*, 1995]. Inactivation of the VHL gene is the most frequent genetic mutation in RCC [Whaley *et al*, 1994, Kaelin & Maher, 1998]. When Zbar and associates used RFLP analysis to study studied tumour tissue from 18 patients with sporadic nonhereditary renal carcinoma, loss of heterozygosity of chromosome 3p was detected in all the tumour tissues [Zbar *et al* 1996]. The VHL gene has been shown to be silenced by CpG islands being methylated in RCC [Herman, 1994]. It has also been shown that reintroduction of either 30-kDa (pVHL₃₀) or 19-kDa (pVHL₁₉) pVHL into VHL⁻ renal carcinoma cells suppresses their ability to form tumours in nude mice [Schoenfield *et al*, 1998, Iliopoulos *et al*, 1995, Gnarra *et al*, 1996]. This observation might account for the fact that VHL mutations invariably map C-terminal to residue 54. Mutations that affect the first 54 amino acids would in principle allow the production of the wild-type pVHL₁₉.

1.4.4 Localization of pVHL

Localisation of the pVHL has shown that pVHL₃₀ is predominantly cytoplasmic, although a significant amount has been found in the nucleus and in association with the endoplasmic reticulum [Schraml *et al* 2003]. This means that the pVHL can shuttle back and forth between the nucleus and cytoplasm in response to certain signals [Iliopoulos *et al* 1995, Lee *et al*, 1996]. VHL was shown to contain a nuclear export domain. Nuclear export of VHL is dependent on at least four independent factors; an ATP hydrolysis step, the presence of GTP, synthesis of polyadenylated ATP and the small GTPase Ran [Groulx *et al* 2000]. Several cancer-causing substitutions found in sporadic RCC do not affect the ability of VHL to assemble with elongin B/C and Cul-2 and appear to inactivate a yet undescribed tumour suppression function [Stebins *et al* 1999]. These inactivating substitutions may prevent the nuclear export of VHL. Reasons for this ability to shuttle between the nucleus and the cytoplasm have yet to be discovered, but may be related to its role as an E3 ligase. This nuclear cytoplasmic shuttling has recently been shown to correlate with HIF-1 α levels, linking the previously described ability of VHL to shuttle between nuclear and cytoplasmic compartments with the ubiquitination and degradation of HIF-1 α [Groulx & Lee 2002].

While its interaction with some proteins has been understood VHL has been shown to inhibit vascular endothelial growth factor in RCC by blocking protein kinase C pathways [Pal *et al*, 1997].

Combined nuclear and cytoplasmic pVHL expression was associated with low histological grade, early tumour stage and better prognosis in RCC patients. Implying that subcellular trafficking is of potential relevance for the aetiology of RCC [Schraml *et al* 2003].

1.4.5 VHL associated proteins

The pVHL tumour suppressor has been shown to associate with a number of other proteins, giving it a wide variety of functions that are only now being categorised and understood, and are summarised below (see fig 1.3).

1.4.5.1 Elongins B and C.

Elongins B and C were first identified by virtue of their ability to stimulate the transcriptional elongation activity of elongin A [Garrett *et al* 1994]. This trimeric (elongin-SIII) complex enhances the rate of elongation by RNA polymerase II, suppressing the basal transcription machinery from pausing or stalling on the DNA template [Aso *et al*, 1995]. These two transcriptional elongation factors were subsequently shown to bind in vitro and in vivo to a short co-linear region of the VHL protein (pVHL) [Kibel, *et al*, 1995, Duan *et al* 1995]. Binding of pVHL to the elongin B/C complex occurs between the C-terminal residues 157-172 termed the α binding site. The α site is the only region of pVHL that is similar to elongin A. The homologous region of elongin A likewise interacts with the B/C complex, establishing this set of amino acid residues as an elongin B/C binding motif [Kibel *et al*, 1995], which stabilises the structure of elongin C [Botuyan *et al*, 1999]. The elongin B/C binding domain on pVHL is a frequent site of mutations, suggesting that elongin binding contributes to tumour suppression by pVHL. Polymorphism's or mutations in this region may affect the binding between the pVHL and B/C complex and predispose individuals to an increased risk of tumour formation

1.4.5.2 Cullin-2.

pVHL binds to cullin-2 (Cul-2), a member of the cullin family. Immunofluorescence studies showed Cul-2 to be a cytosolic protein that can be translocated to the nucleus by pVHL [Pause *et al*, 1997]. It was shown that pVHL mutants that were unable to bind to complexes containing elongin C and Cul-2 were likewise unable to inhibit the accumulation of hypoxia-inducible mRNAs [Lonergan *et al* 1998].

Cullins were first identified in *Caenorhabditis elegans* during a screen for mutants that displayed excess post-embryonic cell divisions. Cul-1 and Cul-2 bear a striking similarity to yeast Cdc53, which plays a role in ubiquitination, and hence degradation of certain proteins. There is significant homology between the Skp1-Cul-2-F-box protein (SCF) complex and the pVHL-elongin-Cul-2 (VEC) complex suggests that both complexes may possess a number of similar functions. Recent elucidation of the crystal structure of the VHL-Elongin C-Elongin B has increased the knowledge of the similarities of the SCF complex and the VEC complex [Stebbins *et al*, 1999].

The Rbx1 and Cul-2 proteins were identified as part of the VCB multi-component complex mediating ubiquitination of target proteins [Kamura *et al* 1999].

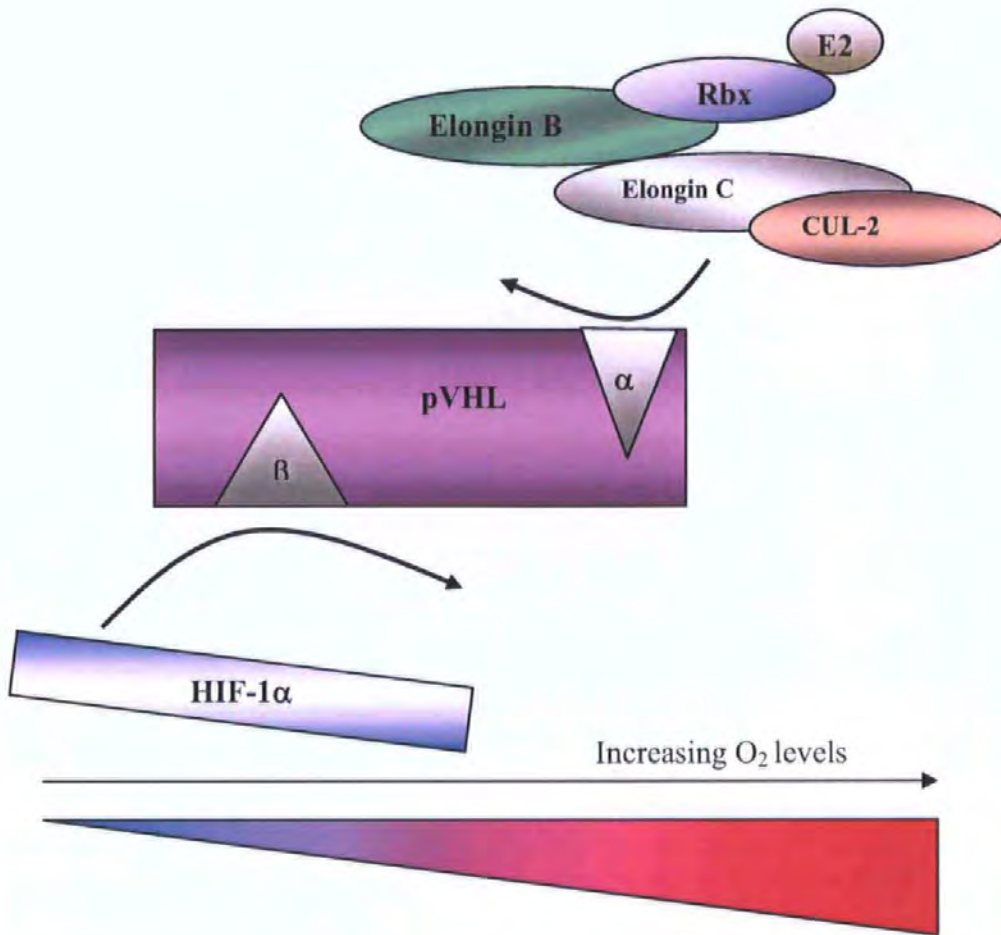


Fig 1.3 The von Hippel-Lindau protein (pVHL) and accessory proteins.

This fig shows the pVHL and the various proteins that are in complex with it. These include the elongins B and C, cullin-2 and Rbx, E2. This complex of proteins forms the VCB complex which is an E3 ubiquitin ligase. Also shown is the HIF-1 α subunit which binds to the β site of the VHL protein.

1.4.6 Downstream targets of pVHL.

pVHL is able to regulate, at least indirectly, the abundance of a variety of mRNAs. These include the mRNAs encoding VEGF, PDGF-B, glucose transporter 1 (GLUT-1), TGF- α , and carbonic anhydrases (CA) 9 and 12 [Maher & Kaelin, 1997]. It has been demonstrated that VHL^{-/-} tumour cells produce high levels of hypoxia-inducible mRNAs, such as the VEGF and GLUT-1 mRNAs, under hypoxic and normoxic conditions. Reintroduction of wild-type pVHL into these cells inhibited the production of hypoxia-inducible mRNAs under normoxic conditions [Maher & Kaelin, 1997]. The hypervascular nature of VHL associated tumours may therefore be linked to the expression and overproduction of hypoxia-inducible mRNAs.

The role of pVHL on VEGF has not been fully elucidated, with conflicting data describing the effect as being either transcriptional or post-transcriptional [Gnarra *et al*, 1996].

Figure 1.3 shows the hypothesised relationship between hypoxia inducible factor one alpha (HIF-1 α) and the VEC complex. It appears that pVHL binds to the HIF-1 α subunit and directs it to the proteasome for ubiquitin-mediated proteolysis (see fig 1.4) [Maxwell *et al*, 1999]. Currently it is not known which other proteins are targeted for ubiquitin mediated proteolysis in this manner, and may be important in elucidating any other functions of pVHL. The number of proteins associated with pVHL is increasing all the time and includes Rbx1 (RING box protein1) [Kamura *et al*, 1999], and E2 protein [Lisztwan *et al*, 1999].

Apart from binding to HIF- α subunits, pVHL has been shown to bind protein kinase C (PKC) and may serve as an E3 ubiquitin ligase for PKC- λ [Pal *et al* 1997, Pal *et al* 1998].

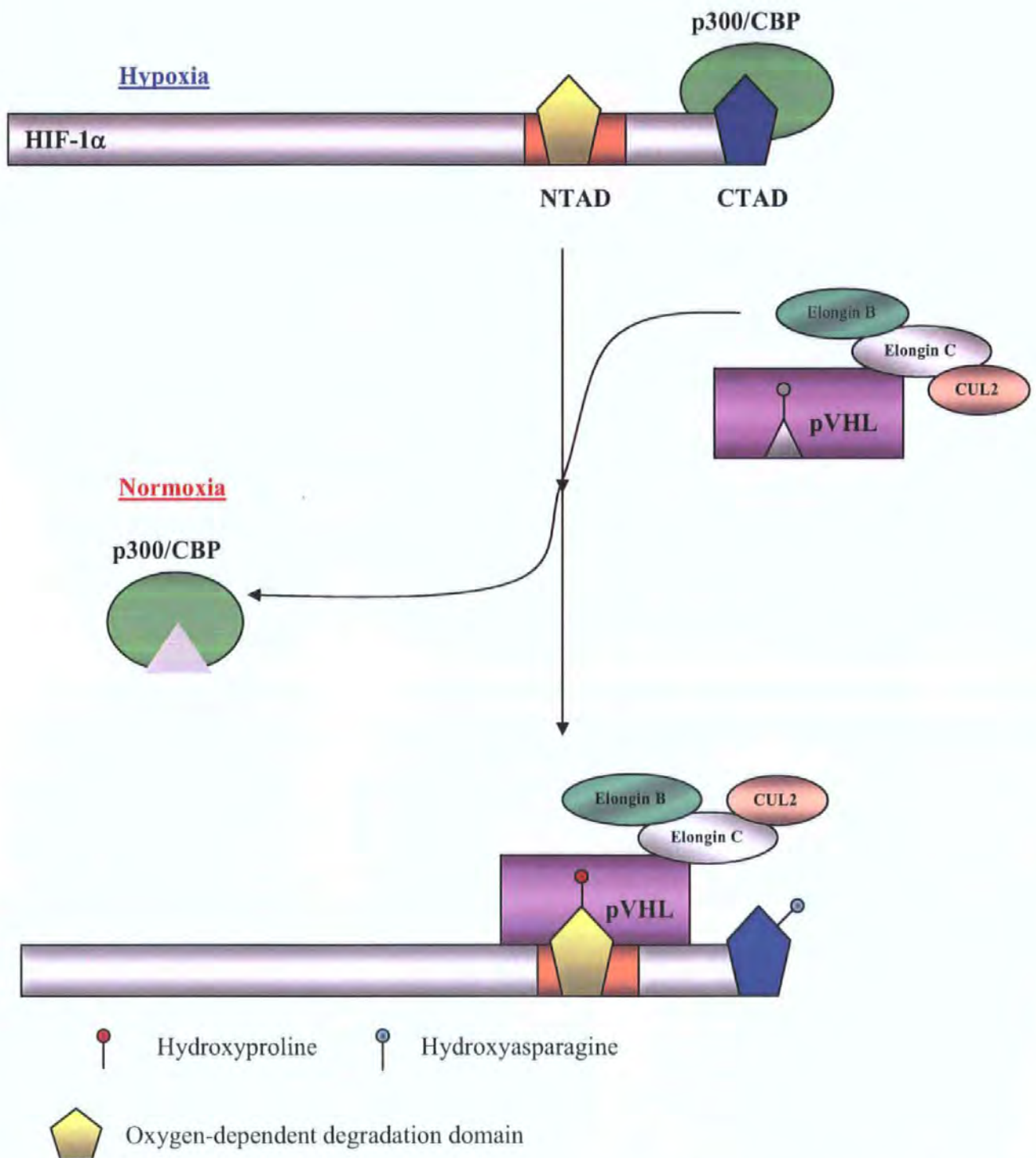


Fig 1.4 Regulation of HIF-1 α by pVHL under normoxic and hypoxic conditions. Under hypoxic conditions, transcriptional activators p300 and cAMP-response-binding protein (CREB)-binding protein (CBP) bind to the carboxy-terminal transactivation domain (CTAD) of hypoxia-inducible factor- α activating transcription of HIF regulated genes. Under normoxic conditions an asparagine in the CTAD is hydroxylated by factor inhibiting HIF-1 (FIH1), preventing the binding of p300/CBP. Proline residues in the oxygen-dependent degradation domain, which is found within the amino-terminal transactivation domain (NTAD), are hydroxylated by EGLN proteins. This aids the binding of the pVHL-elongin-cullin 2 complex, that polyubiquitylates HIF- α , and targets it for destruction by the proteasome.

pVHL can also bind to the transcription factor Sp1 suppressing its activity [Cohen *et al* 1999, Mukhopadhyay *et al* 1997]. In addition to this pVHL interacts with a cytosolic protein-folding complex called CCT [Feldman *et al* 1999, Hanen *et al* 2002].

pVHL may also associate with the endoplasmic reticulum (ER), although there is no evidence that the protein is secreted into the ER lumen [Ohh *et al* 1998, Schoenfeld *et al* 2001]. Fibronectin molecules that are malformed or malprocessed may undergo retrograde transport to the cytosolic surface of the ER membrane, which makes them available to interact with pVHL. Cells lacking pVHL secrete fibronectin, but are defective with respect to fibronectin-matrix assembly [Ohh *et al* 1998]. VHL^{-/-} renal carcinoma cells were not able to assemble exogenous fibronectin into a matrix, linking this to abnormalities of integrin assembly on their cell surface [Schoenfeld *et al* 2001]. Tissue inhibitors of metalloproteinases (TIMPs) and matrix metalloproteinases (MMP) are regulated by pVHL, possibly via HIF and are implicated in extracellular-matrix turnover [Koochelpour *et al* 1999].

1.5 Vascular Endothelial Growth Factor

In 1983 investigators at Beth Israel Hospital in Boston, reported the identification and partial purification (from conditioned medium of a guinea-pig tumour cell line) of a protein that induced vascular leakage [Senger *et al* 1983]. It was proposed that it could mediate the hyperpermeability of tumour blood vessels and was termed tumour vascular permeability factor (VPF).

1.5.1 Isolation and purification of VEGF

Complete purification of Vascular endothelial growth factor (VEGF) was by Plouet *et al* (1989) from the mouse pituitary cell line AtT20 and named 'vasculotropin', and Ferrara and Henzel (1989) who isolated the protein from bovine pituitary follicular cells, showing that it is a basic, heparin-binding homodimeric protein of 46-kDa that disassociates into two identical 23 kDa subunits. Vasculotropin proved to be the mouse ortholog of VEGF. The human VEGF gene is located on chromosome 6 at 6p21.3 [Vinceti *et al* 1996].

1.5.2 Alternative splicing of the VEGF gene

cDNA sequence analysis of a variety of human VEGF clones indicated that VEGF may exist as one of at least six different molecular species, having 121, 145, 148, 165, 189, and 206 amino acids respectively (see fig 1.5) [Leung *et al* 1989, Keck *et al* 1989, Tisher *et al* 1991, Houck *et al* 1991, Poltorak *et al* 1997, Whittle *et al* 1999]. Analysis of these clones showed that they contain a classical signal sequence, supporting the hypothesis that VEGF is a secreted protein. A novel spliced variant of 183 amino acids has been identified in human müller cells with an 18-nucleotide deletion, equating to the loss of 6 amino acids

[Jingjing *et al*, 1999]. Identification of a species with 148 amino acids has been reported to have an inhibitory effect, by binding to another of the isoforms but not activating the receptor [Whittle *et al*, 1999]. Each of these isoforms display different characteristics biochemically and physiologically. Addition of the 44 amino acid-long peptide encoded by exon 7 of the VEGF gene distinguishes VEGF₁₆₅ from VEGF₁₂₁ [Park *et al* 1993, Cohen *et al* 1995]. VEGF₁₄₅ is distinguished by the presence of the 21 amino acid peptide encoded by exon 6 [Poltorak *et al* 1997]. The shortest form VEGF₁₂₁ was found to be freely diffusible and does not bind to heparin or extracellular matrix [Park *et al* 1993]. Whilst VEGF₁₆₅ is secreted, some of the protein is sequestered in the extracellular matrix, and VEGF₁₈₉ is almost completely sequestered by the extracellular matrix [Houck *et al* 1991, Park *et al* 1993]. The VEGF₁₈₉ isoform can be released from the extracellular matrix by plasmin following cleavage at the carboxyl terminus [Park *et al* 1993]. This combination of exon splicing and ability to be sequestered by the extracellular matrix provides VEGF with two different mechanisms of availability.

1.5.3 Differential Isoform expression

VEGF₁₆₅ is the predominant isoform secreted by a variety of normal and transformed cells. Wizigmann-Voos *et al* (1995) found that VEGF₁₂₁ and VEGF₁₆₅ are predominately expressed in capillary hemangioblastoma. In RCC, Tomisawa *et al* (1999) found that patients with pathological stage pT3-4 showed expression of VEGF₁₂₁ + 165 + 189 at a significantly higher incidence than those with pT0-2. Their observations suggest that the VEGF₁₈₉ isoform is closely associated with angiogenesis in RCC.

The central role of VEGF in angiogenesis and neovascularization is analogous to the role of erythropoietin (EPO) in erythropoiesis. Induction of VEGF expression in hypoxic cells was similar to that of EPO [Goldberg & Schneider, 1994]. Transcription of the VEGF gene

and stability of VEGF RNA were both increased in hypoxic cells [Goldberg & Schneider, 1994, Liu *et al* 1995].

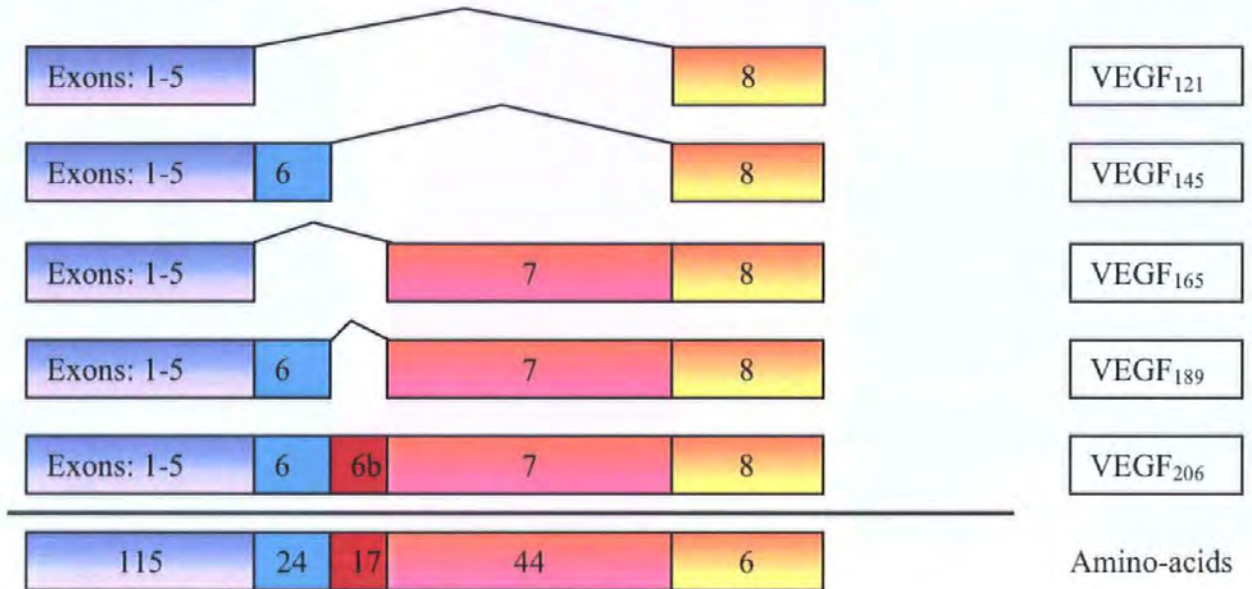


Figure 1.5 Human VEGF gene structure.

Showing the locations of exons 1 to 8 and the various forms of VEGF that arise by alternative splicing. Exon 6, is marked in blue. Exon 6b is red; the orange/pink shading indicates exon 7, present in VEGF₁₈₉ and VEGF₁₆₅.

1.5.4 Role of VEGF in the regulation of blood vessel growth

The development of a vascular supply is a fundamental requirement for organ development and differentiation in multicellular organisms. Blood vessel formation is also necessary for tissue repair and reproductive functions in the adult. Substantial experimental evidence also indicates that neovascularization (angiogenesis) plays an important role in the pathogenesis of a variety of disorders [Folkman, 1991]. VEGF mRNA is known to be correlated with blood vessel growth in the developing mouse embryo, supporting the idea that VEGF is an *in vivo* angiogenic factor [Breier *et al* 1992]. Binding sites of VEGF are largely restricted to the vascular endothelium of large or small vessels [Jakeman *et al* 1992]. VEGF mRNA expression in glioblastoma multiforme correlates with the most ischemic areas of the tumour, indicating that VEGF might be induced by hypoxia [Shweiki *et al* 1992, Plate *et al* 1992]. This was confirmed by culturing cells under hypoxic conditions [Plate *et al* 1992].

1.5.5 Biological activities of VEGF

Whilst VEGF is a potent mitogen for vascular endothelial cells but it is apparently devoid of any appreciable mitogenic activity in other cell types [Ferrara & Henzel, 1989]. Consequently, VEGF has been regarded as an endothelial cell-specific mitogen [Ferrara, 1993]. VEGF is able to induce a marked angiogenic response in a variety of *in vivo* models including the chick chorioallantoic membrane, the rabbit cornea, and the primate iris [Leung *et al*, 1989]. VEGF has been shown to promote angiogenesis in a tri-dimensional *in vitro* model; inducing confluent microvascular endothelial cells to invade a collagen gel and form tube like structures [Pepper *et al*, 1992].

VEGF induces expression of the serine protease's urokinase-type (uPA) and tissue type (tPA) plasminogen activators and also PA inhibitor 1 (PA1) in cultured bovine microvascular endothelial cells [Pepper *et al*, 1991]. It also induces the expression of metalloproteinase interstitial collagenase in human umbilical vein endothelial cells but not in dermal fibroblasts [Unemori *et al*, 1992]. VEGF also induces expression of urokinase receptors in vascular endothelial cells [Mandriota *et al*, 1995].

VEGF has been independently purified and cloned as a vascular permeability factor (VPF) based on its ability to induce vascular leakage in guinea-pig skin [Keck *et al*, 1989]. An increase in microvascular permeability may be a crucial step in angiogenesis associated with tumours and wounds, by inducing plasma protein leakage. This would result in the formation of an extravascular fibrin gel, a substrate for endothelial and tumour cell growth [Dvorak, 1986].

An additional effect of VEGF on the vascular endothelium is the stimulation of hexose transport. Exposure of bovine aortic endothelial cells to VEGF or TNF- α resulted in a significant increase in the rate of hexose transport [Pekala *et al*, 1990].

VEGF also induces tissue factor expression in cultured endothelial cells. The induction of such a procoagulant protein may contribute to the abnormal coagulant properties of tumour vessels in response to TNF- α [Nawroth & Stern, 1988].

VEGF can also induce vasodilatation *in vitro* in a dose dependent fashion, which translates into a transient hypotension *in vivo* [Yang *et al*, 1996].

1.5.6 Regulation of VEGF

Several mechanisms have been shown to involve the regulation of VEGF gene expression. Oxygen tension appears to play a major regulatory part, both *in vitro* and *in vivo* [Minchenko *et al*, 1994]. VEGF mRNA expression is rapidly and reversibly induced by

exposure to low oxygen partial pressure (PO_2) in a variety of cultured cells including retinal pigmented epithelial cells, myoblasts, cardiomyocytes, and tumour cells [Shweiki *et al*, 1992]. In sections of glioblastoma multiforme, VEGF mRNA is highly expressed in ischaemic tumour cells that are juxtaposed to areas of necrosis [Shweiki *et al*, 1992, Plate *et al*, 1992].

1.5.7 Other VEGF family members.

Currently the main focus on VEGF has been on VEGF-A, with less emphasis on the other family members. With the isolation and characterisation of VEGF-B [Olofsson *et al*, 1996], VEGF-C [Joukov *et al*, 1996], VEGF-D [Yamada *et al* 1997] and VEGF-E [Ogawa *et al* 1998] as well as a newly identified factor related to the VEGF family termed, endocrine derived vascular endothelial growth factor (ED-VEGF) [LeCouter *et al*, 2001]. Expression of VEGF-B and C has recently been measured in RCCs [Gunningham *et al* 2001] showing significant up regulation of VEGF-B but not C in neoplastic compared to normal kidney.

1.5.7.1 VEGF-B

VEGF-B is a novel growth factor for endothelial cells, identified as a non-glycosylated highly basic heparin-binding growth factor with structural similarities to VEGF and PlGF. It is widely distributed but mostly abundant in heart, skeletal muscle, and the pancreas. VEGF-B forms disulfide-linked dimers that are secreted but remain bound to cells or the ECM and can be cleaved by heparin [Olofsson *et al* 1996a]. The VEGF-B gene was localized to chromosome 11q13, proximal to the cyclin D1 gene [Paavonen *et al* 1996]. VEGF-B consists of two isoforms obtained by alternative splicing of mRNA from the VEGF-B gene, which spans about 4KB of DNA [Olofsson *et al* 1996b]. The gene consists

of seven exons, and has a similar exon-intron organization to VEGF and PlGF [Grimmond *et al* 1996, Olofsson *et al* 1996b]. The mature proteins are 167 (VEGF-B₁₆₇) and 186 (VEGF-B₁₈₆) amino acid residues respectively. VEGF-B₁₈₆ is generated by an alternative splice acceptor site in exon 6, resulting in an insertion of 101bp between 410 and 411 in the coding sequence of VEGF-B₁₆₇. This insertion introduces a frameshift and a stop codon at the position corresponding to nucleotides 521-523 of the coding region of VEGF-B₁₆₇ cDNA. The two VEGF-B isoforms have an identical NH₂-terminal domain of 115 amino acid residues and different COOH-terminal domains [Grimmond *et al* 1996, Olofsson *et al* 1996]. The secreted VEGF-B₁₆₇ and VEGF-B₁₈₆ polypeptides are 21 kDa and 32 kDa respectively.

The ability of VEGF-B to heterodimerize with VEGF-A is consistent with the conservation of eight cysteine residues involved in inter- and intramolecular disulfide bonding, and co-expression of VEGF-B and VEGF-A in many tissues suggests that VEGF-B-VEGF heterodimers occur naturally. The formation of VEGF-B-VEGF heterodimers suggests there is at least a partial overlap between the cellular signalling receptors (See fig 1.7 showing VEGF family receptors and ligands). VEGF-B specifically binds to VEGFR-1 and endothelial cells respond to VEGF-B by inducing the expression and activity of uPA and PAI-1. It might therefore be involved in endothelial ECM degradation and adhesion [Olofsson *et al* 1998].

VEGF-B was shown to be strongly expressed in the heart and that both isoforms (VEGF-B 167 and VEGF-B 186) bind to neuropilin-1 (NRP-1), suggesting involvement of VEGF-B in NRP-1 mediated signalling [Makinen *et al* 1999].

VEGF-B knockout mice (Vegfb (-/-)) are healthy and fertile unlike VEGF-A knockout mice which die during embryogenesis. The hearts from the VEGF-B knockout mice are reduced in size and show impaired recovery from experimentally induced myocardial

ischemia, suggesting a role for VEGF-B in the development of coronary vasculature [Bellomo *et al* 2000].

VEGF-B 167 accounts for more than 80% of the total transcripts in most tissues. VEGF-B 186 was expressed at lower levels. The VEGF-B 186 is up-regulated in mouse and human tumour cell lines and primary tumours compared to their normal matched tissue [Li *et al* 2001].

It has recently been shown that VEGF-B, through its receptor VEGFR-1, promotes angiogenesis in association with activation of Akt and eNOS-pathways [Silvestre *et al* 2003].

1.5.7.2 VEGF-C

While VEGF-C is homologous to other members of the VEGF / PDGF family, its C terminal half contains extra cysteine-rich motifs. The VEGF-C precursor is more than twice as large as the mature polypeptide [Joukov *et al* 1996]. Proteolytic processing of VEGF-C increases its affinity and activating properties toward VEGFR-3 and it also acquires the ability to bind to VEGFR-2. Synthesis of VEGF-C as a precursor prevents unnecessary angiogenic effects, via VEGFR-2, and allows preferential signalling via VEGFR-3, which is restricted to the venous endothelia during early stages of development and to the lymphatic endothelium during later stages [Joukov *et al* 1997]. VEGF-C binds VEGFR-3 and induces autophosphorylation of VEGFR-3 and VEGFR-2. The critical region for receptor binding and activation by VEGF-C is contained within the first 180 or so amino acid residues [Joukov *et al* 1996]. The VEGF-C dimer consists of polypeptides of 29-31 kDa that are generated by proteolytic cleavage of the precursor peptide between residues 227 and 228 [Joukov *et al* 1997]. Unlike the VEGF gene, the VEGF-C gene promoter does not contain putative binding sites for hypoxia-regulated factors. VEGF-C

was shown to induce specific lymphatic endothelium proliferation and hyperplasia of the lymphatic vasculature in vivo [Jeltsch *et al* 1997].

VEGF-C expression has been correlated with lymph node status, lymphatic invasion, venous invasion, and tumour infiltrating patterns in gastric cancer, with patients who had a high expression of VEGF-C protein having a poorer prognosis than those in the low expression group [Yonemura *et al* 1999]. It was also seen that VEGF-C expression in human prostate carcinoma cells was correlated with the presence of lymph node metastasis [Tsurusaki *et al* 1999].

1.5.7.3 VEGF-D/ FIGF

c-fos-induced growth factor (FIGF) or VEGF-D encodes a 358 amino acid residue long secreted protein. The amino acid sequence indicates that VEGF-D is strongly related to the PDGF/VEGF family of growth factors. VEGF-D contains a central region of eight cysteine residues that are important for dimerization [Orlandini *et al* 1996]. VEGF-D is the only member of the VEGF family that is not induced by hypoxia, but via cell-to-cell contact mediated by the cell surface molecule cadherin-11 [Orlandini *et al* 1996, Orlandini & Oliviero 2001]. It has recently been demonstrated that β -catenin inversely regulates VEGF-D mRNA stability [Orlandini *et al* 2003].

1.5.7.4 Placenta growth factor

A human cDNA coding for a protein related to VEGF was isolated from a term placenta cDNA library and named placenta growth factor [Maglione *et al* 1991]. PlGF, VEGF, and VEGF-B are able to form heterodimers and share a number of biochemical and functional features [Cao *et al* 1996, Olofsson *et al* 1996b, Di Salvo *et al* 1995]. PlGF is active as a

dimeric glycosylated protein, and the formation of an active dimer involves intra and intermolecular disulfide bonds among eight cysteine residues to produce a cysteine knot. Unlike VEGF, which is able to bind VEGFR-1 and VEGFR-2, homodimeric PlGF is able to only recognise the VEGF decoy receptor VEGFR-1 [Park *et al* 1994]. Within the PlGF family, at least three PlGF isoforms (PlGF-1, PlGF-2, and PlGF-3) have been identified, which are all generated by alternative splicing of the same gene [Maglione *et al* 1991, 1993, Cao *et al* 1997]. The two forms named PlGF-1 and 2 (PlGF₁₃₁ and PlGF₁₅₂) differ by the insertion of a highly basic 21 amino acid stretch at the carboxyl end of the protein, conferring the ability of PlGF-2 to bind heparin [Maglione *et al* 1993]. The three-dimensional structure of PlGF at 2.0 Å resolution has been elucidated and shows a high similarity to VEGF even though there is only 42% amino acid homology between the two proteins. PlGF-2 possesses heparin binding ability and can also bind to the neuropilin-1 receptor [Migdal *et al* 1998]. PlGF-1 is less active than PlGF-2 at stimulating endothelial cell growth angiogenesis [Ziche *et al* 1998]. These properties of PlGF-2 could be mediated by the neuropilin-1 receptor, and activation of the receptor increases angiogenic responses mediated by VEGF RTKs support this finding [Soker *et al* 1998]. It is therefore possible that PlGF-1, but not PlGF-2 is a natural antagonist for VEGF when produced as a heterodimer with VEGF.

It has been shown that in tumours expressing high levels of both PlGF and VEGF, functionally inactive heterodimers are formed thus decreasing the activity of VEGF [Eriksson *et al* 2002]. PlGF may play a dual role in the regulation of VEGF-induced angiogenesis and tumour growth as seen in fig 1.6.

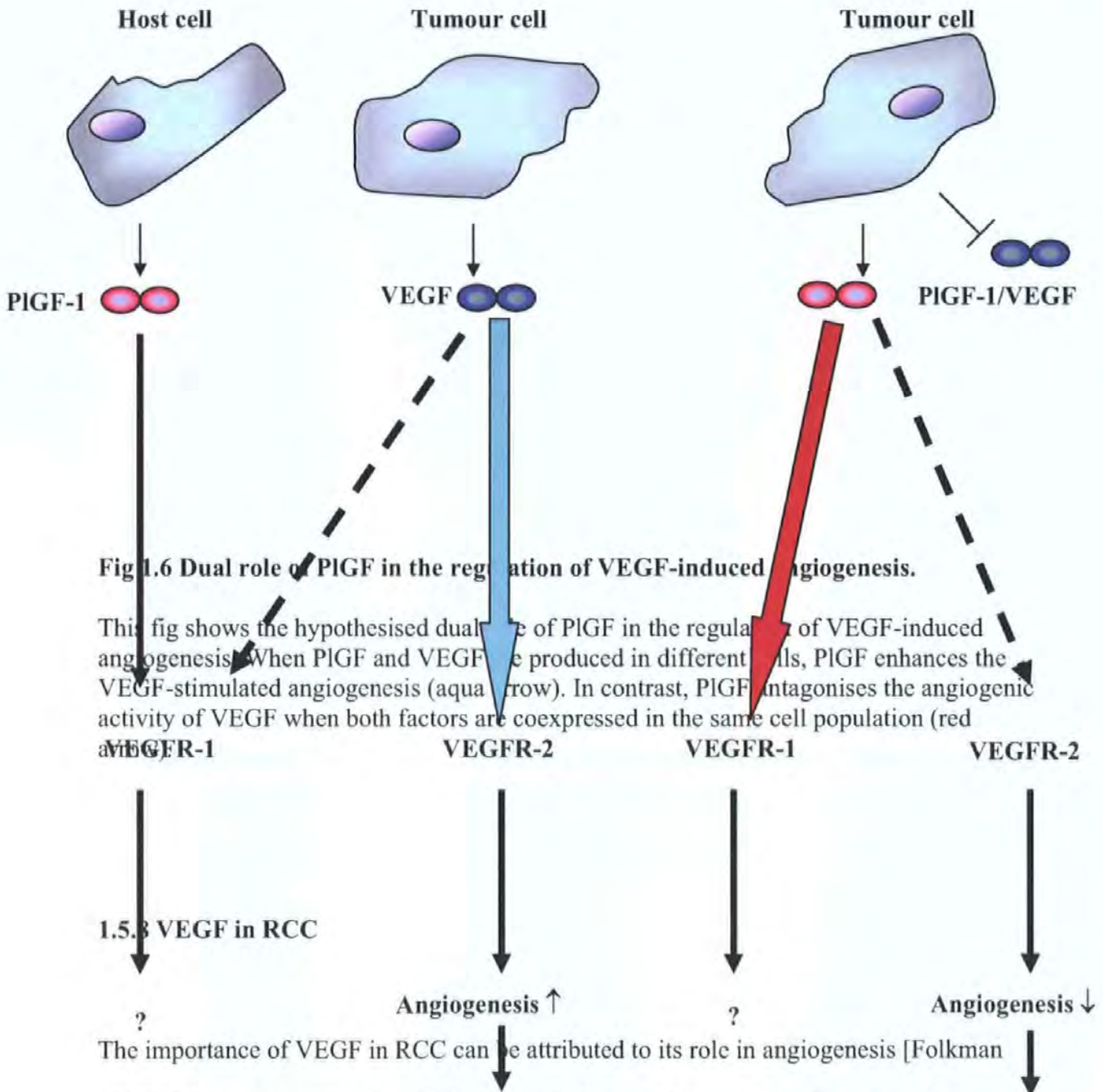


Fig 1.6 Dual role of PlGF in the regulation of VEGF-induced angiogenesis.

This fig shows the hypothesised dual role of PlGF in the regulation of VEGF-induced angiogenesis. When PlGF and VEGF are produced in different cells, PlGF enhances the VEGF-stimulated angiogenesis (aqua arrow). In contrast, PlGF antagonises the angiogenic activity of VEGF when both factors are coexpressed in the same cell population (red arrow).

1.5.3 VEGF in RCC

The importance of VEGF in RCC can be attributed to its role in angiogenesis [Folkman 1971]. Without a supply of new blood vessels the rapidly expanding tumour architecture will not be able to survive. Hence the growth of tumours without additional vasculature are only able to grow to a size of approximately 1mm³ before they require additional vessels to supply O₂ and nutrients and remove waste products [Folkman & Hochberg 1973].

Tumour growth and metastasis vs **Slow growing or dormant tumour**

The role of VEGF in RCC has been shown to be important in the progression of the disease. One of the phenotypic features of RCC is the highly vascularized nature of the tumour [Brown *et al* 1993, Sato *et al* 1994, Takahashi *et al* 1994].

Increased expression of VEGF has been demonstrated because destruction of genes related to the hypoxia sensitive sensing nature of the cell [Iliopoulos *et al* 1996]. VHL mutation and hypermethylation, or loss of function in the ability of pVHL to direct HIF- α subunits to the 28S proteasome can directly contribute to the increase in angiogenesis within this system [Krieg *et al* 2000]. RCC contains a large number of mutations within the VHL gene and inactivation of the protein may therefore lead to increases in hypoxia-inducible proteins [Kovacs *et al* 1991, Yao *et al* 1993, Gnarra *et al* 1994].

A number of isoforms of VEGF have been identified with the role of each of them becoming more apparent. The most common isoform is VEGF 165 and has been shown to be increased in RCC by various groups by methods including, reverse transcriptase polymerase chain reaction (RT-PCR), ribonucleic acid protection assay (RPA) and enzyme linked immunosorbent assay (ELISA) [Nicol *et al* 1997, Tomisawa *et al* 1999, Thelen *et al* 1999, Gunningham *et al* 2001b, Ljungberd *et al* 2003]. Given the wide variety of different sources and methods used to determine the levels of VEGF protein and mRNA expression, it is firmly established that VEGF has an important role to play in RCC.

Association between VEGF and tumour progression has indicated that certain RCCs may be potential targets for anti-angiogenic therapy, after measuring the levels of VEGF mRNA [Ljungberg *et al* 2003]. Association between VEGF and RCC progression has also been found for protein levels [Yang *et al* 2003b], with their results demonstrating that expression of VEGF is proportional to the formation and progression of RCC. It has also been demonstrated by immunohistochemistry that there is a link between VEGF expression and stage, nuclear grade, and microvessel density (MVD) [Zhang *et al* 2002, Jacobsen *et al* 2004]. Mutations in the VHL gene have been correlated with increased expression of VEGF and may in turn be linked to a more aggressive phenotype of RCC [Na *et al* 2003]. VEGF overexpression was shown to correlate with monoallelic and biallelic VHL activation [Igarashi *et al* 2002]. Treatment of Caki-1 cells with antisense-

oligos to VEGF led to a reduction in expressed VEGF levels sufficient enough to impair the proliferation and migration of co-cultured endothelial cells [Shi & Siemann 2002]. Urinary VEGF levels were increased in-patients with RCC (27 patients) [Chang *et al* 2001].

Recent identification of a new form of VEGF termed VEGF165b has opened a whole new area of VEGF biology and function into focus. VEGF 165b was identified as an inhibitory spliced variant which contains 6 alternative amino acids (SLTRKD instead of CDKPRR) at the C-terminal end of the protein [Bates *et al* 2002]. The dimerization [Potgens *et al* 1994] and receptor binding [Keyt *et al* 1996] domains are unaffected by these changes (being in exons 3 and 4 respectively). This spliced variant is unable to activate its cognate receptor (VEGF-R2), and initiate autophosphorylation of the receptor and activate signal transduction molecules down stream. Amounts of this spliced variant have been demonstrated (by RT-PCR) to be reduced in the tumour tissue from patients with RCC in comparison with the normal matched tissue, which had elevated levels of this species [Bates *et al* 2002]. Two mechanisms for the action of this isoform have been postulated; the first being that VEGF 165b binds to the receptor but is unable to activate the autophosphorylation of the TK domain. Second, it may heterodimerize with VEGF 165 and prevents stimulation of the receptor. If VEGF 165 has an isoform such as VEGF 165b then it would be biologically probable that the other VEGF isoforms also have an inhibitory partner. It has been demonstrated that some of these inhibitory partners do exist and are currently undergoing further investigation (personal communication Prof. Peter Mathieson). If these inhibitory isoforms are identified and measured, much of the VEGF literature may need to be revised. This may be due to the fact that various assays to measure VEGF stimulatory isoforms may also be inadvertently be measuring the endogenously expressed isoforms. With the ever-increasing number of anti-angiogenic

molecules currently undergoing trial (see www.angio.org) the ability to express these natural inhibitors to the angiogenic process may provide a new method to tackle disease.

1.5.9 VEGF polymorphisms

The VEGF gene is highly polymorphic, with different polymorphisms distributed across the entire gene, including the 5' and 3' UTR and promoter regions (see table 1.10). Some of these polymorphisms have shown the ability to produce altered levels of the protein [Brogan *et al* 1999, Watson *et al* 2000].

Promoter region polymorphisms have been shown to affect the expression level of various cytokines [Watson *et al* 2000]. Associations have been found with polymorphisms in the 5' UTR of VEGF and diabetic retinopathy in type 2 diabetes [Awata *et al* 2002], as well as acute allograft rejection [Shahbazi *et al* 2002] and preeclampsia [Brenchley *et al* 2002]. A common C/T (at nucleotide position 936) mutation may be an important determinant of VEGF plasma levels [Renner *et al* 2000].

There are ethnic differences between Japanese and Caucasian populations for the G1612A and G702T genotypes. The C705T, C936T, and G1612A polymorphisms in the 3'-UTR are not associated with the risk of RCC [Abe *et al* 2002]. The 3'-UTR of VEGF also increases the stability of the mRNA and is associated with hypoxic induction of the gene [Awata *et al* 2002].

Links between promoter polymorphisms, expression of the protein and promoter activity have been reported by a number of groups [Watson *et al* 2000, Yang *et al* 2003]. The CC genotype of an 18 nucleotide deletion/insertion polymorphism at -2549 is in complete linkage with a SNP (C-2578A) and been shown to be increased in patients with diabetic nephropathy in comparison with uncomplicated patients and normal controls. The C allele is also increased in the diabetic nephropaths in comparison with the uncomplicated patients and normal control groups. The C or A allele and the 18 nucleotide insert in the promoter region was cloned into a luciferase reporter plasmid to analyse changes in transcriptional activity. The construct containing the C allele had an activity of 1.95 fold compared to the

A allele. This suggests that the C allele in the promoter region (minus the 18 nucleotide insert) will lead to enhanced expression of the gene [Yang *et al* 2003].

Recent haplotype analysis of the VEGF promoter has shown that the -460/+405 polymorphism increased the mean induction by phorbol ester from 5-fold to 8.5-fold [Stevens *et al* 2003].

The gene designated as the Hu family binds to the AU rich elements of the 3'-UTR of several genes including VEGF mRNA [Nabors *et al* 2001, Goldberg-Cohen *et al* 2002], with the suggestion that Hu is able to prevent attack by RNAses by conformational changes in mRNA. Any single nucleotide changes in the 3'-UTR may alter the ability of these Hu proteins to stabilize the mRNA and create differences in gene expression.

The CC genotype of the C936T in the 3'-UTR is associated with higher plasma levels in comparison with the CT and TT genotypes [Renner *et al* 2000]. The C to T polymorphism leads to the loss of a potential binding site for AP-4 (a helix-loop-helix transcription factor) although the relevance of this has yet to be elucidated [Renner *et al* 2000]. Although the numbers in this study were small it may be of importance in the understanding of the stabilization of the mRNA.

The G-1154A polymorphism in the 5'-UTR of the gene is associated with higher VEGF production in patients with acute renal rejection or with the risk of prostate cancer [Shahbazi *et al* 2002, McCarron *et al* 2002].

Polymorphism	Description / Association	Reference
G -2578 A	Includes 18-nucleotide insert for the A allele, diabetic nephropathy	Brogan <i>et al</i> 1999, Yang <i>et al</i> 2003
G -1455 A	No current associations	Brogan <i>et al</i> 1999
G -1190 A	No current associations	
G -1154 C	Risk factor for prostate cancer	Brogan <i>et al</i> 1999, McCarron <i>et al</i> 2002
G -1001 C	No current associations	Brogan <i>et al</i> 1999
C -634 G	No current associations	
C -460 T	Not associated with childhood steroid-sensitive nephrotic syndrome (SSNS), calcium oxalate stone disease	Holt <i>et al</i> 2003, Lin <i>et al</i> 2003
-460 / +405	Enhanced production of VEGF in vitro	Stevens <i>et al</i> 2003
A -141 C	Not associated with SSNS	Holt <i>et al</i> 2003,
C -7 T	No current associations	Brogan <i>et al</i> 1999
G +405 C	Not associated with SSNS, Significant correlation between LPS stimulated PBMCs and VEGF protein production	Holt <i>et al</i> 2003,
C +702 T	Not associated with Japanese RCC population, no association with plasma levels	Abe <i>et al</i> 2002
C +936 T	Associated with plasma levels, not associated with Japanese RCC, T allele may decrease susceptibility to sarcoidosis	Renner <i>et al</i> 2000, Abe <i>et al</i> 2002 Morohashi <i>et al</i> 2003
G +1612 A	Not associated with Japanese RCC population	Abe <i>et al</i> 2002

Table 1.10 Polymorphisms located in the VEGF gene and associations with disease

This table shows polymorphisms of the VEGF gene and any associations with disease or expression levels of the protein. Included are polymorphisms that have yet to be associated with any disease.

1.5.10 VEGF receptors

Three VEGF receptors have been identified to date. These are designated VEGFR-1, VEGFR-2, and VEGFR-3, and were previously called *flt-1* (*fms*-like tyrosine kinase), KDR (kinase insert domain-containing receptor)/*flk-1* (fetal liver kinase-1), and *FLT4* respectively [Shibuya *et al* 1990, Shibuya *et al* 1999, Neufeld *et al* 1999, Aprelikova *et al* 1992] (see fig 1.7). Structurally they all consist of seven Ig homology domains in their extracellular part and a tyrosine kinase signalling domain, which is split by a kinase insert. The VEGFR-1 and VEGFR-2 are expressed mainly in the blood vascular endothelium, and VEGFR-3 is found mainly in the lymphatic endothelium. VEGF binds to both VEGFR-1 and VEGFR-2 using symmetrical binding sites at each pole of the dimer for receptor binding [Wiesmann *et al* 1997, Muller *et al* 1997] (see fig 1.7). The second Ig homology domain was found to be essential for ligand binding [Wiesmann *et al* 1997], although to reconstitute full activity the first three domains were required [Davis-Smyth *et al* 1996, Barleon *et al* 1997, Davis-Smyth *et al*, 1998].

VEGFR-1 is a M_r 180,000 transmembrane glycoprotein, although the mRNA can be spliced to produce a shorter soluble form consisting of the first six extracellular Ig homology domains [Shibuya *et al* 1990, De Vries *et al* 1992, Pajusola *et al* 1993].

VEGFR-2 is a M_r 195,000 protein with no spliced variants reported for the receptor [Pajusola *et al* 1993]. VEGFR-3 is a M_r 195,000 which is proteolytically cleaved in the fifth Ig homology domain, resulting in M_r 120,000 and 75,000 chains linked by a disulphide bond [Lee *et al* 1996, Pajusola *et al* 1994].

The promoter for VEGFR-1 contains sequences matching the HIF-1 α consensus binding sites, and HIF-2 α may stimulate VEGFR-2 promoter activity [Gerber *et al* 1997, Ikeda *et al* 1996, Kappel *et al* 1999].

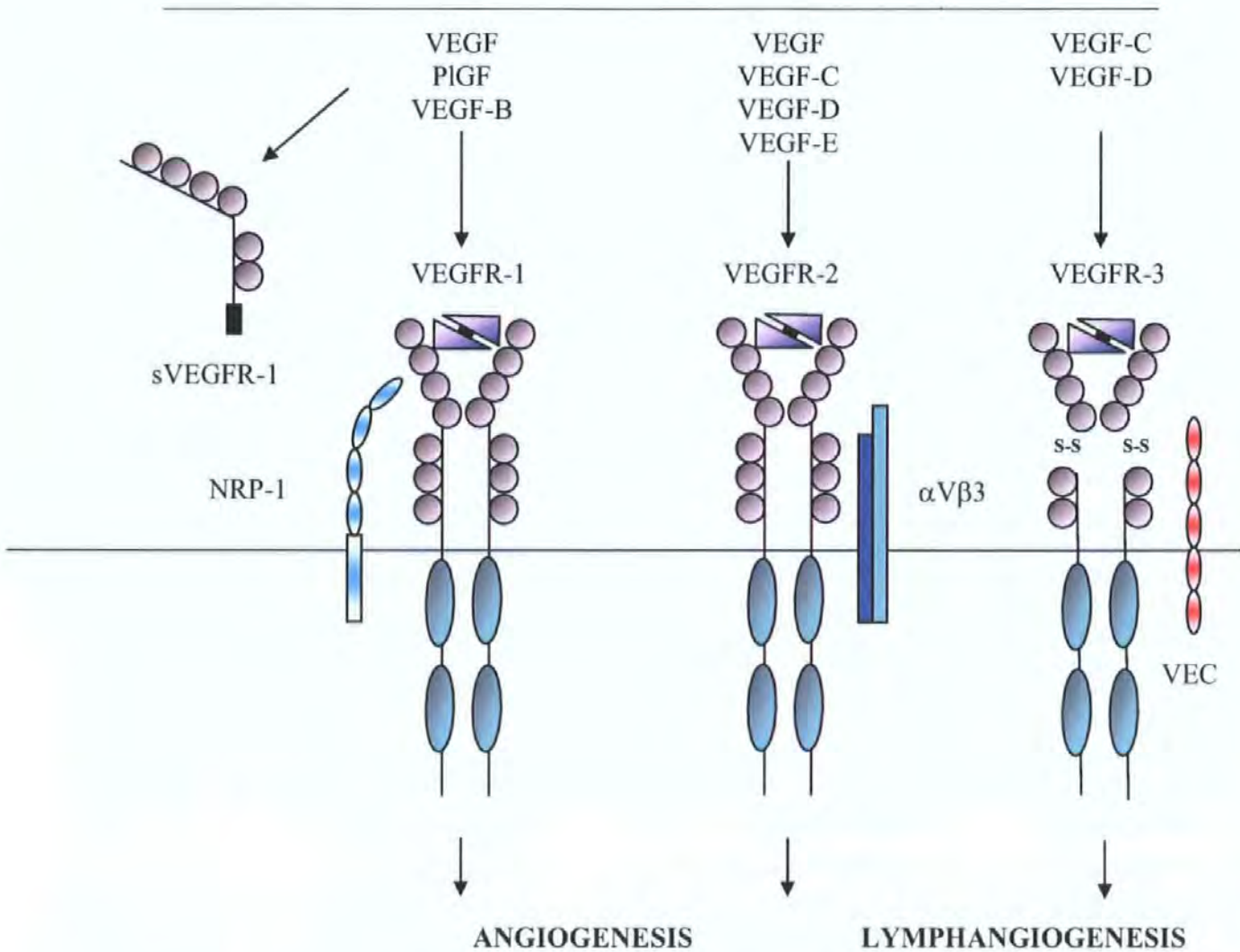


Fig 1.7 The various VEGFs and their receptors.

This fig shows the VEGF receptors 1, 2, 3, and the soluble VEGFR-1. VEGFR-1 and VEGFR-2 have seven immunoglobulin homology domains, in VEGFR-3 the fifth immunoglobulin domain is cleaved on receptor processing into disulphide-linked subunits. The accessory proteins for each receptor are also shown, consisting of neuropilin-1 (NRP-1) with R1, $\alpha V\beta 3$ integrin with R2, and vascular endothelial-cadherin (VEC) with R3. These co-receptors are required for correct activation of the receptor.

1.5.10.1 VEGFR signalling

As a class of receptor tyrosine kinases (RTK), the VEGFR are believed to dimerize and undergo trans-autophosphorylation on ligand binding [Joukov *et al* 1996, Waltenberger *et al* 1994]. VEGFR-1 and VEGFR-2 activate the MAPK pathway in response to VEGF in many types of endothelial cells [Seetharam *et al* 1995, Landgren *et al* 1998, D'Angelo *et al* 1995, Kroll & Waltenberger 1997, Abed & Zachary 1997, Rousseau *et al* 1997, Mukhopadhyay *et al* 1998, Takahashi *et al* 1999] (see fig 1.8).

The role of Ras in the VEGFR-MAPK pathway remains to be elucidated. In VEGFR-2 transfected fibroblasts PKC has been implicated in VEGF-induced MAPK activation [Takahashi & Shibuya 1997]. Phosphorylation of the Ras GTPase-activating protein in endothelial cells after VEGF stimulation has been reported by various groups [Waltenberger *et al* 1994, Seetharam *et al* 1995, Guo *et al* 1995]. In addition to MAPK, the p38 pathway has been shown to be activated by VEGF [Rousseau *et al* 1997].

The PLC- γ -PKC pathway has been implicated in the mitogenic action of VEGF. VEGFR-1 interacts with the PLC- γ -SH domain in the yeast two-hybrid system [Igarashi *et al* 1998]. VEGF induces the phosphorylation and activation of PLC- γ , leading to the hydrolysis of phosphatidylinositol 4,5-biphosphate to diacylglycerol and inositol 1,4,5-triphosphate [D'Angelo *et al* 1995, Xia *et al* 1996]. Inositol 1,4,5-triphosphate is likely to be responsible for the increase in intracellular Ca^{2+} after VEGF stimulation and diacylglycerol activated certain PKC isoforms expressed in target cells [De Vries *et al* 1992].

STATs are latent cytoplasmic transcription factors. All three receptors were shown to be strong activators of STAT3 and STAT5, while STAT1 was not activated by any of the VEGFRs [Korpelainen *et al* 1999].

VEGFR-3 isoforms bind to and phosphorylated adapter protein Sch, while phosphorylation is stronger in cells expressing the longer isoform [Borg *et al* 1995, Fournier *et al* 1995].

The Shc PTB domain is required for ligand-induced Src tyrosine phosphorylation by VEGFR-3 [Fournier *et al* 1999]. Both VEGFR-3 isoforms bind to Grb2 via its SH2 domain in an inducible manner [Pajusola *et al* 1994, Fournier *et al* 1995].

The VEGF receptors have been shown to associate with cell surface adhesive proteins (see fig 1.7). It has been shown that activated VEGFR-2 was found in complex with integrin $\alpha_v\beta_3$, which is an adhesion molecule expressed on angiogenic endothelium [Soldi *et al* 1999]. $\alpha_v\beta_3$ binds to pericellular matrix proteins containing an arginine-glycine-aspartic acid (RDG) peptide motif, and has been shown to be involved in the cell cycle and survival of endothelial cells [Brooks *et al* 1994, Scatena *et al* 1998].

Another endothelium-specific cell-cell adhesion protein that has been implicated in the molecular interactions with the VEGF receptors is VE-cadherin. In normal endothelial cells VEGFR-2 co-localized with VE-cadherin [Carmeliet *et al* 1999], similarly VEGFR-3, but not VEGFR-1 is co-immunoprecipitated with VE-cadherin.

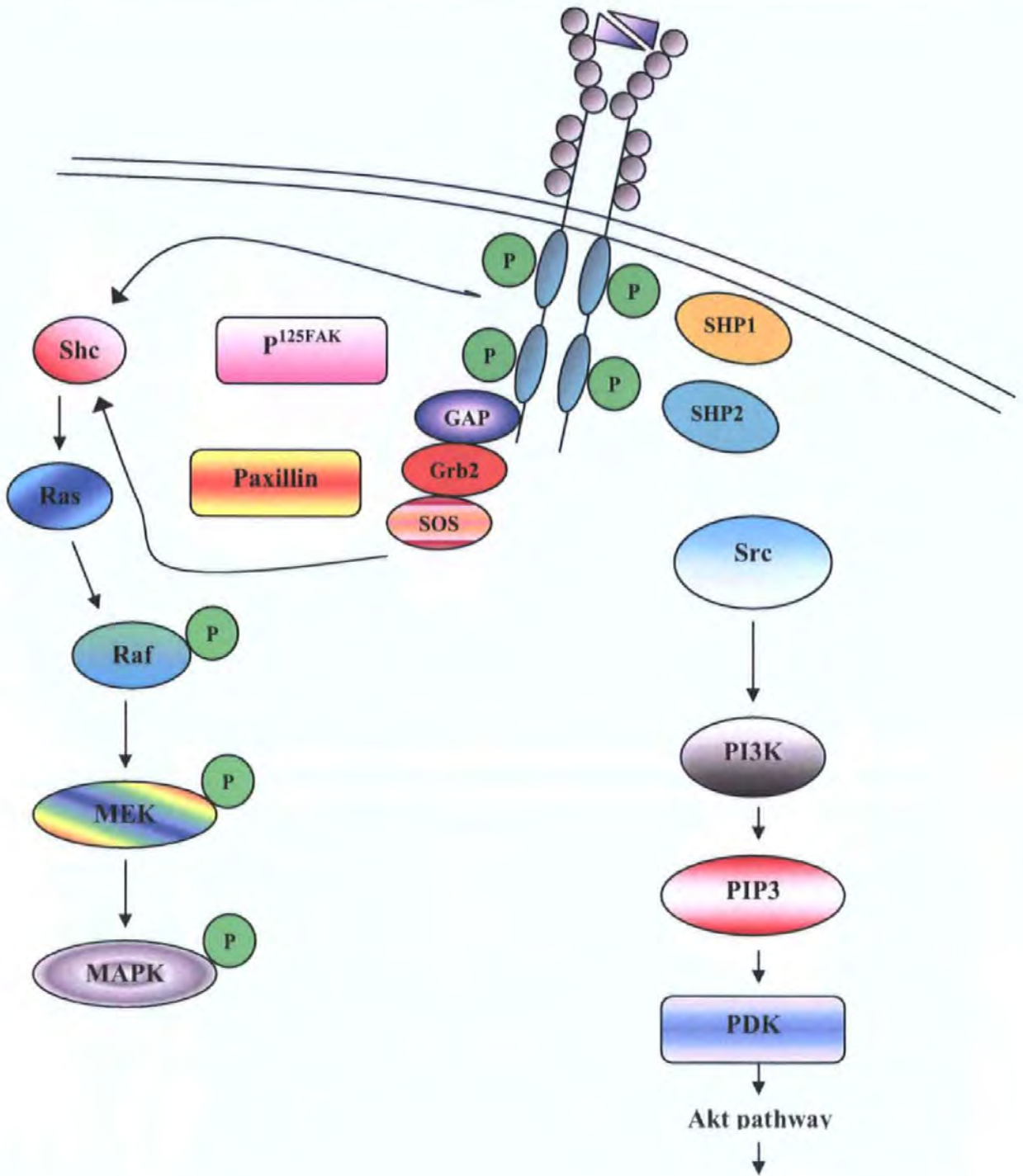


Fig 1.8 Signal transduction pathway for the VEGF receptor

This fig shows the signal transduction pathway for the VEGF receptor via the MAPK and Akt pathways. Phosphorylation occurs via the MAPK or Akt pathways and leads to signal transduction in the cell nucleus.

Newly formed tumour vessels are often 'leaky', VEGF has been shown to be one of the most potent inducers of vascular permeability [Dvorak *et al* 1995]. VEGF-C induces vascular permeability which is not transduced via its receptor VEGFR-3 [Joukov *et al* 1998]. PlGF is unable to induce permeability via its receptor (VEGFR-1) [Park *et al* 1994]. This implies that either VEGFR-2 or an unidentified receptor mediates the permeability response. To test this, the third variable domain in the VEGF polypeptide was substituted with the analogous region of PlGF, creating a chimeric molecule with reduced binding to VEGFR-2. This mutant still induced vascular permeability with an activity similar to that of wild type VEGF [Stacker *et al* 1999]. Most of the signal transduction studies have been carried out using endothelial cells that express more than one type of VEGFR.

Disruption of any of the three VEGFRs leads to embryonic death. The various VEGFRs are expressed in the mouse embryonic mesoderm between embryonic day (E) 7.5 and E9.5 [Dumont *et al* 1995]. No blood vessels are formed with embryos that have targeted null mutations of VEGFR-2 [Shalaby *et al* 1995]. When VEGFR-1 was homozygous for a targeted mutation an excess of cells in both embryonic and extra-embryonic locations were seen, but the endothelial cells fail to organize into normal vascular channels [Fong *et al* 1995]. When only the tyrosine kinase domain of VEGFR-1 was deleted, the mice developed normal vessel and survived [Hiratsuka *et al* 1998]. During embryogenesis VEGFR-1 acts as a VEGF sink, regulating the amount of free VEGF available for vascular development. Loss or disruption of VEGFR-3 led to cardiovascular failure after E9.5 [Dumont *et al* 1998], suggesting that VEGFR-3 is required for maturation of the primary vascular plexus into a hierarchy of large and small vessels. VEGFR-3 has a general blood vascular function during early development and only later becomes restricted to the lymphatic endothelium.

1.6 Hypoxia Inducible Factor (HIF)

Molecular oxygen (O_2) is required by mammals for essential metabolic processes, including oxidative phosphorylation, which O_2 serves as an electron acceptor during ATP formation. Low levels of O_2 is termed hypoxia and there are a number of adaptive responses to this including, erythropoiesis [Jelkman *et al* 1992], neovascularization [White *et al* 1992] and glycolysis in cells cultured at reduced O_2 tension [Wolfe *et al* 1993]. These adaptive responses either increase O_2 delivery or activate alternative metabolic pathways which do not require O_2 .

Erythropoietin (EPO) was the first gene investigated which mediated a response to hypoxia. Cis-acting DNA sequences were identified in the 3' flanking region [Semenza 1994].

HIF is a heterodimeric transcription factor, which consists of, α and β subunits, it was discovered in studies of hypoxia-inducible expression of the haematopoietic growth factor erythropoietin [Semenza & Wang 1992] (see fig 1.9). The HIF-1 β subunit is constitutively expressed, while the expression and transcriptional activity of HIF-1 α subunits is precisely regulated by the cellular O_2 concentration [Wang *et al* 1995]. The HIF system is key to the response of a variety of cell types to hypoxia [Semenza 2000]. The HIF-1 α sub-unit is a novel polypeptide of 826 amino acids [Wang & Semenza 1995]. HIF-1 α contains a basic helix-loop-helix (bHLH) domain, PAS domain, ID domain, oxygen dependent-degradation domain (ODD), N terminal transactivation domain (NTAD), and C terminal transactivation domain (CTAD). These different domains serve as the regulatory domains for HIF-1 α activation. The bHLH and PAS domains aid dimerization with HIF-1 β and DNA binding [Salceda *et al* 1997, Huang *et al* 1998, Maxwell *et al* 1999, Berra *et al* 2001].

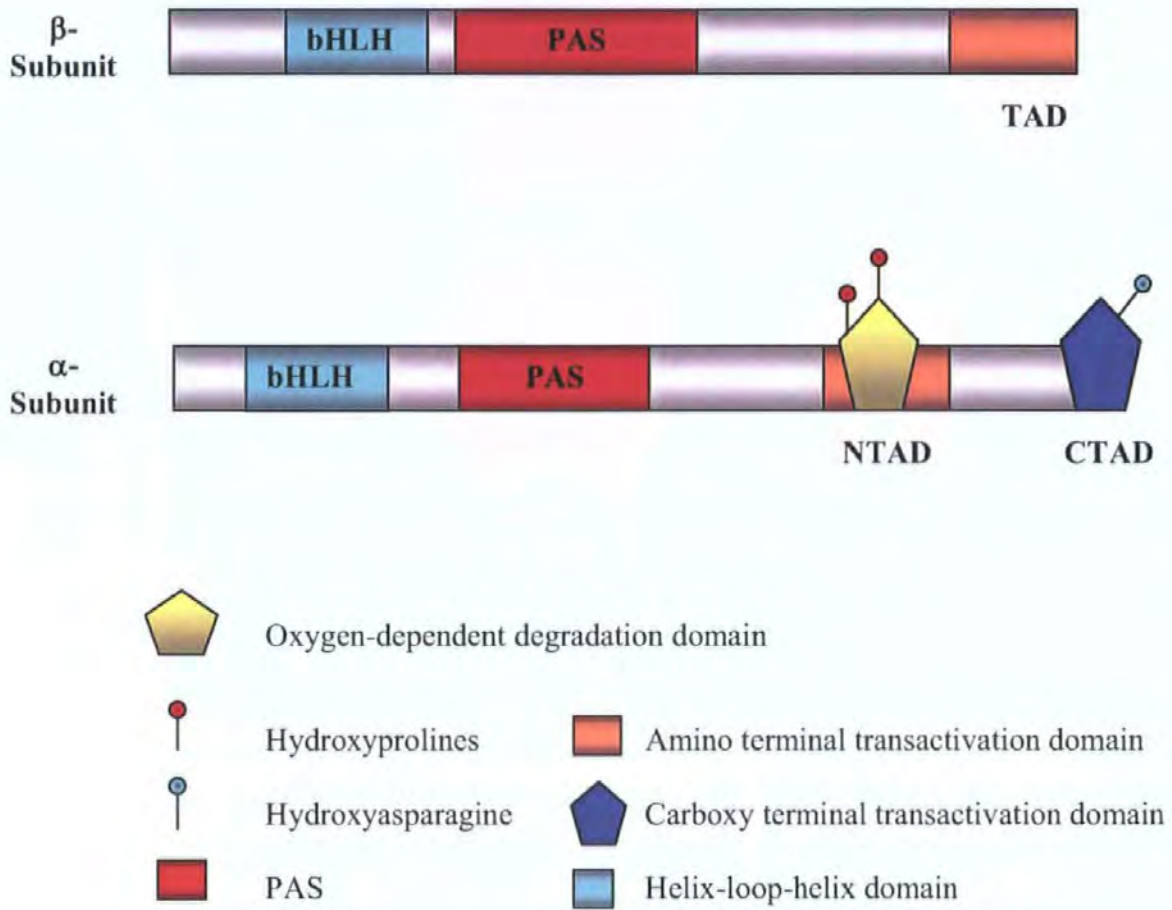


Figure 1.9 Structure of the HIF-1.

The figure shows the oxygen-regulated α subunit and the constitutively expressed β subunit, proline and asparagine hydroxylation sites, the amino-terminal transactivation domain and carboxy-terminal transactivation domain along with the basic-helix-loop-helix and Per-Arnt-Sim domains.

1.6.1 Hypoxic response of HIF

Local hypoxia is often due to a vascular aetiology and is a component of ischaemic processes whereby cellular O₂ delivery, energy substrate delivery and metabolite removal are all affected. Hypoxia plays a significant role in the pathophysiology of many diseases including renal disease. It is also a major pathogenic factor in other vascular related disease processes, including retinal and tumour neovascularization.

Regardless of aetiology, hypoxia ultimately has its effects at the cellular level by depriving cells of adequate O₂ to meet their metabolic demands [Semenza, 1998]. Individual cells sense hypoxia and respond by altering the expression of specific genes, that are involved in the maintenance of O₂ homeostasis under normoxic conditions. Homeostatic responses to hypoxia can be classified as systemic, local or cellular in nature. Cellular responses include the transition from oxidative phosphorylation to glycolysis as the pathway for ATP generation. HIF-1 α is a transcriptional activator of genes encoding proteins that play major roles in cellular, local and systemic homeostatic responses to hypoxia [Ratcliffe *et al*, 1998]. Hypoxia can be systemic or local in its aetiology [Semenza, 1996]. A number of causes of systemic hypoxia include decreased ambient O₂ concentration (high altitude), decreased O₂ exchange, decreased blood O₂ carrying capacity, decreased cardiac output (cardiomyopathy), right-to-left shunt, and decreased O₂ unloading in peripheral tissues [Semenza 1996].

1.6.2 Transcriptional activation of HIF

HIF-1 α recognizes the hypoxia response element (HRE) (5' (G/C/T)-ACGTGC-(G/T) 3') which is present in the enhancers of several genes and leads to their expression [Semenza *et al* 1996]. Erythropoietin (EPO), VEGF, and various glycolytic enzymes have HRE sequences in their promoters/enhancers. The transcriptional activation of HIF is linked to its ability to recruit various coactivator proteins including, p300, CREB-binding protein (CBP), steroid receptor coactivator-1, and translation initiation factor 2 [Arany *et al* 1996, Ema *et al* 1999, Carrero *et al* 2000]. The amino acid composition of the helix-loop-helix (HLH) and basic domain determines the transcription factors specificity toward a particular DNA sequence [Semenza *et al* 1996]. NMR and X-ray crystallographic structures have shown that the HLH domain dimerizes to form a parallel four-bundle left handed helix, with the contacts occurring within a hydrophobic core region [Michel *et al* 2000].

Genes	Reference
Growth factor related	
Endocrine gland-derived VEGF	LeCouter <i>et al</i> 2001
Erythropoietin	Jiang <i>et al</i> 1996a
IGF-II	Feldser <i>et al</i> 1999
TGFB ₃	Canniggia <i>et al</i> 2000
VEGF	Carmeliet <i>et al</i> 1998; Iyer <i>et al</i> 1998a
Receptors	
FLT-1	Gerber <i>et al</i> 1997
α 1B-adrenergic receptor	Eckhart <i>et al</i> 1997
Transferrin receptor	Tacchini <i>et al</i> 1999
Glucose metabolism	
Aldolase A	Iyer <i>et al</i> 1998a; Ryan <i>et al</i> 1998
Aldolase C	Iyer <i>et al</i> 1998a; Ryan <i>et al</i> 1998
Enolase	Iyer <i>et al</i> 1998a
Glucose transporter 1	Ryan <i>et al</i> 1998; Wood <i>et al</i> 1998
Glucose transporter 3	Iyer <i>et al</i> 1998a
Hexokinase 1	Iyer <i>et al</i> 1998a
Phosphoglycerate kinase 1	Carmeliet <i>et al</i> 1998; Iyer <i>et al</i> 1998a
Lactate dehydrogenase A	Iyer <i>et al</i> 1998a; Ryan <i>et al</i> 1998
Genes related to vascular growth and development	
Adenylate kinase 3	Wood <i>et al</i> 1998
Endothelin-1	Hu <i>et al</i> 1998
Heme oxygenase-1	Lee <i>et al</i> 1997
LDL receptor-related protein	Wykoff <i>et al</i> 2000b
Plasminogen activator inhibitor 1 (PAI-1)	Kietzmann <i>et al</i> 1999
Prolyl-4-hydroxylase α 1	Takahashi <i>et al</i> 2000
Transferrin	Rolfs <i>et al</i> 1997

Table 1.11 Genes regulated by HIF-1

This table shows a number of the different types of genes that under the regulatory control of the HIF-1 gene. Adapted from Choi *et al* 2003.

Immunoblot analysis of HIF-1 α and HIF-1 β protein expression in Hep3B cells revealed low levels of HIF-1 proteins in cells at 20% O₂ and a marked increase in cells at 1% O₂ with peak levels at 4-8h of continuous hypoxia [Wang *et al* 1995]. The major intracellular process that consumes O₂ is oxidative phosphorylation, which is the primary metabolic pathway for ATP generation in most cell types. When O₂ is limited, expression of genes encoding components of the electron transport chain is down regulated, whereas transcription of genes encoding glucose transporter 1 and enzymes of the glycolytic pathway are activated [Stein *et al* 1995, Webster *et al* 1990]. This metabolic adaptation allows cells to continue to synthesise ATP by an O₂ independent pathway. HIF-1 appears to play a major role in the co-ordinate transcriptional activation of genes encoding glycolytic enzymes [Semenza *et al* 1994, Minchenko *et al* 2002]. The induction of multiple glycolytic enzymes in response to hypoxia appears inconsistent with long-held theories of rate limiting enzymatics [Kashiwaya *et al* 1994].

A growing body of experimental evidence supports the hypothesis that HIF-1 co-ordinates systemic, local, and cellular homeostatic responses to hypoxia through the transcriptional activation of specific genes, many of which remain to be identified. To date at least 100-300 genes have been found with hypoxia response elements (HREs) in their promoter regions, ranging from genes involved in glucose metabolism [Iyer *et al* 1998a] to angiogenic molecules [Carmeliet *et al* 1998] and genes involved in vascular development [Kietzmann *et al* 1999] see table 1.11.

1.6.3 Prolyl hydroxylation of HIF-1 α and subsequent breakdown

The regulation of HIF-1 α activity has come under increasing scrutiny over the last few years (see fig 1.10). Two proline residues (P402 and P564) in HIF-1 α are constitutively hydroxylated under normoxic conditions [Ivan *et al* 2001, Jaakkola *et al* 2001].

Hydroxylation of these proline residues allows binding and subsequent degradation by pVHL [Maxwell *et al* 1999,]. Other residues have also been found to be critical for pVHL-mediated degradation (tyrosine 564 and Ile 565 in mice) [Pereira *et al* 2003]. This site-specific hydroxylation of proline residues under normoxic conditions takes place by a family of enzymes called prolyl hydroxylases (PHD1, PHD2, and PHD3), which catalyse proline hydroxylation by using Fe²⁺ and ascorbate as co factors [Epstein *et al* 2001, Bruick & McKnight 2001]. Prolyl hydroxylases use molecular oxygen and 2-oxoglutarate as substrates in addition to the hydroxyl acceptor proline residue. This occurs at specific prolines within a conserved motif of LXXLAP. [Kivirikko & Pihlajaniemi 1998].

Treatment of cardiac myocytes, smooth muscle cells and endothelial cells with hypoxia or a hypoxia mimetic (i.e. CoCl₂) results in a significant time dependent increase in PHD3, but not PHD1 or 2 mRNA levels, which correlated with an increase in HIF-1 α protein expression [Cioffi *et al* 2003]. The PHD-pathway may play a key role in phenotypic changes that are observed in a hypoxic myocyte, and may suggest a strategy to pharmacologically induce protection in the heart [Wright *et al* 2003]. Localisation of the three PHD enzymes by confocal fluorescence microscopy showed that PHD1 was exclusively present in the nucleus, PHD2 and FIH-1 mainly were mainly located in the cytoplasm, and PHD3 was distributed in the cytoplasm and nucleus [Metzer *et al* 2003].

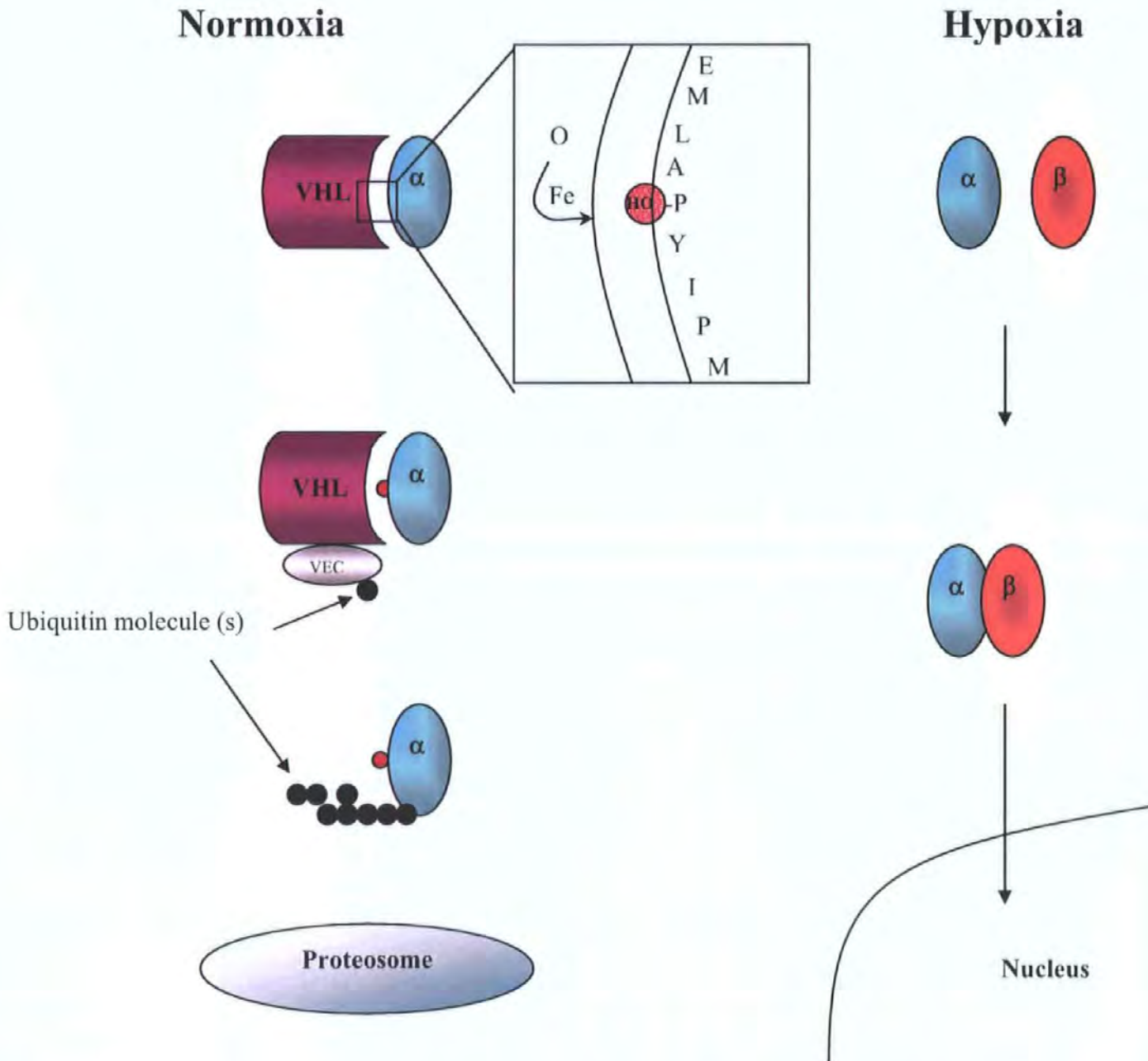


Figure 1.10 oxygen-dependent regulation of the HIF-1 α transcription factor

This fig shows the addition of an OH moiety to the proline 564 that aids binding to the VHL protein and leads to the subsequent degradation of the HIF-1 α subunit, via addition of ubiquitin molecules.

1.6.4 Factor inhibiting HIF-1 (FIH-1)

Fe^{2+} -dependent enzymes that use molecular oxygen to oxidize both 2-oxoglutarate and either polypeptide or metabolic substrates are found throughout nature. Several members of this family have been identified, revealing a conserved double stranded β -helix composing the enzyme core [Roach *et al* 1995, Clifton *et al* 2001]. A critical His-X-Asp/Glu dyad and C-terminal His residue lies within this fold and is responsible for binding the iron atom [Hegg & Que 1997].

HIF transcriptional activity is suppressed under normoxic conditions by hydroxylation of an asparagine residue within its C-terminal transactivation domain, blocking association with p300/CBP [Mahon *et al* 2001] (see fig 1.11).

The protein factor inhibiting HIF-1 (FIH-1), which has previously been shown to interact with HIF, is an asparaginyl hydroxylase, and like other hydroxylase enzymes FIH-1 is a Fe^{2+} -dependent enzyme that uses molecular oxygen to modify its substrate [Dann *et al* 2002].

An inhibitory protein that interacted with residues 757-826 at the C-terminus of HIF-1 α , encompassing part of the inhibitory domain and C-TAD [Mahon *et al* 2001]. Homodimer formation is required for substrate recognition [Dann *et al* 2002]

Asparagine-803 in the C-TAD is hydroxylated by FIH-1 under normoxic conditions causing abrogation of the HIF-1 α /p300 interaction [McNeil *et al* 2002].

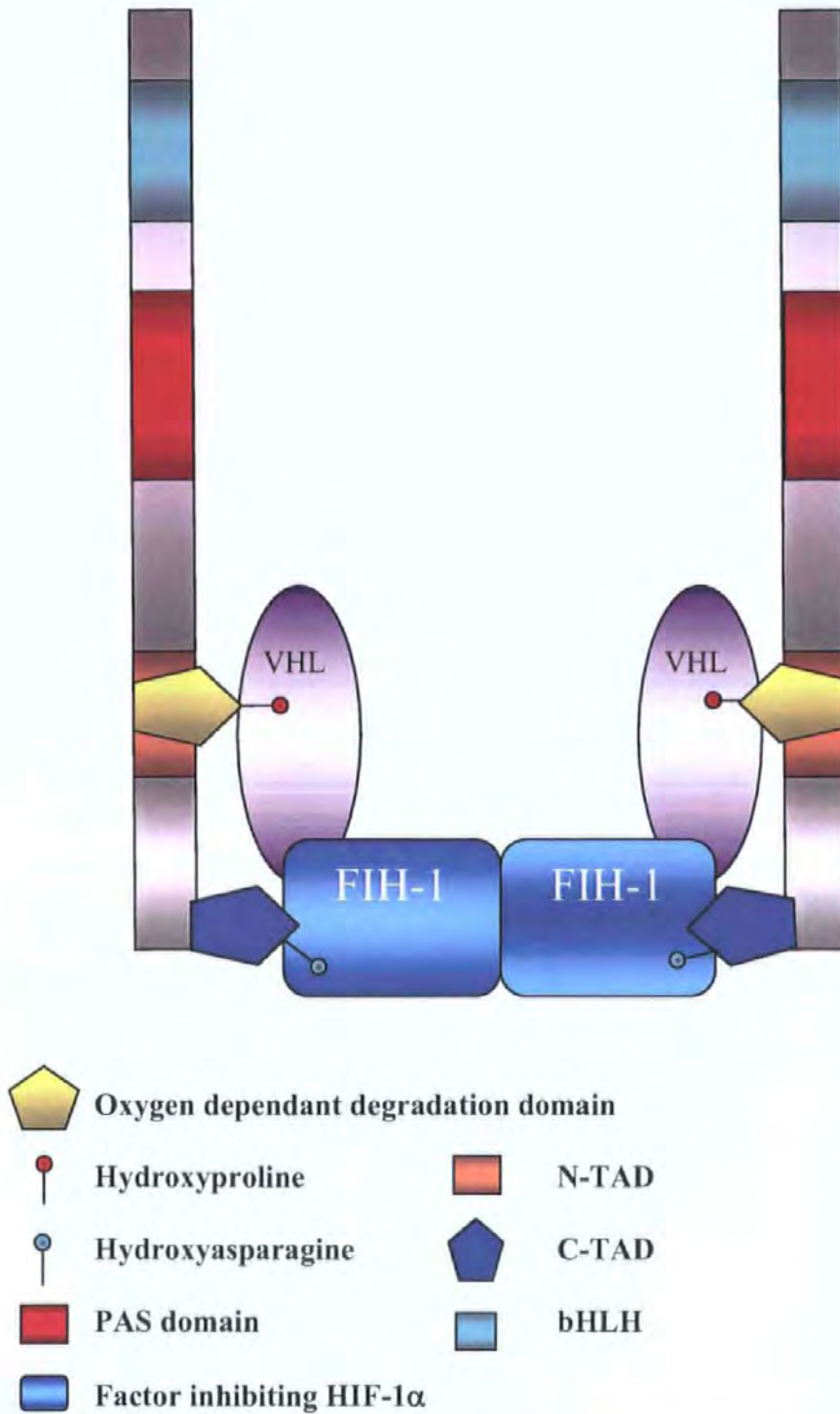


Fig 1.11 Hypothetical model of the complex formation by the FIH-1 dimer, VHL and HIF-1 α .

This model represents the FIH-1 dimer acting as a bridge for the association between VHL and HIF-1 α under normoxic conditions.

To achieve full induction of the hypoxic response pathway after exposure to low O₂ levels requires both stabilisation of the HIF- α transcription factor and activation of the N-TAD [Lando *et al* 2002]. Each of these events depends on the abrogation of a hydroxylase enzyme that negatively regulates HIF under normoxic conditions. Although the two modes of regulation can be uncoupled via construction of chimeras containing either the ODD or C-TAD, the machinery regulating both appears to be linked [Mahon *et al* 2001]. Under normoxic conditions HIF- α C-TAD association with the CH1 domain of the co activator p300 is blocked by hydroxylation of a conserved asparagine residue [Lando *et al* 2002]. Asparagine-803 in the C-TAD of HIF-1 α is hydroxylated under normoxic conditions causing abrogation of the HIF-1 α / p300 association [McNeil *et al* 2002]. Hydroxylases that catalyse the post-translational modification of aspartic acid / asparagine residues have been previously described [Stenflo *et al* 1989, Lavaisiere *et al* 1996].

The crystal structure of FIH-1 at 2.8 Å resolution shows that it is composed of a central β -barrel surrounded by eight α -helices. The prominent feature in the FIH-1 structure is a distinctive groove that extends from the active site towards the interconnecting loop between domains I and II. The FIH-1 structure suggests a mechanism for the physiological regulation of HIF-1 activity by revealing three-dimensional locations of regions in FIH-1 that are implicated in the interactions with HIF-1 α C-TAD and VHL. The distinctive binding sites for HIF-1 α C-TAD, and VHL on the surface of FIH-1 support the possibility that the ternary complex and cooperation of the three proteins may be required in the hydroxylation of HIF- α by FIH-1 [Lee *et al* 2003].

1.6.5 HIF-2 α

Isolation and characterization of a cDNA for a novel Per-Arnt/Ahr-Sim basic-helix-loop-helix (bHLH-PAS) factor that interacts with the Aryl-receptor nuclear translocator (Arnt), and its predicted amino acid sequence was shown to exhibit significant similarity to the hypoxia-inducible factor-1 α . This HIF-1 α like factor (HLF) encoded by the isolated cDNA bound the hypoxia-response element (HRE) found in enhancers of genes for erythropoietin, VEGF, and various glycolytic enzymes, and activated the transcription of a reporter gene the HRE. Although the transcription-activating properties of HLF were similar to those reported for HIF-1 α their expression patterns were quite different. HLF mRNA was most abundantly expressed in the lung, followed by the heart, liver and various organs under normoxic conditions, whereas HIF-1 α mRNA was ubiquitously expressed at much lower levels. HLF mRNA was closely correlated with that of VEGF mRNA [Ema *et al* 1997]. Cloning and chromosomal localization for HIF-2 α led to chromosome 11p15 [Wolting & McGlade 1998]. HIF-2 α was shown to be strongly expressed, and strongly induced by hypoxia at the level of protein but not mRNA. Although differences between the abundance of HIF-2 α and HIF-1 α were seen in different cell types, the differences in the inducible response was more subtle with HIF-2 α protein being slightly more evident in normoxic and mildly hypoxic cells [Wiesener *et al* 1998]. A comparison of the oxygen-regulated and transactivating domains in HIF-1 α and HIF-2 α produced two domains in HIF-2 α similar to HIF-1 α , a C-terminal domain (amino acids 828-870) and a larger internal domain (amino acids 517-682) demonstrating a similar organization for both proteins. HIF-2 α sequences were less inducible than those of HIF-1 α , and was strikingly reduced as their expression level increased [O'Rourke *et al* 1999]. HIF-2 α may play an important role in vascular remodelling, as mutant embryos that were derived from HIF-2 α ^{-/-} embryonic stem cells developed severe vascular defects both in the yolk sac and embryo

proper and died between embryonic day (E) 9.5. and E12.5. Normal blood vessels were found by vasculogenesis but they either fused improperly or failed to assemble into larger vessels later during development [Peng *et al* 2000]. Using immunohistochemistry the expression and distribution of HIF-1 α and HIF-2 α was seen in the nucleus of a number of solid tumours including, bladder, brain, breast, colon, ovarian, pancreatic, prostate, and renal carcinoma [Xia *et al* 2001, Leek *et al* 2002, Onita *et al* 2002, Khatua *et al* 2003].

HIF-2 α was also strongly expressed by subsets of tumour-associated macrophages, sometimes in the absence of any expression in tumour cells [Talks *et al* 2000].

In the presence of functional VHL protein, HIF-1 α mRNA levels are elevated, whereas HIF-2 α mRNA expression is increased only in VHL deficient RCC cell lines lacking a functional VHL gene product. On a protein level, in VHL deficient cell lines both HIF-1 α and HIF-2 α subunits are constitutively expressed, whereas re-introduction of a functional VHL gene restores the instability of HIF-1 α and HIF-2 α proteins under normoxic conditions to direct the HIF- α subunits for destruction in the proteasome [Krieg *et al* 2000]. Using human breast cancer cell lines it has been shown that there was an inverse correlation between HIF-1 α and HIF-2 α induction under hypoxia. Cell line with reduced induction of HIF-1 α or HIF-2 α showed high basal levels of VEGF and improved survival under hypoxia. Despite the fact that HIF proteins are necessary for optimal tumour growth and angiogenesis *in vivo*, over expression of these molecules seem detrimental to tumour growth [Blancher *et al* 2000].

Complete cDNA of HIF-2 α was cloned and transfected to cells from the 293 Tet-Off fetal kidney cell line and expression pattern of VEGF and transferrin receptor (TfR), target genes of HIF-1 α examined by reverse transcriptase polymerase chain reaction (RT-PCR). Endogenous VEGF was increased by the introduction of HIF-2 α cDNA at both the mRNA and protein level. This suggests that endogenous VEGF can be upregulated by HIF-2 α that may be involved in the angiogenesis of RCC [Xia *et al* 2001].

Increased expression of HIF-1 α and HIF-2 α mRNA has also been shown in primary human bladder tumours compared with normal bladder specimens [Jones *et al* 2001].

Tumour associated macrophages (TAMs) preferentially migrate to hypoxic areas within tumours and strongly express HIF-2 α . By correlating HIF-2 α with microvessel density (MVD) as a marker of angiogenesis it suggested that TAM HIF-2 α signalling may be a useful target for future anti-angiogenic strategies [Leek *et al* 2002].

Under hypoxic conditions heart, lung, brain, kidney, liver, pancreas, and intestine of rats showed marked induction of HIF-2 α . Immunohistochemistry revealed nuclear accumulation indicating the HIF-2 α plays an important role in the transcriptional response to hypoxia *in vivo* [Wiesener *et al* 2003].

1.6.6 HIF-3 α

The HIF-3 α transcription factor is a 662 amino acid protein with a predicted molecular weight of 73kDa, and was expressed in adult thymus, lung, brain, heart, and kidney. The N-terminal bHLH-PAS domain of this protein shares amino acid sequence identity with that of HIF-1 α and HIF-2 α (57% and 53% identity respectively, see fig 1.12). It was shown that HIF-3 α dimerizes with ARNT, and this resultant heterodimer recognized the HRE core sequence, TACGTG. Transient transfection experiments showed that the HIF-3 α -ARNT dimer can occur *in vivo*, and that the activity of HIF-3 α is up regulated in response to cobalt chloride and low oxygen tension [Gu *et al* 1998]. Sequence analysis revealed that, like mouse HIF-3 α , human HIF-3 α has high similarity with HIF-1 α and HIF-2 α in the bHLH and PAS domains, but lack structures for transactivation found in the C-terminus of HIF-1 α and 2 α . Reporter gene analysis showed that HIF-3 α suppresses hypoxia-inducible HIF-mediated gene expression, and HIF-3 α might be a negative regulator of hypoxia-inducible gene expression in the human kidney [Hara *et al* 2001].

The inhibitory PAS (Per/Arnt/Sim) domain protein, IPAS, functions as a dominant negative regulator of hypoxia-inducible factors by forming complexes with those proteins that fail to bind to hypoxia response elements of target genes. Sequencing of the mouse IPAS genomic structure revealed that IPAS is a spliced variant of the HIF-3 α locus. In addition to three unique exons (1a, 4a, and 6) IPAS shares three exons (2, 4, and 5) with HIF-3 α as well as alternatively spliced variants of exons 3 and 6. Using mice exposed to normoxia or hypoxia (6% O₂ for 6 hrs) the alternatively spliced HIF-3 α transcript was observed in the heart and lung. The alternatively spliced transcript was only observed under hypoxic conditions, this mechanism may establish negative feedback loop regulation of adapted responses to hypoxia/ischemia in tissues [Makino *et al* 2002].

Recently multiple splice variants of the HIF-3 α locus have been reported, HIF-3 α 1-6 (with the HIF-3 α 2 also known as IPAS domain protein). The common oxygen-dependent degradation domain of HIF-3 α 1-3 splice variants is targeted for ubiquitination by pVHL complex in vitro and in vivo. This activity is enhanced in the presence of prolyl hydroxylase and is dependent on a proline residue at position 490. It was also shown that ubiquitin conjugation occurs on lysine residues at position 465 and 568 within the ODD, demonstrating additional targets of the pVHL complex and suggests a growing complexity in the regulation of hypoxia-inducible genes by the HIF family of transcription factors [Maynard *et al* 2003]. In contrast to HIF-1 α , 1 β , and 2 α levels of HIF-3 α mRNA increased after 2 hours of hypoxia, suggesting that induction of HIF-3 α at the transcriptional level may represent a rapidly reacting component of the HIF system to protect against hypoxic damage [Heidbreder *et al* 2003].

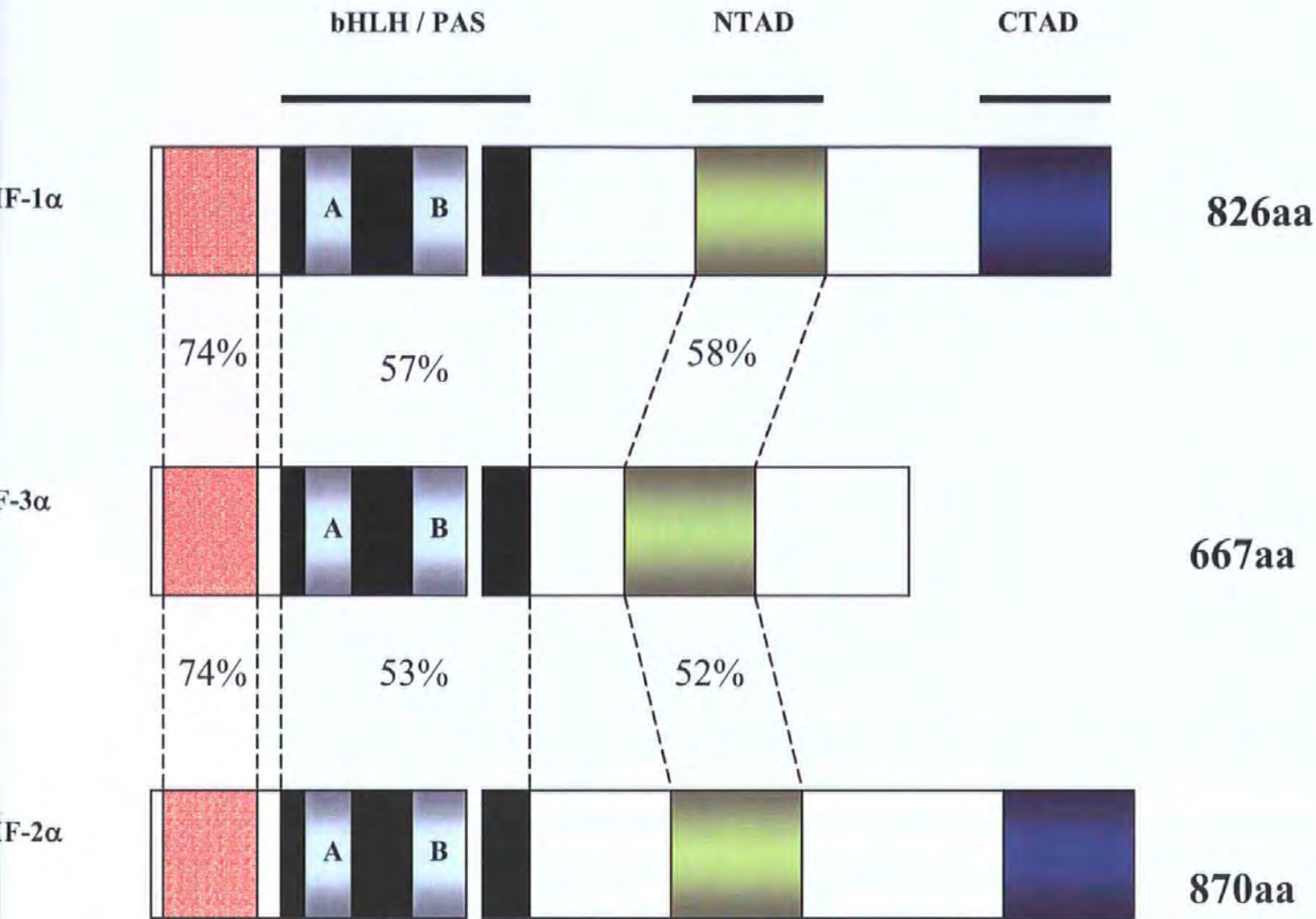


Fig 1.12 Structural similarities between the three HIF- α species

There is structural similarity between the various domains of the HIF- α subunit, with homology between the PAS, bHLH, NTAD and CTAD domains of the protein.

1.6.7 Antisense HIF

A novel species has been identified, termed antisense hypoxia inducible factor (aHIF) which is a natural antisense transcript from the HIF-1 α gene sequences encoding the 3' UTR of HIF-1 α mRNA [Trash-Bingham & Tartof, 1999]. It is thought to bind to the HIF mRNA and provide a link between HIF and VHL [Neckers, 1999]. aHIF is widely expressed in normal foetal and adult tissues as in tumour tissues. Foetal aHIF expression is higher than adult expression and high enough to effect the HIF-1 α mRNA/aHIF transcript ratio. It is thought that aHIF could expose AU rich elements present in the 3' UTR of HIF-1 α and increase the degradation speed of HIF-1 α mRNA. It has also been shown that the aHIF promoter contains several putative HREs, which may explain the overexpression of aHIF under hypoxic conditions, creating a loop of regulation for HIF-1 α expression [Rossignol *et al* 2002].

1.7 Hypothesis

The highly vascularized nature of renal cell carcinoma makes it an ideal disease to study genes that are involved in the angiogenesis of tumours. There are a number of genes involved in the regulation of renal cell carcinoma, and the angiogenesis of the tumour.

Polymorphic variants of hypoxia-inducible factor-1 alpha (HIF-1 α) that change the coding region of the gene have been shown to alter transactivation activity, which in conjunction with polymorphisms in the glucose transporter-1 (GLUT-1) promoter may effect the progression of renal cell carcinoma (RCC). We have shown, for the first time, that these polymorphisms are associated with RCC [Ollerenshaw *et al* 2004].

The expression of genes related to tissue hypoxia (HIF-1 α , GLUT-1, and VEGF) will be analysed using a ribonuclease protection assay (RPA). RPA analysis was chosen as a method to measure the levels of mRNA in the samples because of the advantages over other assay methods. The assay is performed in a liquid interface within a single tube reducing user error, it is able to detect partially degraded mRNA and was a technique which had been optimised in our laboratory.

1. Samples for the study will be obtained from a variety of sources; tumour samples will be taken prospectively from all nephrectomy patients. DNA will also be obtained retrospectively from paraffin embedded sections to elucidate any role polymorphisms in either the VEGF or HIF-1 α genes play in the progression or severity of RCC.
2. The expression of the different isoforms of VEGF has been poorly studied to date in RCC, and may provide important clues as to the severity of the disease. Are the levels of mRNA elevated in the tumour verses normal tissue of patients with RCC? Expression of the VEGF 165 and 189 isoforms of the VEGF gene will be quantitatively analysed using a ribonuclease protection assay (RPA). Particular isoforms have been associated with cancer including VEGF₁₈₉, and VEGF₁₆₅ (the

predominately expressed isoform secreted by normal and transformed cells). Will the levels of these isoforms relate to the tumour stage?

3. Recent identification of the polymorphic nature of the VEGF gene has led to a number of studies of diseases in relation to these polymorphisms. Polymorphisms have been described in the promoter region of the VEGF gene, which I intend to analyse in conjunction with the expression of the mRNA to look for any correlations. DNA from paraffin embedded RCC tissue will be used for subsequent PCR analysis of VEGF promoter polymorphisms. The C-2578A (containing an 18 nucleotide insertion at – 2550 which is in complete linkage with the A allele) will be evaluated for any possible role in RCC. Will it be possible to attribute in part these polymorphisms to the outcome or severity of RCC or the expression levels of the VEGF mRNA in the tumour and non-neoplastic tissue in patients with RCC? Using the Robson stage and Fuhrman grade will it be possible to determine any links between these polymorphisms and a particular stage or grade
4. Recently identified polymorphisms in the HIF-1 α gene (C1772T and G1790A) have been linked to the transactivation of the protein. These polymorphisms in the HIF-1 α gene will be studied to identify any differences between the control and patient populations. Will any of the alleles be linked to patients with RCC, and will there be a segregation of the alleles according to the severity of disease?

These experiments will hopefully lead to an increased knowledge of the role of hypoxia inducible genes in RCC, and angiogenesis of the tumour. Can polymorphisms in the VEGF gene lead to differences in the expression levels of mRNA produced by the gene? If there are differences in expression levels of mRNA can they be linked to severity of disease?

Initially using the whole population of RCC patients and then using the CC RCC subgroup to see if there are any correlations within this group and the polymorphisms studied.

Subgroups of RCC will be measured for all the assays, and then divided into just CC RCC to see if any significant differences can be found.

2.0 Materials

2.1 General reagents and stock solutions

2.1.1 Water

Double distilled tap water was used to make up all stock, general purpose and specialist solutions. Sterile water (Parkfields Pharmaceuticals Ltd, UK) was used to make oligonucleotide probe and primer solutions and used for PCR.

2.1.2 Diethyl pyrocarbonate (DEPC) treated water

1ml of diethyl pyrocarbonate (DEPC)(Sigma, UK) was added to 1L of double distilled tap water, and allowed to dissolve overnight, and was subsequently used for all RNA work.

2.2 Proprietary solutions, and reagents

All reagents used were analytical grade or equivalent.

2.2.1 Stock solutions

- i. Tris/borate electrophoresis buffer (TBE)
10X solution: 0.89mM Tris base, 0.89M Boric acid, 2mM EDTA (pH8)
- ii. Ethidium bromide
10mg ml⁻¹ in H₂O
- iii. Orange G
- iv. Sodium Chloride
- v. Tris

2.3 Autoclaving

All solutions, glassware and plastic ware used in the techniques of DNA/RNA analysis were autoclaved at a temperature of 121°C and a pressure of 15 p.s.i for 30 minutes in a steam autoclave (Prior Clave, UK).

2.4 Patients

Patients for this study were selected from those undergoing surgery for radical nephrectomy for suspected RCC. Histological analysis of the tumour was carried out in the histopathology department at Derriford hospital, Plymouth by a consultant histopathologist. Tumours were graded using the Fuhrman grade and staged by Robson stage, which is based on the Tumour Node Metastasis (TNM) staging of the tumour. An experienced consultant pathologist in Derriford hospital removed tumour and normal tissue from the RCC sample, taking care to remove sections of tumour from non-necrotic looking areas of the tumour mass (approximate size of these sections was 1 x 1 x 2-cm). Patient notes were reviewed to obtain the TNM staging and any other information regarding the patient was extracted from the notes.

2.5 Ethics

Ethics for the project were obtained for the removal and analysis of patients DNA, RNA, and protein from the tumour and normal tissue. This was carried out in accordance to the local regional ethics committee (LREC) approved protocol. The project and any relevant background information was explained to each patient, by an SHO or registrar in urology before informed consent could be given.

2.6 Control population

Controls for the genetic polymorphism study were obtained from sequential cord bloods taken from deliveries at the Obstetrics and Gynaecology department, Derriford hospital, Plymouth. DNA was then prepared using standard salt extraction protocols, with only the sex of the sample being known.

Intro to Methods

Methodological approaches used include polymerase chain reaction, ribonuclease protection assay, DNA extraction, RNA extraction.

These methods were used to quantify the levels of mRNA and frequency of polymorphisms in the genes studied. RPA was chosen as a method to study the expression of mRNA because of: ease of use, experience in this method in our laboratory, ability to detect partially degraded mRNA, and it is a single tube experiment eliminating user error through transfer of the material.

2.7 Polymerase chain reaction (PCR).

Polymerase chain reaction or PCR has become a highly used assay in the toolbox of all molecular biologists. PCR is a very powerful tool that can be used in a wide variety of assays. Primarily for the purposes of this thesis it was used to amplify stretches of subject DNA. The principles behind PCR are as follows: Short complementary oligonucleotides are used to bind to the DNA that serves as a binding site for a thermostable DNA polymerase. Amplification of genomic DNA using oligonucleotides that bind to complementary regions of the DNA in the 5' to 3' direction thus allowing elongation of the DNA by thermostable DNA dependent DNA polymerases, i.e *Taq* polymerase from the bacterium *Thermus Aquaticus*.

The DNA is denatured at 94-95°C for 30 seconds, the oligonucleotide primers annealed at temperatures ranging from 50-65°C for 1.30 to 2 minutes, and then the DNA extended at

72°C for 1 to 2 minutes. This cycle of denaturation, annealing, and extension is then repeated for 30-35 cycles. Conditions for each PCR are optimised according to the primers T_m and $MgCl_2$ concentration required. Primers can either be designed by hand/eye or a computer package such as “DNA Star”. A schematic representation of PCR can be seen in fig 2.1. A wide variety of thermostable DNA dependent DNA polymerases have become available, *Taq* polymerase (originating from the bacterium *Thermus Aquaticus*) has been used in the PCR assays here.

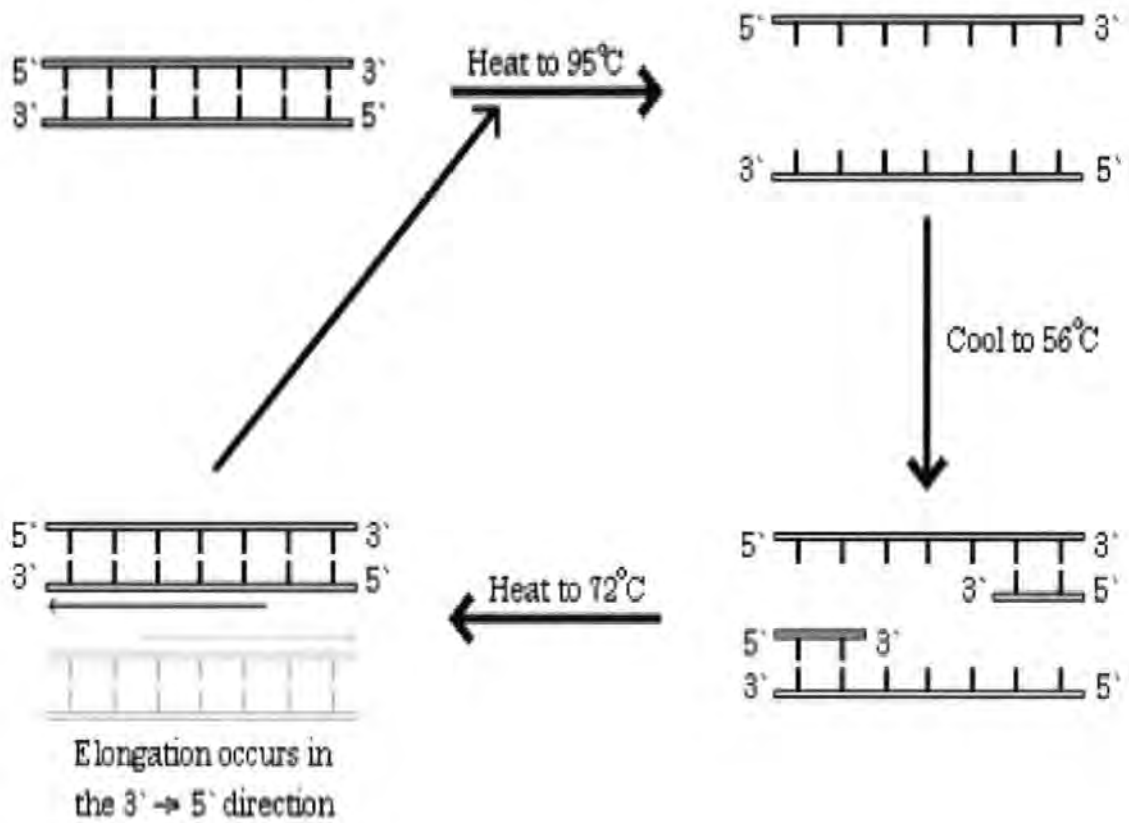


Fig 2.1 Polymerase chain reaction

This fig shows a cycle of the polymerase chain reaction, consisting of a denaturation step followed by annealing of the primers and finally extension of the DNA

2.7.1 PCR for the polymorphisms in the VEGF gene

2.7.1.1 PCR for the C to A @-2578 in the VEGF gene promoter

Primers were designed to produce a fragment which would be 212 bp long for the C allele, and 230 bp long for the A allele (which contains the 18 nucleotide insertion).

The conditions for the PCR reaction were: Denaturation at 95°C for 4 mins, followed by 30 cycles of denaturation at 95°C, anneal at 60°C for 2 mins, and extension at 72°C for 2 mins, followed by a final extension of 72°C for 4 mins.

VEGF sense primer for C to A @ -2578

5'- TTC CCT CTT TAG CCA GAG CCG GGG -3'

VEGF antisense primer for C to A @ -2578

5' CTG AGA GCC GTT CCC TCT TTG CTA -3'

The PCR products were separated by agarose gel electrophoresis (150V) using a 3-4% agarose gel

2.7.2 Polymorphisms in the hypoxia-inducible factor-1 α

2.7.2.1 PCR for the C to T @ nucleotide 1772 in codon 582 in the hypoxia inducible factor 1 α exon 12.

The C(1772)T nucleotide substitution creates a change from a proline to serine at codon 582. Primers were designed to produce a fragment which would be 346 bp long for the T allele, and 229, and 118 bp long for the C allele. The substitution from a C to a T abolishes a Hph I cut site.

The conditions for the PCR reaction were: Denaturation at 95°C for 4 mins, followed by 30 cycles of denaturation at 95°C for 30 secs, anneal at 57°C for 2 mins, and extension at 72°C for 2 mins, followed by a final extension of 72°C for 10 mins. Samples were digested overnight using 2U of Hph I at 37°C.

The forward primer is 5'- AAG GTG TGG CCA TTGTAA AA ACTC – 3'

The reverse primer is 5'- GCA CTA GTA GTT TCT TTA TGT ATG – 3'

2.7.2.2 PCR for the G to A @ nucleotide 1790 in codon 588 in the hypoxia inducible factor 1 α exon 12.

The G(1790)A nucleotide substitution creates a change from a alanine to threonine at codon 588.

The conditions for the PCR reaction were: Denaturation at 95°C for 4 mins, followed by 30 cycles of denaturation at 95°C for 30 secs, anneal at 57°C for 2 mins, and extension at 72°C for 2 mins, followed by a final extension of 72°C for 10 mins. Samples were digested overnight using 2U of Aci I at 37°C.

The forward primer is 5'- AAG GTG TGG CCA TTGTAA AAA CTC - 3'

The reverse primer is 5'- TGA CTC AAA GCC ACA GAT AAC ACG - 3'

This produces a fragment of 475 base pairs in length.

Digestion of the PCR product with the restriction enzyme Aci I (New England Biolabs) produces a single cut site to give two bands of 331 and 144 base pairs respectively for the G allele. The change to an A allele abolishes the cut site and the fragment produced is 475 base pairs.

2.8 Extraction of high molecular weight DNA from whole blood using standard kits

DNA was extracted using a Nucleon BACC kit following standard procedure. 3-10ml of blood was placed in a 50ml Falcon tube (Becton Dickinson, UK) and 4x volume of Nucleon A added and shaken for 4 minutes. The solution was centrifuged for 4 mins at 1300g. The supernatant was discarded and 2ml of Nucleon B was added to pellet vortexed and incubated at 37°C for 10-15 minutes. 500µl of 5M Na Perchlorate was added and inverted 10-15 times, centrifuged at 1300g for 3 minutes. 200µl of Silica suspension layered onto the red layer without disturbing the phases. Centrifuged at 1300g for 3 minutes the upper aqueous phase was transferred to a fresh 15ml Falcon. 2x volume of ethanol was added (-20°C) and inverted a few times. The DNA was hooked out with a glass rod, washed in 70% ethanol and dissolved in 500µl of sterile water or TE buffer.

aaggtgtggccattgtaaaaactcatgtatttgctgttttaagg
 acacagatttagacttggagatgtagctccctat
 atcccaatggatgatgacctccagttacgttccttcgatcagttg
 tcaccattagaaagcagttccgcaagccctgaaag
 cgcaagtcctcaaagcacagttacagtattccagcagactcaa
 atacaagaacctactgctaatgccaccactaccactg
 ccaccactgatgaattaaaaacagtgacaaaagaccgtatgg
 aagacattaaaatattgattgcatctccatctcctacc
 cacatacataaagaaactactagtgcacatcatccatatag
 agatactcaaagtcggacagcctcaccaaacagagc
 aggaaaaggagtcatagaacagacagaaaaatctcatcaa
 gaagccctaacgtgttatctgtcgtttgagtca

Figure 2.2 Hypoxia-inducible factor 1 α exon 12 PCR amplimers and polymorphisms.
 cca to ca = Proline to Serine (Hph I), C allele = 229, 118 bp bands (wild type), T allele =
 346 bp bands. gca to aca = Alanine to Threonine (Aci I), G allele = 331 bp & 144bp (wild
 type), A allele = 475 bp.

2.8.1 Extraction of high molecular weight DNA from kidney tumour samples using standard kits

Tumour and normal tissue were subjected to a homogenisation procedure before DNA extraction. Tissue was ground in a pestle with a mortar, in the presence of liquid nitrogen. DNA was extracted using a Nucleon BACC kit following standard procedure.

2.8.2 Extraction of high molecular weight DNA from paraffin embedded sections of kidney from patients with renal cell carcinoma (Using the Nucleospin C & T kit, Macherey-Nagel)

10µm sections of tumour and normal tissue from RCC patients was taken and placed in a sterile 1.5ml eppendorf. The sections were trimmed of any excess paraffin before being placed in 1ml of xylene for 30 minutes (vortex every 5 mins) to remove the remaining paraffin.

180µl of BUFFER T1 (tissue lysis mix), and 25µl of proteinase k (20mg/ml) (which is stored at -20°C) was added to the eppendorf containing the paraffin section and placed in a shaking incubator at 55°C. Remove samples and vortex after 30 minutes and return to the incubator overnight.

Preheat BUFFER B2 to 70°C for 5 mins. Add 1 volume of B2 to 4 volumes B1, and pipette to mix (this is BUFFER B3).

Pre-heat ELUTION BUFFER BE to 70°C.

For each sample, place a Nucleospin column into a 2ml collection tube. Add 200µl BUFFER B3 to the lysed paraffin sample, vortex IMMEDIATELY for 15 seconds to mix, incubate at 70°C for 10 mins. Add 210µl ethanol to the sample mix, vortex IMMEDIATELY and QUICKLEY transfer sample to Nucleospin column, centrifuge at

8200 rpm for 1 min. Discard eluate and replace column in collection tube. If some liquid or solidified paraffin remains in the spin column, transfer the column to a fresh 1.5ml tube and incubate at 70°C for 1 min. Transfer the column back to the ml collection tube QUICKLEY and centrifuge at 8200 rpm for 1 min.

Add 500µl of BUFFER B5 to the column, and centrifuge at 8200 rpm for 1 min. Discard the eluate and replace column in collection tube. Repeat by adding 500µl BUFFER B5 to the column and centrifuge at 8200 rpm for 3 mins. Discard the collection tube.

Place the Nucleospin column into a fresh 1.5ml tube, add 50µl ELUTION BUFFER BE (70°C) and incubate at 70°C for 1 min. Centrifuge at 8200 rpm for 1 min, discard the column and close 1.5ml tube.

2.8.3 Extraction of high molecular weight DNA from fresh frozen tumour and normal kidney tissue from patients with renal cell carcinoma

After snap freezing of the tumour and normal kidney tissue and storage at -196°C in liquid nitrogen, samples were removed and a small section sliced off the end of the tissue block. This section was minced using a scalpel until a paste like consistency was made.

2.9 Isolation and Culturing of Peripheral Blood Mononuclear cells (PBMCs) from whole blood

20mls of peripheral blood was collected into 5% EDTA Vacutainers (Becton-Dickinson). The blood was diluted 1:1 in phosphate buffered saline and the PBMCs harvested following standard density gradient centrifugation. The PBMCs were washed twice and re-suspended at a concentration of 0.5×10^6 /ml in RPMI 1640 (11mM D-glucose) supplemented with 1% penicillin/streptomycin (Gibco GRL, UK), 10% fetal calf serum (Gibco GRL, UK) and separated into 2 flasks.

2.10 Extraction of total and messenger RNA

Using a guanidine phenol extraction kit (RNA-Stat 60™).

At the end of the incubation period the cells were pelleted by centrifugation the supernatant removed and the cells re-suspended and lysed in RNA Stat 60™. The solution was then transferred to an RNase-free eppendorf, 200µl of chloroform was added (Sigma Chemicals, UK), vortexed and centrifuged at 13000g for 15 minutes at 4°C. The upper aqueous phase was transferred to a clean eppendorf, mixed with 500µl of isopropanol and centrifuged. The supernatant was discarded and the pellet was washed in 75% ethanol and re-suspended in 50-100µl DEPC treated water by vortexing. The approximate amount of total RNA extracted was determined using a Cecil 5500 spectrophotometer scanning 240-280nm (Cecil Instruments Ltd, UK).

2.11 Preparation of first strand cDNA.

RNA and primers were mixed and heated to 70°C for 10 mins, and then placed on ice for 1 minute (all reaction were undertaken using a thermal cycler to obtain correct temperatures). 10mM dNTP mix (10mM each dATP, dCTP, dGTP, dTTP) was added along with MgCl₂ (25mM), PCR buffer, and DRR (0.1M) and incubated at 42°C for 5 mins. 1µl of SUPERSCRIPT II RT was added to the mix and incubated at 42°C for 50 mins. The reaction was terminated at 70°C for 15 mins and then chilled on ice. 1µl of RNase H was added and incubated at 37°C for 20 mins. The products were then stored at -20°C or used for PCR amplification of RPA probes (see fig 2.3).

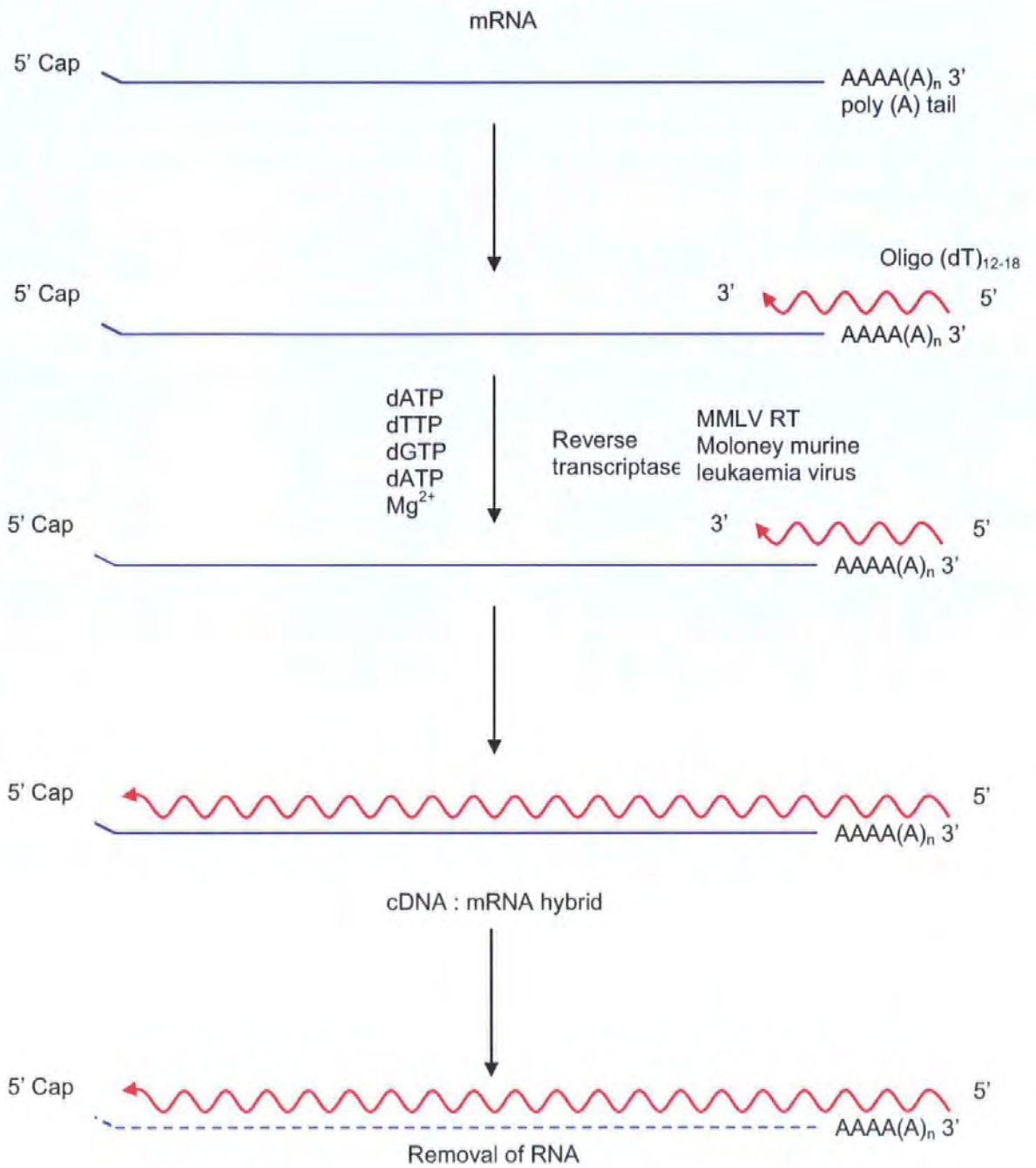


Fig 2.3 Preparation of first strand cDNA.

This fig shows a schematic representation of the preparation of first strand cDNA using a reverse transcriptase

2.12 Ribonuclease protection assay (RPA)

In the case of PCR for RPA the primers were designed so that the fragment would equate to the size of RNA probe that would be subsequently transcribed, using an antisense primer that has had a T7 promoter inserted at the 5' end.

2.12.1 Preparation of antisense RNA probes

High molecular weight DNA was prepared from 10ml of peripheral blood using Nucleon extraction kits (Scotlab, Paisley, Scotland). An aliquot of this DNA was amplified using the polymerase chain reaction (PCR). 3 primers were designed to amplify exons of the gene to be analysed. The first round amplification reaction was performed in 30 μ l volumes, containing the amplimers for the gene of interest.

10mM dNTPs (Amersham Pharmacia Biotech, Bucks, UK), 10X buffer solution, 10mM MgCl₂, 1 U *Taq* polymerase (HT Biotech UK). The samples were subjected to an initial cycle of denaturation for 4 mins at 94⁰C. This was followed by 30 cycles of amplification that consisted of denaturation for 30 sec's at 94⁰C, annealing for 2 min at 57⁰C, and extension for 2 min at 72⁰C, with a final extension for 2 mins at 72⁰C in a icycler (BioRad, UK). A second round of amplification was then employed using the antisense amplimer with a RNA polymerase T7 adapter and stuffer nucleotides, 10mM dNTPs, 10X buffer solution, 10mM MgCl₂, 1 U *Taq* polymerase. The samples were then subjected to DNA amplification as described above. 2.5 μ g of the product was used to generate the radioactive RNA probes by incorporating α^{32} P UTP (Amersham Pharmacia Biotech, Bucks, UK) using the MAXIscript System (Ambion, Texas, USA).

An equal volume of gel loading buffer (Ambion, Texas, USA) was added to the reaction and heated to 85-95⁰C for 3-5 mins and then loaded onto a 5% acrylamide/8M urea gel and

run at approx. 100-200 volts for 40mins - 1 hour. The gel was wrapped in SaranWrap and exposed to Kodak X-Omat film for 10 - 30 sec's. The radioactive band was marked and the gel re-aligned to enable the radioactive probe to be excised and immersed in 350µl of elution buffer (Ambion, Texas, USA) and then incubated overnight at 37⁰C to get 95% recovery.

2.12.2 Hybridisation of RPA probe to RNA sample:

The RNA sample and the RPA antisense probe were mixed together and precipitated with ethanol and ammonium acetate 1/10 volume of 5M NH₄OAC (Ambion, Texas, USA) and 2.5 X volumes of ethanol (Rathburn, Scotland) were added. The sample was mixed thoroughly and then placed in -20⁰C for 15 mins. The RNA was pelleted by centrifugation at a max of 4⁰C for 15 mins. The supernatant was drawn off and the pellet was dissolved in 20µl of hybridisation buffer (Ambion, Texas, USA) vortexed for 5-10 seconds and then centrifuged to collect at the bottom of the tube. The sample was then incubated at 92⁰C for 3-4 mins, vortexed, centrifuged and then incubated overnight at 42⁰C to allow hybridisation.

RNase Digestion:

The sample was spun briefly after hybridisation and 200µl of diluted RNase mix (Ambion, Texas, USA) was added and spun briefly. The reaction was incubated for 30 mins at 37⁰C, after which 300µl of RNase inactivation/precipitation solution (Ambion, Texas, USA) was added, vortexed and centrifuged. The reaction was then transferred to -20⁰C for at least 15 mins.

Separation and detection of protected fragments:

A 5% denaturing polyacrylamide gel was prepared as before. The sample was centrifuged for 15 mins at 4⁰C and the supernatant was removed. The pellet was re-suspended in gel

loading buffer and vortexed for 1 min. The sample then was heated for 3-4 mins at 94⁰C, vortexed and spun briefly. The gel was run for approx. 40mins – 1 hour at 100 – 200 volts. The gel was then transferred to filter paper (Amersham Pharmacia Biotech, USA), covered with saran (Dow) and exposed to Kodak X-Omat film (Kodak) at –20⁰C to –80⁰C overnight. The bands were analysed using a phosphor-imager (BioRad, UK).

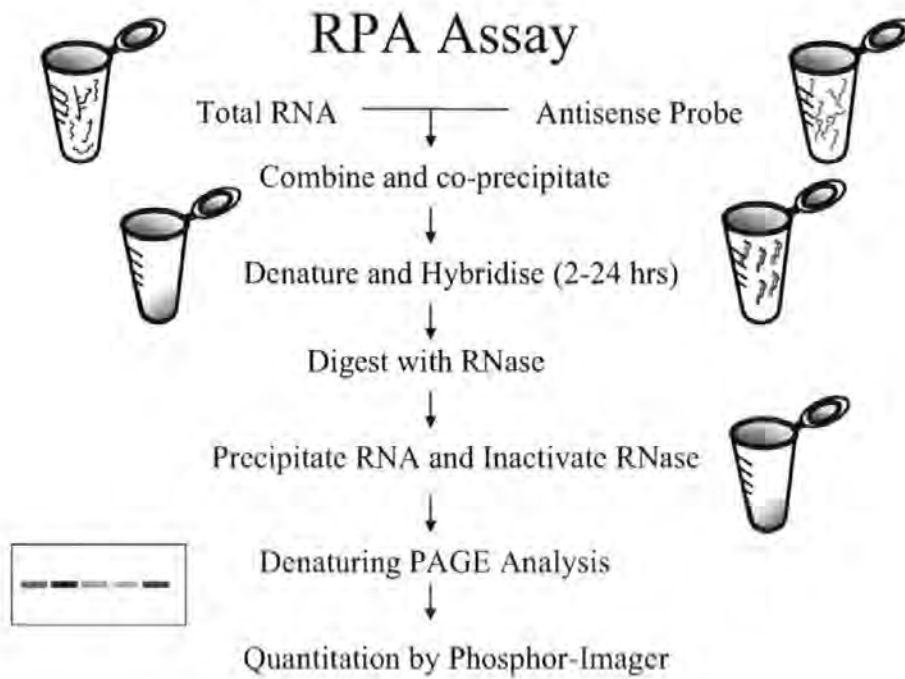


Figure 2.4 Schematic representation of the ribonuclease protection assay (RPA) Total RNA and antisense probe are combined in a single tube, hybridized and after digestion with an appropriate RNase run on a PAGE gel and quantitated using a Phosphor-imager.

VEGF RPA Primers		
Sense	Anti-sense	T7 Tag + Anti-sense
5' -GGG CAG AAT CAT CAC GAA GTG GTG- 3'	5' -ACG CTC CAG GAC TTA TAC CGG- 3'	5' -TAA TAC GAC TCA CTA TAG GGA GGC TTT ACG CTC CAG GAC TTA TAC CGG- 3'
HIF-1α RPA Primers		
Sense	Anti-sense	T7 Tag + Anti-sense
5' - GTG CCA CAT CAT CAC CAT ATA GAG - 3'	5' - GCT GCA TGA TCG TCT GGC TGC - 3'	5' - TAA TAC GAC TCA CTA TAG GGA GGC TTT GCT GCA TGA TCG TCT GGC TGC - 3'
GLUT-1 RPA Primers		
Sense	Anti-sense	T7 Tag + Anti-sense
5' - GGG TCG CCT CAT GCT GGC TGT GGG - 3'	5' - CCC CCA ACA GAA AAG ATG GCC - 3'	5' - TAA TAC GAC TCA CTA TAG GGA GGC TTT CCC CCA ACA GAA AAG ATG GCC - 3'
18SrRNA RPA Primers		
Sense	Anti-sense	T7 Tag + Anti-sense
5' - CGG CTA CCA CAT CCA AGG AGG GCA - 3'	5' - CGC CGA GAG GGC AAG GGG GGG - 3'	5' - TAA TAC GAC TCA CTA TAG GGA GGC TTT CGC CGA GAG GGC AAG GGG GGG - 3'

Table 2.1 RPA primers for various RNA species

This table shows the RPA primers used for VEGF, HIF-1 α , GLUT-1 and 18SrRNA

Probe	Size	Isoform detection
VEGF	395, and 323	189, 165 spliced variants
HIF-1 α	280	
GLUT-1	201	
18SrRNA	305	

Table 2.2 RPA probe sizes and isoform detection

This table shows the sizes of the RPA probes protected fragments and isoform detected.

Probe	MgCl ₂ Concentration (mMol)	Annealing Temperature (°C)
VEGF	6	57
GLUT-1	6	63
HIF-1 α	9	52
18SrRNA	6	66

Table 2.3 PCR conditions for the RPA probes.

This table shows the MgCl₂ concentration and annealing temperature of the PCR reactions for the RPA probes

2.13 Statistical Analysis

Expression of mRNA in the RPA assay was analysed by using a Phospho-Imager (BioRad) to measure the levels of mRNA. This data was then normalized by dividing by the 18SrRNA value of that sample. These values were analysed using a non-parametric test, Mann-Whitney (Wilcoxon W) test, to assess any differences between the tumour and normal tissue expression values. All statistical analysis was performed using 'Excel' (Microsoft) and 'Statgraphics' (Statistical Graphics Corp) statistical packages.

Genotypes and alleles for the VEGF and HIF-1 α polymorphisms were analysed using 2X2 contingency tables and a *P* value of <0.05 was taken to be significant. Bonferroni corrections were used to correct for the number of observations made in the analysis of multiple groups.

3.0 RESULTS

3.1 Patients for ribonuclease protection assay (RPA)

Patients undergoing radical nephrectomy were recruited into the study, after diagnosis of a probable renal cell carcinoma being the cause of the tumour mass in the kidney. Tumour and normal tissue from 44 patients was used to measure HIF-1 α and GLUT-1 mRNA, while tumour and normal tissue from 34 patients was used to measure the levels of VEGF isoforms 165 and 189.

3.1.1 RPA for VEGF isoforms 165 and 189, HIF-1 α and GLUT-1

Expression of VEGF isoforms 165 and 189 in the tumour and normal tissue can be seen in fig. 3.1. The two VEGF RNA species are labelled as well as the internal control 18SrRNA. For the differences in mRNA expression to be described as real differences and not just errors in loading it is important to include an internal control. For the internal control to be valid it needs to be a species of RNA that is expressed constitutively in the cell under hypothesis. For the purposes of these experiments we chose 18SrRNA as the internal control.

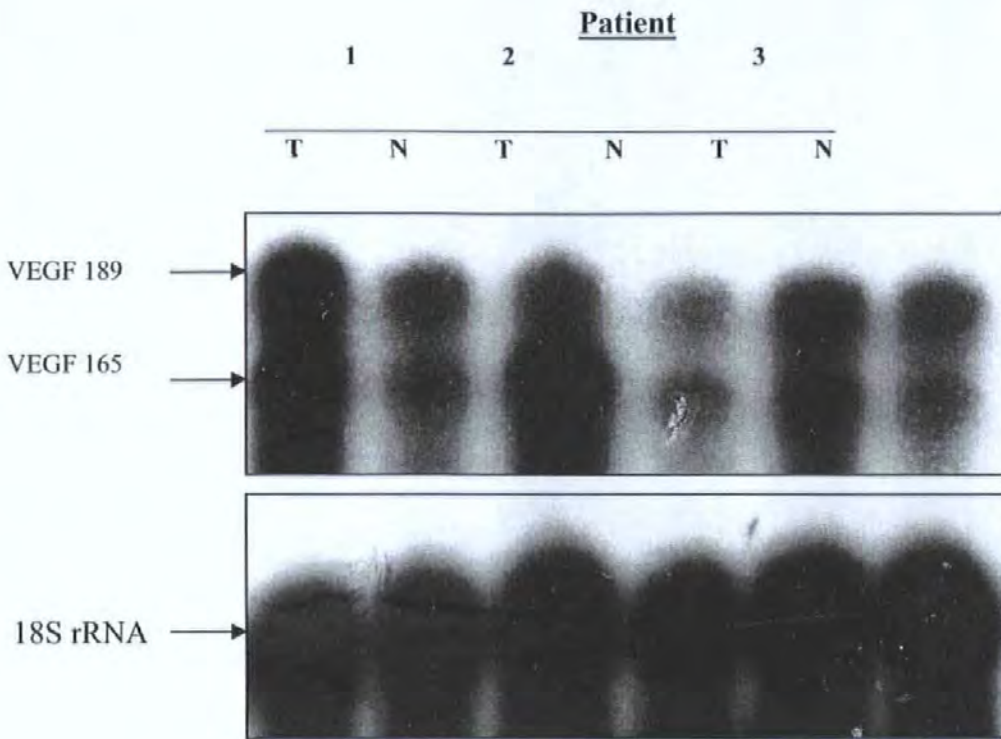


Figure 3.1. Expression of VEGF 189 and 165 in tumour (T), and normal (N) tissue of renal cell carcinoma patients.

This fig shows the expression of VEGF 165 and VEGF 189 along with 18S rRNA which was used as a control and is shown in the lower band.

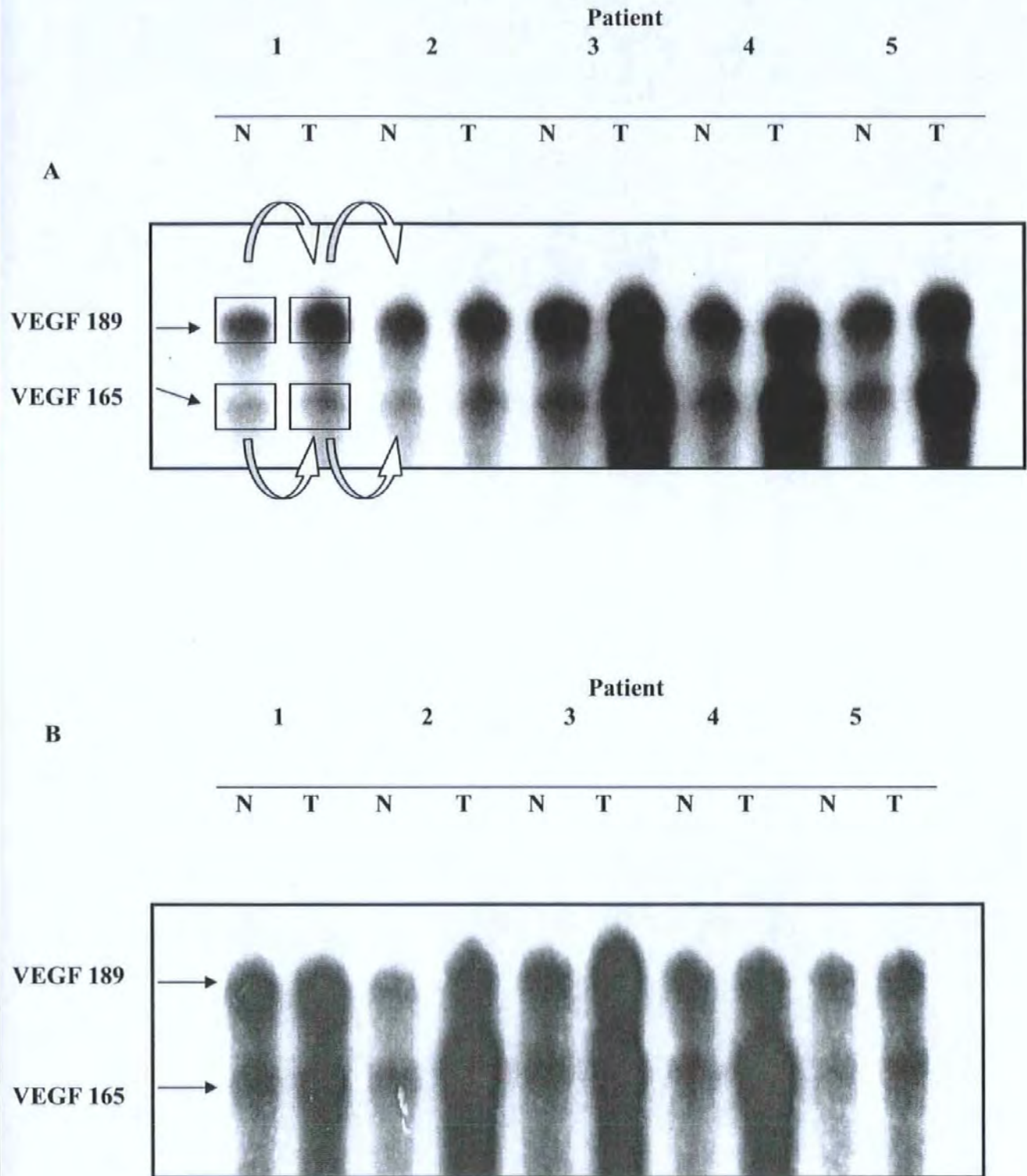


Fig 3.2 RPA assay for VEGF 165 and VEGF 189

Figs A and B show a number of patients measured for VEGF 165 and 189 mRNA transcripts. The boxes demonstrate the size used to measure the intensity of the bands, which are then moved from band to band. Five patients are shown in both A and B.

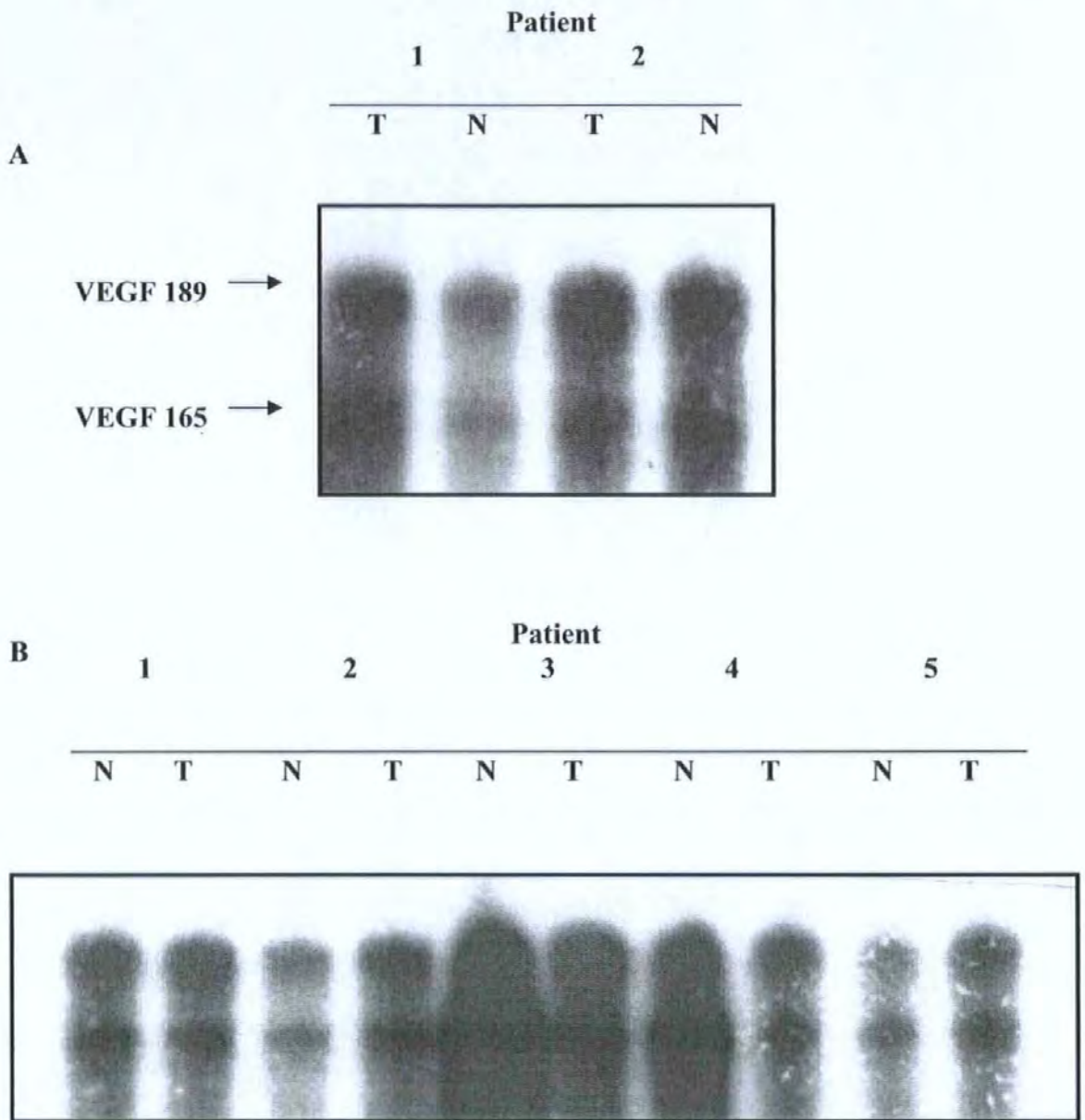


Fig 3.3 RPA assay for VEGF 165 and VEGF 189

Figs A and B show a number of patients measured for VEGF 165 and 189 mRNA transcripts. Two patients are shown in A and five patients in B.

Variable	HIF-1 α & GLUT-1 RPA %(n=44)	VEGF isoforms RPA %(n=34)
Mean Age (years)	64	63
Range	33-87	33-82
Gender		
Male	72.7 (32)	70.6 (24)
Female	27.3 (12)	29.4 (10)
Robson Stage		
I	34.1 (15)	32.3 (11)
II	27.3 (12)	26.5 (9)
III	20.4 (9)	14.7 (5)
IV	18.2 (8)	26.5 (9)
Histology		
Clear cell	86.4 (38)	82.3 (28)
Papillary	6.8 (3)	11.8 (4)
Chromophobe	4.5 (2)	5.9 (2)
Leiomyosarcoma	2.3 (1)	0.0 (0)
Fuhrman grade*		
G1	0.0 (0)	0.0 (0)
G2	33.3 (12)	31.0 (9)
G3	66.7 (24)	62.1 (18)
G4	0.0 (0)	6.9 (2)

Table 3.1.1 Characteristics of the patients recruited for ribonuclease protection assays.

This table shows the frequency of gender, Robson stage, histology and Fuhrman grade for the RPA assay for both HIF-1 α /GLUT-1 and VEGF165 and VEGF189. The value n represents the number of patients in each group, and the number in parenthesis represents the number of patients in each subgroup.

*Fuhrman grades unobtainable for 8 and 5 patients for the HIF-1 α /GLUT-1 and VEGF isoforms respectively

Table 3.1.1 shows that of the patients analysed for mRNA expression the average age for the HIF-1 α and GLUT-1 RPA was 64 (range 33-87) and the average age for the VEGF isoforms was 63 (range 33-82). The distribution between sex was similar for both HIF-1 α /GLUT-1 and VEGF isoforms (72.7% male 27.3% female for HIF-1 α /GLUT-1 and 70.6 male and 29.4% female for VEGF isoforms respectively). The most common Robson tumour stage for patients analysed for HIF-1 α and GLUT-1 were stage I, followed by II, III, and IV. Whilst for the VEGF isoforms it was Robson stage I, followed by stage II, IV, and III.

The clear cell histology was the most prevalent with a frequency of 86.4% for the HIF-1 α / GLUT-1 and 82.3% for the VEGF isoforms, with the other subtypes making up only 13.6% and 17.7% for the HIF-1 α / GLUT-1 and VEGF isoforms respectively. No Fuhrman grade 1 patients were found for either cohort, with the majority being grade 3 followed by grade 2 (66.7% for HIF-1 α / GLUT-1 and 62.1% for VEGF isoforms), showing a similar distribution for Fuhrman grade for both the HIF-1 α /GLUT-1 and VEGF isoforms. For technical reasons it was not possible to analyse all the patients for each assay.

RCC patients		
	Tumour Tissue	Normal Tissue
VEGF 165 (34)	^a 3.05 ± 0.40	1.56 ± 0.22
VEGF 189 (34)	^b 2.41 ± 0.32	1.43 ± 0.22
HIF-1α (44)	^c 1.34 ± 0.11	1.10 ± 0.08
GLUT-1 (44)	^d 1.99 ± 0.21	1.63 ± 0.20

Table 3.1.2 Expression of VEGF isoforms, HIF-1α, and GLUT-1 in patients with renal cell carcinoma

This table shows the expression levels for VEGF165, VEGF189, HIF-1α, and GLUT-1 in both the tumour and normal tissue of patients with RCC. Comparisons between the tumour and normal tissue were made for each group using the Mann-Whitney W test for non-parametric data and a value of <0.05 was considered as significant. The number of patients is shown in parenthesis. The data is expressed as a mean of the target mRNA/18SrRNA ± SE. ^a*P*=VEGF165 tumour vs. normal, *P*=0.00002, ^b*P*=VEGF189 tumour vs. normal, *P*=0.0002, ^c*P*=HIF-1α tumour vs. normal, *P*=0.01, ^d*P*=GLUT-1 tumour vs. normal, *P*=0.003.

A highly significant increase in mRNA expression was seen for VEGF 165 with expression in tumour tissue being 3.05 in comparison with the normal tissue which was 1.56 (sample RNA/18SrRNA internal control) $P=0.00002$ (Table 3.1.2). A significant difference was also found between VEGF 189 expression in the tumour and normal tissue (2.41 vs. 1.43 expression between tumour and normal tissue) $P=0.0002$. There was no preferential expression of VEGF 165 to VEGF 189 in the tumour or normal tissue.

There was a significant difference in the expression of HIF-1 α and GLUT-1 in the tumour compared to matched normal tissue ($P=0.01$ and $P=0.003$ respectively) as shown in table 3.1.2 with the increases in HIF-1 α being from 1.10 in the normal to 1.34 in the tumour tissue and for GLUT-1 1.63 in the normal to 1.99 in the tumour.

Regression analysis of the expression levels of both HIF-1 α and GLUT-1 showed that there was a significant correlation between the increasing levels of HIF-1 α and GLUT-1.

There was no significant difference in the expression of 18SrRNA between the tumour tissue and the normal tissue, showing that comparable quantities of RNA had been used between samples.

^c Histology (n=34)	VEGF isoform			
	VEGF 165		VEGF 189	
	Tumour	Normal	Tumour	Normal
CC (28)	^a 3.2 ± 0.46	1.6 ± 0.26	^b 2.5 ± 0.38	1.4 ± 0.26
CH (2)	3.9 ± 1.24	1.8 ± 0.75	2.0 ± 0.28	1.8 ± 0.38
P (4)	1.9 ± 0.71	1.4 ± 0.51	1.9 ± 0.76	1.5 ± 0.61

Table 3.1.3 VEGF expression vs. histology in RCC patients.

This table shows the expression levels of VEGF165 and VEGF189 according to histology. The number in parenthesis represents the actual number of patients in each group. The data is expressed as a mean of the VEGF isoform/18SrRNA control value ± SE. ^a*P*=VEGF165 Tumour vs. Normal CC, *P*=0.001, ^b*P*=VEGF189 T vs. N CC, *P*=0.02. ^c*P*= Histology CC vs. CH vs. P for VEGF165 tumour and normal and VEGF189 tumour and normal. *P*= N/S CC clear cell, P papillary, CH chromophobe.

The RCC patients which were measured for expression of VEGF 165 and VEGF 189 fell into three histological subtypes; clear cell, papillary, and chromophobe with 28, 2, and 4 patients respectively (see table 3.1.3)

Levels of VEGF 165 were significantly increased in the tumour vs. normal tissue of patients with clear cell RCC (3.2 vs. 1.6) *P*=0.001. This significant increase was also seen in the tumour vs. normal tissue for VEGF 189 of patients with clear cell RCC (2.5 vs. 1.4) *P*=0.02. Between patients with papillary and chromophobe RCC there was no difference between tumour vs. normal tissue for VEGF 165 or VEGF 189. There was no significant difference between the levels of VEGF 165 or VEGF 189 in any of the histological subtypes (CC vs. CH vs. P) *P*=N/S.

Fuhrman grade (n=29)	VEGF isoform			
	VEGF 165		VEGF 189	
	Tumour	Normal	Tumour	Normal
2 (9)	^a 4.84 ± 1.11	2.44 ± 0.56	3.70 ± 0.80	2.11 ± 0.59
3 (18)	^b 2.53 ± 0.37	1.25 ± 0.25	1.87 ± 0.38	1.11 ± 0.25
4 (2)	2.25 ± 0.22	0.89 ± 0.18	2.92 ± 0.76	1.17 ± 0.43

Table 3.1.4 VEGF expression vs. Fuhrman grade in RCC patients.

This table shows the expression levels of VEGF165 and VEGF189 according to Fuhrman grade. The number of patients in each group is in parenthesis. The data is expressed as a mean of the VEGF isoform/18SrRNA control value ± SE. ^a*P*=VEGF165 T vs. N Fuhrman grade 2, *P*=0.04, ^b*P*=VEGF165 T vs. N Fuhrman grade 3, *P*=0.01, ^c*P*= Fuhrman grade 2 vs. 3 vs. 4 for VEGF165 tumour and normal and VEGF189 tumour and normal, *P*=N/S.

Significant differences were seen for the expression levels of VEGF 165 tumour vs. normal in Fuhrman grade 2 (4.84 vs. 2.44) *P*=0.04, but not VEGF 189 (3.70 vs. 2.11) *P*=N/S.

Fuhrman grade 3 also showed significant differences for VEGF 165 tumour vs. normal (2.53 vs. 1.25) *P*=0.01, but not VEGF 189 tumour vs. normal (1.87 vs. 1.11) *P*=N/S.

Increases were seen in Fuhrman grade 4 for both VEGF 165 (2.25 vs. 0.89) and VEGF 189 (2.92 vs. 1.17) although it was not statistically significant *P*=N/S (see table 3.1.4)

No patients studied had Fuhrman grade 1 tumours. Fuhrman grade 2 had the highest expression levels of both isoforms 4.84 and 3.70 (expressed as VEGF isoform mRNA / 18SrRNA) for 165 and 189 isoforms. The expression levels of VEGF 165 decreased as the stage increased with grade 3 being 2.53 and stage 4 being 2.25. In comparison VEGF 189 decreased from stage 2 to 3 (1.87) but increased in patients with Fuhrman grade 4 to 2.92

°Robson stage (n=34)	VEGF isoform			
	VEGF 165		VEGF 189	
	Tumour	Normal	Tumour	Normal
1 (11)	^a 3.67 ± 1.04	1.84 ± 0.41	2.80 ± 0.79	1.64 ± 0.40
2 (9)	2.47 ± 0.65	1.34 ± 0.49	1.61 ± 0.57	1.16 ± 0.51
3 (5)	2.53 ± 0.43	1.32 ± 0.57	2.15 ± 0.63	1.33 ± 0.59
4 (9)	^b 3.18 ± 0.47	1.56 ± 0.40	2.87 ± 0.39	1.50 ± 0.39

Table 3.1.5 VEGF expression vs. Robson stage in RCC patients.

This table shows the expression of VEGF165 and VEGF189 in relation to Robson stage. The data is expressed as a mean of the VEGF isoform/18SrRNA control value ± SE. The numbers of patients are shown in parenthesis. ^a P =VEGF165 Tumour vs. Normal Robson stage 1, P =0.05, ^b P =VEGF165 T vs. N Robson stage 4, P =0.03, ^c P = Robson stage 1 vs. 2 vs. 3 vs. 4 for VEGF165 tumour and normal and VEGF189 tumour and normal, P =N/S.

VEGF 165 was significantly increased in Robson stage 1 for tumour vs. normal (3.67 vs. 1.84) P =0.05. While it was increased in tumour vs. normal for VEGF 189 it was not significant. Robson grade 2 tumours showed increases of both VEGF 165 and VEGF 189 compared to tumour tissue (2.47 vs. 1.34 and 1.61 vs. 1.16) but neither were significant. Increases were seen for VEGF 165 and VEGF 189 in Robson stage 3 although those were not statistically significant. Robson stage 4 showed significant increases for VEGF 165 (3.18 vs. 1.56) P =0.03, although VEGF 189 was increased (2.87 vs. 1.50) it was not significant. Robson stage 1 vs. 2 vs. 3 vs. 4 showed no significant difference for VEGF 165 or VEGF 189 tumour or normal (table 3.1.5)

There was no correlation between the expression of VEGF and the pathological stage of the tumour in relation to Robson stage for either VEGF 165 or VEGF 189 expression

(table 3.1.6). The highest levels of expression for VEGF 165 were in Robson stage 1 followed by 4, 3, and 2, with no significant differences between the stages.

VEGF Isoform				
^c Gender (n=34)	VEGF 165		VEGF 189	
	Tumour	Normal	Tumour	Normal
Male (24)	^a 2.98 ± 0.53	1.39 ± 0.25	^b 2.31 ± 0.41	1.31 ± 0.24
Female (10)	3.23 ± 0.50	1.96 ± 0.44	2.65 ± 0.54	1.72 ± 0.49

Table 3.1.6 Expression of VEGF isoforms 165 and 189 vs. Gender.

This table shows the expression levels of VEGF165 and VEGF189 according to gender. The data is expressed as a mean of the VEGF isoform/18SrRNA control value ± SE. The numbers of patients are shown in parenthesis. ^a*P*=VEGF165 Tumour vs. Normal for males, *P*=0.002, ^b*P*=VEGF189 T vs. N for males, *P*=0.04, ^c*P*=Male vs. female for both VEGF isoforms and tumour and normal tissues, *P*=N/S.

Significant increases for VEGF 165 and VEGF 189 were seen in tumour vs. normal for males (2.98 vs. 1.39, and 2.31 vs. 1.31) *P*=0.002 and *P*=0.04. VEGF 165 and VEGF 189 tumour vs. normal for females was increased (3.23 vs. 1.96 and 2.65 vs. 1.72) but was not statistically significant *P*=N/S. VEGF 165 and VEGF 189 were increased in females vs. males for tumour and normal tissue although none of these increases were significant (see table 3.1.6).

^a Robson Stage (34)	Gender	
	^b Male %(n=24)	^c Female %(n=10)
1 (11)	37.5 (9)	20.0 (2)
2 (9)	25.0 (6)	30.0 (3)
3 (5)	12.5 (3)	20.0 (2)
4 (9)	25.0 (6)	30.0 (3)

Table 3.1.7 Distribution of Robson stage with Gender for RCC patients

This table shows the distribution of Robson stage with respect to gender for VEGF isoforms. Values are expressed as frequencies with the number of patients in parenthesis. ^a P = Male vs. female for all Robson stages, P =N/S. ^b P =Robson stage 1 vs. 2 vs. 3 vs. 4 for males, P =N/S. ^c P =Robson stage 1 vs. 2 vs. 3 vs. 4 for females, P =N/S.

Robson stage 1 was the most common stage in males, followed by 2 and 4 equally and then 3. There were no statistically significant differences between the distribution of Robson stage vs. males P =N/S. Robson stages 3 and 4 were the most common in females followed by stages 1 and 3. No significant differences were found between male and female for any of the Robson stages 1 to 4 (see table 3.1.7).

^a Fuhrman grade (29)	Gender	
	^b Male %(n=20)	^c Female %(n=9)
2 (9)	20.0 (4)	55.6 (5)
3 (18)	70.0 (14)	44.4 (4)
4 (2)	10.0 (2)	0.0 (0)

Table 3.1.8 Distribution of Fuhrman grade with Gender for RCC patients

This table shows the distribution of Fuhrman grade with respect to gender for VEGF isoforms. Values are expressed as frequencies with the number of patients in parenthesis. ^a P = Male vs. female for all Fuhrman grades, $P=N/S$. ^b P =Fuhrman grade 2 vs. 3 vs. 4 for males, $P=0.00009$. ^c P =Fuhrman grade 2 vs. 3 for females, $P=N/S$.

No Fuhrman grade 1 was found for either male or female in the population. Fuhrman grade 3 was the most common in the male population (70.0) followed by Fuhrman grades 2 and 4 (20.0 and 10.0 respectively). Fuhrman grade 2 vs. 3 vs. 4 for the male population was statistically significant $P=0.00009$. Fuhrman grade 2 was the most common in the female population (55.6) followed by Fuhrman grade 3 (44.4). Fuhrman grade 2 vs. 3 for the female population was not significant. Comparing Fuhrman grade vs. gender produced no significant differences (table 3.1.8).

VEGF Isoforms				
^c Age (34)	VEGF 165		VEGF 189	
	Tumour	Normal	Tumour	Normal
<50 (6)	^a 1.86 ± 0.30	0.71 ± 0.16	1.51 ± 0.51	0.79 ± 0.24
51-70 (16)	^b 3.76 ± 0.74	1.83 ± 0.36	2.89 ± 0.58	1.68 ± 0.36
71> (12)	2.71 ± 0.44	1.62 ± 0.36	2.21 ± 0.42	1.42 ± 0.38

Table 3.1.9 Expression of VEGF isoforms with age distribution

This table shows the expression of VEGF165 and VEGF189 with respect to age. The data is expressed as a mean of the VEGF isoform/18SrRNA control value ± SE. The numbers of patients are shown in parenthesis. ^a P =VEGF165 Tumour vs. Normal <50, P =0.01, ^b P =VEGF165 T vs. N 51-70, P =0.02, ^c P = Age <50 vs. 51-70 vs. 71> for VEGF165 tumour and normal and VEGF189 tumour and normal, P =N/S.

VEGF 165 tumour vs. normal was increased for patients in age groups, <50 (1.86 vs. 0.71) P =0.01, and 51-70 (3.76 vs. 1.83) P =0.02. VEGF 189 tumour vs. normal was increased for all age groups <50 (1.51 vs. 0.79), 51-70 (2.89 vs. 1.68), and 71> (2.21 vs. 1.42) but was not significantly increased. There was no significant for <50 vs. 51-70 vs. 71> for VEGF 165 and VEGF 189 tumour or normal tissue (see table 3.1.9).

VEGF 165 and VEGF 189			
^a Robson stage (34)	Age		
	^b <50 (6)	^c 51-70 (16)	^d 71> (12)
1 (11)	33.3 (2)	25.0 (4)	41.7 (5)
2 (9)	33.3 (2)	37.5 (6)	8.3 (1)
3 (5)	33.3 (2)	12.5 (2)	8.3 (1)
4 (9)	0.0 (0)	25.0 (4)	41.7 (5)

Table 3.1.10 Distribution of Robson stage with age for RCC patients

This table shows the distribution of Robson stage with respect to age for VEGF 165 and VEGF 189 in patients with RCC. The number of patients are shown in parenthesis.

^a P =Age <50 vs. 51-70 vs. 71> for all Robson stages, P =N/S. ^b P =Age <50 Robson 1 vs. 2 vs. 3 vs. 4, P =N/S. ^c P =Age 51-70 Robson 1 vs. 2 vs. 3 vs. 4, P =N/S. ^d P =Age 71> Robson 1 vs. 2 vs. 3 vs. 4, P =N/S.

There were equal numbers of patients in Robson stages 1, 2, and 3 for the <50 years old group. Robson stage 2 had the most patients for the 51-70 age group followed by groups 1 and 4 equally and then Robson stage 3. The 71> age group had the most patients in Robson stages 1 and 4 followed by 2 and 3 equally. There were no significant differences between Robson stages and any of the groups (see table 3.1.10).

Histology (44)	HIF-1 α		GLUT-1	
	Tumour	Normal	Tumour	Normal
CC (38)	^a 1.33 \pm 0.12	1.12 \pm 0.09	^b 2.06 \pm 0.23	1.67 \pm 0.23
P (3)	1.16 \pm 0.06	1.08 \pm 0.17	1.46 \pm 0.27	1.56 \pm 0.36
CH (2)	1.63 \pm 0.82	0.62 \pm 0.06	0.97 \pm 0.01	1.07 \pm 0.40
L (1)	1.52	1.29	2.60	1.71

Table 3.1.11 HIF-1 α and GLUT-1 expression vs. histology in RCC patients.

This table shows the expression of HIF-1 α and GLUT-1 according to histology. The data is expressed as a mean of the HIF-1 α and GLUT-1 mRNA/18SrRNA control value \pm SE. The numbers of patients are shown in parenthesis. ^a P =HIF-1 α Tumour vs. Normal for CC, P =0.03, ^b P =GLUT-1 T vs. N for CC, P =0.003, ^c P = Histology CC vs. P vs. CH for HIF-1 α and GLUT-1 tumour and normal, P =N/S.

CC clear cell, P papillary, CH chromophobe, L leiomyosarcoma.

HIF-1 α was significantly increased in the tumour vs. normal tissue for clear cell RCC patients (1.33 vs. 1.12) P =0.03, whilst it was increased in the papillary, chromophobe and leiomyosarcoma patients it was not significant. GLUT-1 was significantly increased in the tumour vs. normal tissue for the clear cell patients (2.06 vs. 1.67) P =0.003. There were no other significant differences for any of the other subtypes (see table 3.1.18).

The chromophobe patients had the highest expression levels of HIF-1 α (1.63) followed by a patient with leiomyosarcoma (1.52), and clear cell (1.33) with the papillary patients having the lowest expression of HIF-1 α (1.16).

The patient with leiomyosarcoma had the highest expression of GLUT-1 (2.60), followed by the clear cells (2.06), and papillary (1.46) with the chromophobe being the lowest at 0.97 (table 3.1.11).

^b Fuhrman grade (n=36)	HIF-1 α		GLUT-1	
	Tumour	Normal	^c Tumour	Normal
2 (12)	1.63 \pm 0.20	1.27 \pm 0.22	2.85 \pm 0.55	2.28 \pm 0.57
3 (24)	1.27 \pm 0.16	1.05 \pm 0.10	^a 1.71 \pm 0.21	1.34 \pm 0.19

Table 3.1.12 HIF-1 α and GLUT-1 expression vs. Fuhrman grade in RCC patients.

This table shows the expression of HIF-1 α and GLUT-1 according to Fuhrman grade. The data is expressed as a mean of the HIF-1 α and GLUT-1 mRNA/18SrRNA control value \pm SE. The numbers of patients are shown in parenthesis. ^a*P*= GLUT-1 Tumour vs. Normal for Fuhrman grade 3, *P*=0.03, ^b*P*= Fuhrman grade 2 vs. 3 for HIF-1 α tumour and normal and GLUT-1 normal tissue, *P*=N/S, ^c*P*= Fuhrman grade 2 vs. 3 for GLUT-1 tumour, *P*=0.05.

HIF-1 α was increased in both Fuhrman grades 2 and 3 (1.63 vs. 1.27 and 1.27 vs. 1.05) although these were not significant, *P*=N/S. GLUT-1 was increased in the tumour vs. normal but was not significant for Fuhrman grade 2. Fuhrman grade 3 for GLUT-1 was significantly increased in the tumour vs. normal (1.71 vs. 1.34) *P*=0.03. Fuhrman grade 2 vs. grade 3 for HIF-1 α tumour and normal and GLUT-1 normal was not significant *P*=N/S, but Fuhrman grade 2 vs. grade 3 for GLUT-1 tumour was significant (2.85 vs. 1.71) *P*=0.05 (see table 3.1.12).

No Fuhrman grades 1 or 4 were seen for the patients studied, with only grades 2 and 3 found. The expression levels of both HIF-1 α and GLUT-1 decreased from grade 2 to 3. A decrease from 1.63 to 1.27 was seen for the HIF-1 α tumour mRNA, and a decrease from 2.85 to 1.71 seen for GLUT-1 tumour mRNA.

^b Robson stage (n=44)	HIF-1 α		GLUT-1	
	Tumour	Normal	Tumour	Normal
1 (15)	1.47 \pm 0.27	1.11 \pm 0.18	2.04 \pm 0.45	1.64 \pm 0.39
2 (12)	1.16 \pm 0.14	1.12 \pm 0.13	1.51 \pm 0.28	1.23 \pm 0.26
3 (9)	^a 1.27 \pm 0.10	1.02 \pm 0.08	2.00 \pm 0.25	1.69 \pm 0.28
4 (8)	1.42 \pm 0.21	1.14 \pm 0.24	2.57 \pm 0.54	2.15 \pm 0.70

Table 3.1.13 HIF-1 α and GLUT-1 expression vs. Robson stage in RCC patients.

This table shows the expression of HIF-1 α and GLUT-1 according to Robson stage. The data is expressed as a mean of the HIF-1 α and GLUT-1 mRNA/18SrRNA control value \pm SE. The numbers of patients are shown in parenthesis. ^a P = HIF-1 α Tumour vs. Normal for Robson stage 3, P =0.001, ^b P = Robson stage 1 vs. 2 vs. 3 vs. 4 for HIF-1 α and GLUT-1 tumour and normal tissue, P =N/S.

The highest levels of expression for HIF-1 α were seen in Robson stage 1 (1.47), preceded by Robson 4, 3, and 2 (1.42, 1.27, and 1.16 respectively).

The highest levels for GLUT-1 were seen in Robson stage 4 (2.57) followed by Robson 1, 3 and 2. There were no significant differences between the levels of expression and Robson stage.

HIF-1 α tumour vs. normal tissue was increased in all Robson stages although only Robson stage 3 was significant (1.27 vs. 1.02) P =0.001. Although GLUT-1 was increased in the tumour vs. normal tissue for all Robson stages this was not significant. There were no significant differences between Robson stage 1 vs. 2 vs. 3 vs. 4 for HIF-1 α or GLUT-1 tumour or normal tissue (see table 3.1.13).

^c Gender (n=44)	^a HIF-1 α		^b GLUT-1	
	Tumour	Normal	Tumour	Normal
Male (32)	1.34 \pm 0.14	1.09 \pm 0.09	1.90 \pm 0.24	1.65 \pm 0.21
Female (12)	1.34 \pm 0.11	1.13 \pm 0.20	2.19 \pm 0.38	1.59 \pm 0.49

Table 3.1.14 Expression of HIF-1 α and GLUT-1 vs. Gender.

This table shows the expression levels of HIF-1 α and GLUT-1 according to gender. Values are expressed as a mean of the HIF-1 α and GLUT-1 mRNA / 18SrRNA value \pm SE. The numbers of patients are shown in parenthesis. ^a P = HIF-1 α Tumour vs. Normal for male and females, P =N/S, ^b P = GLUT-1 T vs. N for male and females, P =N/S, ^c P = Male vs. Female for HIF-1 α and GLUT-1 tumour and normal tissue, P =N/S.

Increases for HIF-1 α in tumour vs. normal for males and females was seen (1.34 vs. 1.09 and 1.34 vs. 1.13) although these were not significant increases P =N/S. GLUT-1 was increased in tumour vs. normal tissue for males and females (1.90 vs. 1.65 and 2.19 vs. 1.59) though not significant P =N/S. The levels of HIF-1 α mRNA were exactly the same in both male and female (1.34). There was a slightly higher level of GLUT-1 expression seen in the females in comparison to the males of 2.19 vs. 1.91, although this was not a significant increase (see table 3.1.14).

^b Age (n=44)	HIF-1 α		GLUT-1	
	Tumour	Normal	Tumour	Normal
<50 (4)	0.93 \pm 0.22	0.68 \pm 0.20	0.93 \pm 0.38	0.77 \pm 0.51
51-70 (22)	1.40 \pm 0.14	1.20 \pm 0.12	2.31 \pm 0.35	2.04 \pm 0.34
71> (18)	1.35 \pm 0.19	1.08 \pm 0.12	^a 1.83 \pm 0.21	1.32 \pm 0.18

Table 3.1.15 Expression of HIF-1 α and GLUT-1 with age distribution

This table shows the expression levels of HIF-1 α and GLUT-1 according to age. Values are expressed as a mean of the HIF-1 α and GLUT-1 mRNA / 18SrRNA value \pm SE. The numbers of patients are shown in parenthesis. ^a P = GLUT-1 Tumour vs. Normal for 71>, P =0.01, ^b P = Age <50 vs. 51-70 vs. 71> for HIF-1 α and GLUT-1 tumour and normal tissue, P =N/S.

Although HIF-1 α expression in the tumour vs. normal was increased for all age groups it was not significant. Similarly GLUT-1 expression in tumour vs. normal tissue was increased for all age groups, only the 71> group was statistically significant (1.83 vs. 1.32) P =0.01. There was no statistical significance for any of the other age groups (see table 3.1.15).

^a Robson Stage (44)	Gender	
	Male %(n=32)	Female %(n=12)
1 (15)	37.5 (12)	25.0 (3)
2 (12)	25.0 (8)	33.3 (4)
3 (9)	21.9 (7)	16.7 (2)
4 (8)	15.6 (5)	25.0 (3)

Table 3.1.16 Distribution of Robson stage with Gender for RCC patients

This table shows the distribution of Robson stage with respect to gender for HIF-1 α /GLUT-1. ^a*P*= Male vs. female for all Robson stages, *P*=N/S.

Robson stage 1 contained the most number of males followed by stages 2, 3, and 4. (frequencies of 37.5, 25.0, 21.9, and 15.6 respectively). The distribution of Robson stage with respect to gender produced no significant differences between any of the Robson stages. In the female population Robson stage 2 contained the most patients followed by stages 1 and 4 equally and then stage 3 (33.3, 25.0, and 16.7 respectively). Even though there were slight differences between the abundance of Robson stage in male vs. females these were not significant (see table 3.1.16).

Gender		
^aFuhrman grade (36)	^bMale %(n=25)	^cFemale %(n=11)
2 (12)	16.0% (4)	72.7% (8)
3 (24)	84.0 %(21)	27.3% (3)

Table 3.1.17 Distribution of Fuhrman grade with Gender for RCC patients

This table shows the distribution of Fuhrman grade with respect to gender for HIF-1 α /GLUT-1. ^a P = Male vs. female for all Fuhrman grades, $P=0.003$, ^b P = Male Fuhrman grade 2 vs. 3, $P=0.000006$, ^c P = Female Fuhrman grade 2 vs. 3, $P=0.003$

No Fuhrman grades 1 or 4 were found for either male or female in the population.

Fuhrman grade 3 was the most common in the male population (84.0) followed by Fuhrman grade 2 (16.0). Fuhrman grade 2 vs. 3 for the male population was statistically significant $P=0.000006$. Fuhrman grade 2 was the most common in the female population (72.7) followed by Fuhrman grade 3 (27.3). Fuhrman grade 2 vs. 3 for the female population was significant $P=0.003$. Comparing Fuhrman grade 2 and 3 vs. gender produced significant differences $P=0.003$ (see table 3.1.17).

Patients analysed for HIF-1 α GLUT-1 mRNA			
Robson stage	Age		
	<50 (4)	51-70 (24)	71> (16)
1 (15)	50.0% (2)	20.8% (5)	50.0% (8)
2 (12)	25.0% (1)	37.5% (9)	12.5% (2)
3 (9)	25.0% (1)	20.8% (5)	18.7% (3)
4 (8)	0.0% (0)	20.8% (5)	18.7% (3)

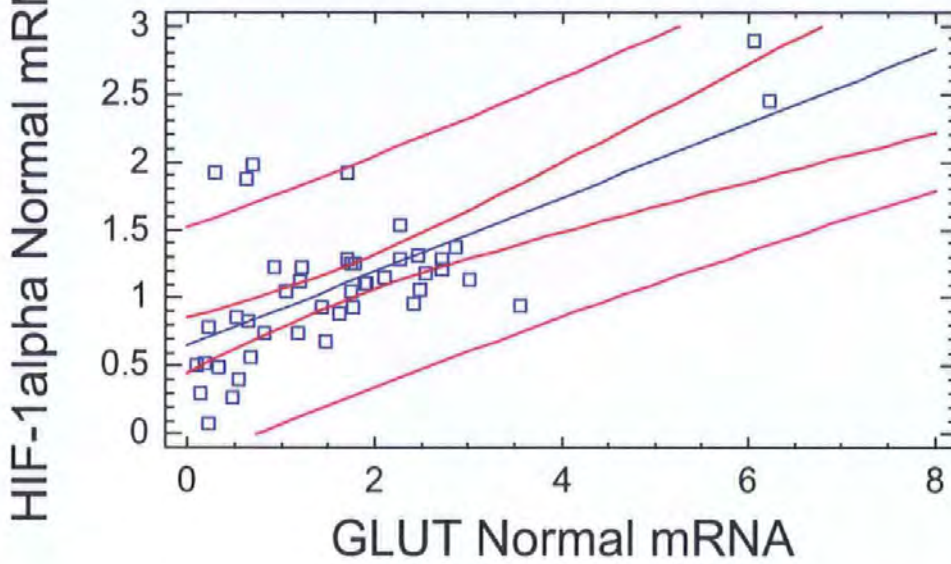
Table 3.1.18 Distribution of Robson stage with age for RCC patients

This table shows the distribution of Robson stage with respect to age for HIF-1 α /GLUT-1 in patients with RCC. The number of patients are shown in parenthesis. ^a P =Age <50 vs. 51-70 vs. 71> for all Robson stages, P =N/S.

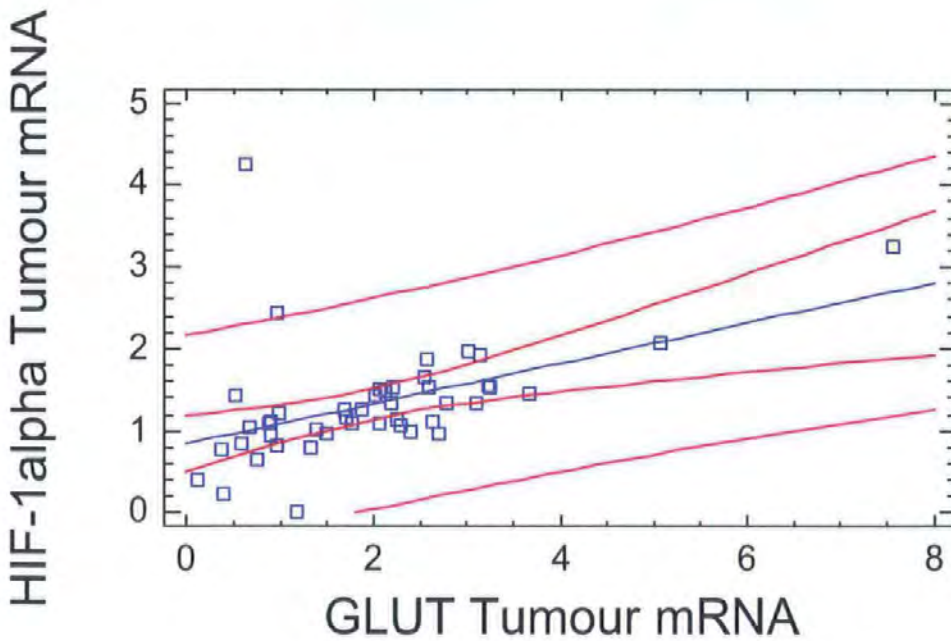
The largest age group was the 51-70 years of age followed by the 71> and <50 had the least number of patients (24 vs. 16 vs. 4 respectively). The distribution of Robson stage with respect to age was carried out and no significant differences were found.

Robson stage 1 had the most patients for the <50 age group, followed by stages 2 and 3 equally. Robson stage 2 had the most patients for the 51-70 age group followed by groups 1, 3 and 4 equally. Age group 71> had the most patients in Robson stage 1 followed by 3 and 4 equally, with the least patients in stage 2. There were no significant differences between Robson stages vs. age P =N/S. There was no significant difference between Robson stage 1 to 4 in each age group (<50, 51-70, 71>) P =N/S (see table 3.1.18).

HIF-1 alpha vs. GLUT-1 Regression Analysis



A

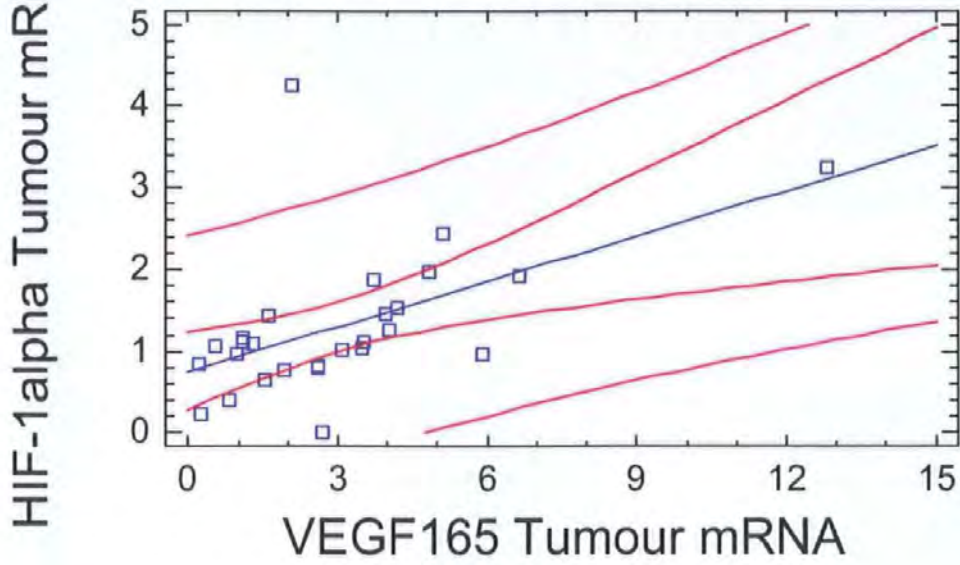


B

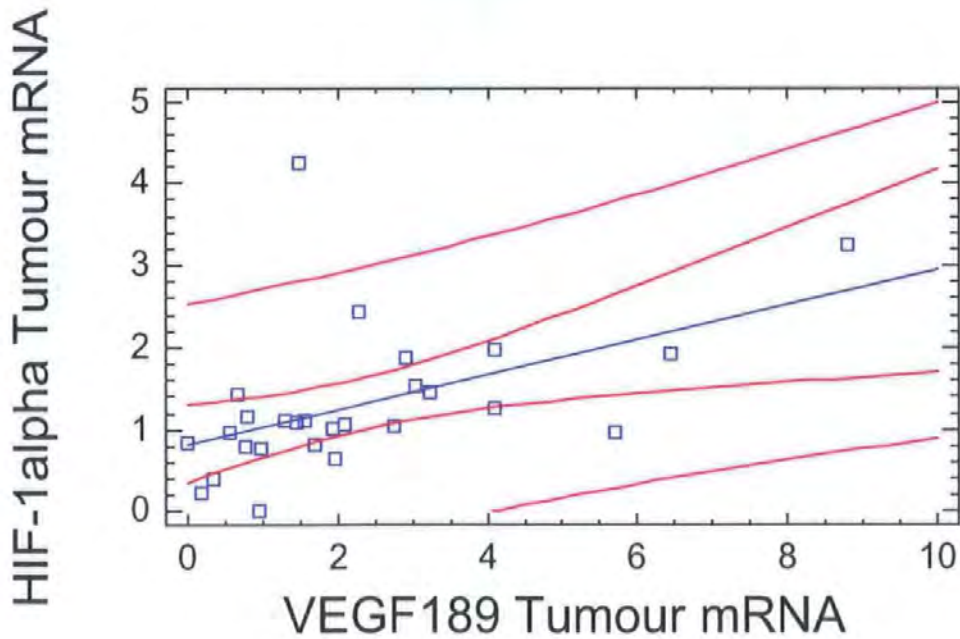
Fig 3.4 Regression analysis for HIF-1 α in relation to GLUT-1.

This fig shows the relationship between HIF-1 α and GLUT-1 in the normal (A) and tumour (B) tissue of patients with RCC. Fig A P =HIF-1 α normal vs. GLUT-1 normal, P <0.00001. Fig B P =HIF-1 α normal vs. GLUT-1 normal, P =0.001.

HIF-1 alpha vs. VEGF Regression analysis



A

 $r^2 = 23.80$

B

Fig 3.5 Regression analysis for HIF-1 α in relation to VEGF 165 and VEGF 189

This fig shows the relationship between HIF-1 α and VEGF 165 (A) and VEGF 189 (B) in the tumour tissue of patients with RCC. Fig A P =HIF-1 α tumour vs. VEGF 165 tumour, P =0.004. Fig B P =HIF-1 α tumour vs. VEGF 189 tumour, P =0.01.

Regression analysis was used to look for any correlations between VEGF 165, VEGF 189 and GLUT-1 expression levels in relation to HIF-1 α . The value of HIF-1 α increases in relation to an increase in the value (expression) of GLUT-1 in both the tumour and normal tissue, fig 3.2 A and B. Indicating the HIF-1 α levels are correlated to increasing levels of GLUT-1 at the mRNA level. VEGF 165 tumour vs. HIF-1 α tumour expression produced a significant relationship between the two species, $P=0.004$ shown in fig 3.3 A. This relationship was also seen for VEGF 189 tumour vs. HIF-1 α although it was not as strong, $P=0.01$ fig 3.3 B. Showing that the value of VEGF 165 and VEGF 189 mRNA in the tumour increases with HIF-1 α .

3.2 VEGF promoter polymorphism

The VEGF polymorphism studied was a C to A single nucleotide polymorphism (SNP) at position -2578 in the promoter region (identified by Brogan *et al*, 1999). This polymorphism was of interest because the A allele also contains an insertion of 18 nucleotides at position -2549, The control population was typed for this polymorphism with the results shown in table 3.2.1.

With the polymorphism at -2578 either containing or not containing an 18-nucleotide insertion it was possible to separate the two alleles on a standard agarose gel. Figure 3.4 shows the pattern produced by the different combination of alleles.

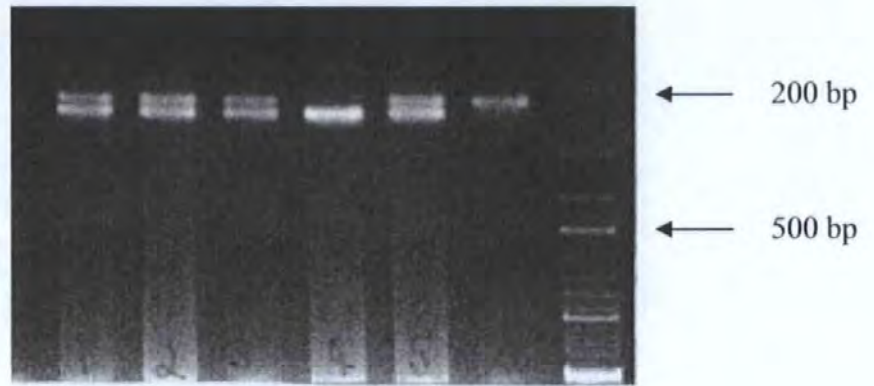


Figure 3.6 Polymerase chain reaction for the 18-nucleotide insertion at -2578 into the VEGF promoter and a C to A single nucleotide polymorphism.

Lanes 1-3 and 5 show heterozygous C/A, with a homozygous A/A in lane 4 and homozygous C/C in lane 6. Agarose gel showing the C → A polymorphism at -2578 relative to the translation start site. Two bands can be seen in the heterozygotes, while one band is present in the homozygotes. Lane 7 contains a 100bp ladder.

3.2.1 VEGF C-2578A polymorphism

Variable	Patients %(n =173)	Controls (n =142)
Age (years)	64.6	
Range	35-96	
Gender		
Male	71.1 (123)	45.1 (64)
Female	28.9 (50)	54.9 (78)
Robson Stage		
I	32.9 (57)	
II	22.6 (39)	
III	32.9 (57)	
IV	11.6 (20)	
Histology		
Clear Cell	90.2 (156)	
Papillary	5.2 (9)	
Chromophobe	2.3 (4)	
Others	2.3 (4)	
Fuhrman Grade		
G1	1.9 (3)	
G2	34.4 (54)	
G3	56.7 (89)	
G4	7.0 (11)	

Table 3.2.1 Characteristics of patients with renal cell carcinoma and healthy controls for the 18 nucleotide insertion C-2578A polymorphism in the VEGF gene.

This table shows the frequency of gender, Robson stage, Fuhrman grade, and histology of patient and control groups for the 18N insert C-2578A polymorphism. The actual number of subjects are shown in parenthesis.

The mean age of the patients was 64.6 years with a range from 35 to 96 years. The distribution of gender for the patients was 71.1% male to 28.9% female, whilst the controls distribution was 45.1% male to 54.9% female.

There were equal numbers of patients for both Robson stage I and III with 32.9% of patients falling into either of these categories. Robson stage II contained 22.6% of the patients and only 11.6% in Robson stage IV.

The majority of patients were in the clear cell subgroup accounting for 90.2% of the total, followed by 5.2% for the papillary and the chromophobe and others making up the remaining 4.6%.

Fuhrman grade 3 was the most prevalent with 56.7% followed by 34.4% in grade 2, 7% in grade 4, and under 2% in grade 1 (see table 3.2.1).

C-2578A	RCC % (n=173)	Normal controls % (n=142)
^a VEGF Genotype		
C A	66.5 (115)	62.0 (88)
C C	22.0 (38)	21.1 (30)
A A	11.6 (20)	16.9 (24)
^b Allele		
C	55.2 (191)	52.1 (148)
A	44.8 (155)	47.9 (136)

Table 3.2.2 VEGF 18 nucleotide insert C-2578A polymorphism genotype and allele frequencies.

This table shows the frequency of the genotypes and alleles for the C-2578A polymorphism in RCC and control patients. The number of subjects is given in parentheses. ^a P =VEGF genotypes patients vs. controls, P =N/S, ^b P =VEGF alleles patients vs. controls, P =N/S.

The heterozygous C/A -2578 genotype was the most common in both the patient and control groups (66.5% vs. 62.0% respectively), followed by the CC genotype (22% vs. 21.1%). The AA genotype was the least prevalent with only 11.6% for the patients and 16.9% for the controls.

There were no significant differences between the patient and control groups for any of the genotypes. Although the AA genotype was slightly increased in the controls vs. patients (16.9% vs. 11.6%) this was not significant. The C allele was the most common for both patient and control groups with 55.2% and 52.1% respectively, although there was no significant difference between the patient and control groups for either allele (see table 3.2.2).

^a Genotype	Fuhrman grade			
	1 (n=3)	2 (n=54)	3 (n=89)	4 (n=11)
CC	33.3 (1)	24.1 (13)	16.8 (15)	27.3 (3)
CA	66.7 (2)	61.1 (33)	74.2 (66)	63.6 (7)
AA	0.0 (0)	14.8 (8)	9.0 (8)	9.1 (1)
^b Alleles	(n=6)	(n=108)	(n=178)	(n=22)
C	66.7 (4)	54.6 (59)	53.9 (96)	59.1 (13)
A	33.3 (2)	45.4 (49)	46.1 (82)	40.9 (9)

Table 3.2.3 Distribution of the C-2578A alleles according to Fuhrman grade.

This table shows the frequency of alleles (C-2578A) according to Fuhrman grade. The number of subjects is given in parentheses. ^a P =Genotype vs. Fuhrman grade, P =N/S. ^b P =Alleles vs. Fuhrman grade, P =N/S.

^a Genotype	Robson stage			
	1 (n=57)	2 (n=38)	3 (n=56)	4 (n=20)
CC	29.8 (17)	18.4 (7)	17.9 (10)	20.0 (4)
CA	57.9 (33)	63.2 (24)	73.2 (41)	75.0 (15)
AA	12.3 (7)	18.4 (7)	8.9 (5)	5.0 (1)
^b Alleles	(n=114)	(n=76)	(n=112)	(n=40)
C	58.8 (67)	50.0 (38)	54.5 (61)	57.5 (23)
A	41.2 (47)	50.0 (38)	45.5 (51)	42.5 (17)

Table 3.2.4 Distribution of the C-2578A alleles according to Robson stage.

This table shows the frequency of alleles (C-2578A) according to Robson stage. The number of subjects is given in parentheses. ^a*P*=Genotype vs. Robson stage, *P*=N/S.

^b*P*=Alleles vs. Robson stage, *P*=N/S.

Fuhrman grade 3 was the most common for the C-2578A genotype, followed by grade 2, 4 and 1. There were no significant differences between the Fuhrman grades for the three genotypes. The most common genotype in all Fuhrman grades was CA followed by the CC with the AA genotype being the most rare. The C allele was the most common for all Fuhrman grades. There were no significant differences between Fuhrman grade and alleles (see table 3.2.3).

Robson stage 1 was the most common with 57 patients followed by stage 3 (56), stage 2 (3) and stage 4 (20). The CA genotype increased from Robson stage 1 to 4 (57.9, 63.2, 73.2, and 75.0 for stages 1, 2, 3, and 4 respectively), while the AA genotype decreased as the stage increased (12.3 to 5.0). The CC genotype was near 30% for Robson 1 and 20% for Robson stage 2 to 4. Although there were some trends they were not significant. The C allele was the most common for Robson stages 1, 3 and 4, with Robson stage 2 being equal between the C and A alleles. No significant differences were found between the alleles and Robson stage (see table 3.2.4).

^a VEGF C-2578A	Age (years at operation)		
	<50 %(n=18)	51-70 %(n=98)	71> %(n=57)
CC	16.7 (3)	23.5 (23)	21.1 (12)
CA	61.1 (11)	64.3 (63)	71.9 (41)
AA	22.2 (4)	12.2 (12)	7.0 (4)
^b Alleles	(n=36)	(n=196)	(n=114)
C	47.2 (17)	55.6 (109)	57.0 (65)
A	52.8 (19)	44.4 (87)	43.0 (49)

Table 3.2.5 Distribution of C-2578A alleles with age at operation.

This table shows the frequencies of the C-2578A genotypes with respect to the age of patient at operation. The number of patients is shown in parenthesis. ^a*P*=Genotype vs. age, *P*=N/S. ^b*P*=Alleles vs. age, *P*=N/S.

Most of the population were in the 51-70 age group followed by the 71> and then the <50. The frequency of the AA genotype decreased as the age increased, from 22.2 in the <50 age group to 7.0% in 71> age group. The frequency of the CA genotype increased slightly with age (61.1 vs. 64.3 vs. 71.9) although these increases were not statistically significant. The A allele was the most common in the <50 age group, with the C allele being the most prevalent in the other groups. These differences were not significant (see table 3.2.5).

VEGF isoform		
^a VEGF C-2578A genotype (n=34)	VEGF 165	VEGF 189
CA (19)	2.9 ± 0.37	2.3 ± 0.38
CC (7)	^b 4.7 ± 0.45	^c 3.7 ± 1.03
AA (8)	2.1 ± 0.58	1.4 ± 0.40

Table 3.2.6 Expression of VEGF isoforms in comparison with the C-2578A polymorphism.

This table shows the expression levels of the VEGF isoforms 165 and 189 and the VEGF polymorphism in the promoter region (C-2578A). Values are expressed as means ± SE. ^a P =VEGF C-2578A CA vs. CC vs. AA, P =N/S, ^b P =VEGF165 C-2578A CC vs AA, P =N/S, ^c P =VEGF189 C-2578A CC vs. AA, P =0.0515.

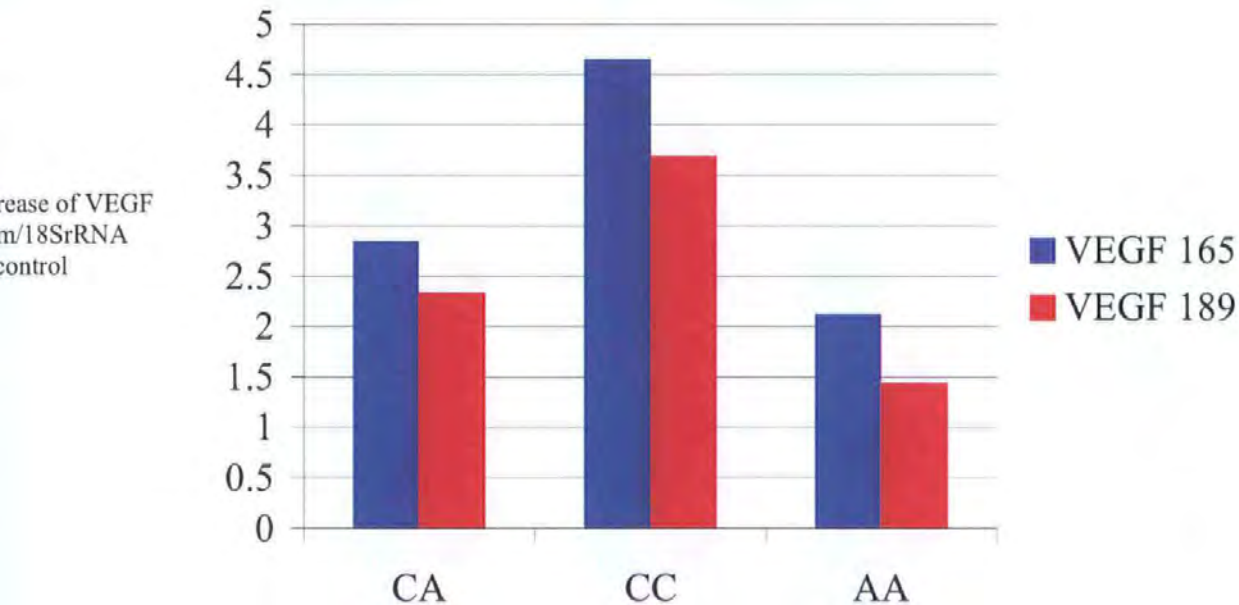


Fig 3.7 Expression of VEGF isoforms 165 and 189 for the VEGF polymorphism C-2578A.

This fig shows the expression levels of VEGF 165 and VEGF 189 according to VEGF C-2578A genotype.

Expression of VEGF isoforms with the VEGF polymorphism C-2578A shows that the CC genotype had the highest expression levels for both VEGF 165 and VEGF 189 (4.7 and 3.7 respectively). The AA genotype had the lowest expression for VEGF 165 and VEGF 189 (2.1 and 1.4 accordingly). The value of the CA genotype fell between these two values for both VEGF 165 and VEGF 189 (2.9 and 2.3 respectively).

Although differences were seen for the three genotypes there was no significant differences for VEGF 165 CA vs. CC vs. AA, or VEGF 189 CA vs. CC vs. AA. Comparisons between CC vs. AA for VEGF 165 was also not significant, and although VEGF 189 CC vs. AA was not significant it was nearly significant $P=0.0515$ (see table 3.2.6 and Fig 3.5).

Variable	Patients %(n =156)	Controls (n =142)
Age (years)	64	
Range	35-96	
Gender		
Male	71.2 (111)	45.1 (64)
Female	28.8 (45)	54.9 (78)
Robson Stage		
I	29.5 (46)	
II	22.4 (35)	
III	35.3 (55)	
IV	12.8 (20)	
Fuhrman Grade		
G1	2.0 (3)	
G2	32.7 (49)	
G3	58.0 (87)	
G4	7.3 (11)	

Table 3.2.7 Characteristics of patients with clear cell renal cell carcinoma and healthy controls for the 18 nucleotide insertion C-2578A polymorphism in the VEGF gene.

This table shows the frequency of gender, Robson stage, Fuhrman grade, and histology of clear cell RCC patient and control groups for the 18N insert C-2578A polymorphism. The actual number of subjects are shown in parenthesis.

The mean age of the patients was 64 years, with a range of 35 to 96 years old. Of the patients with clear cell RCC 71.2% were male with 28.8% female (the controls were 45.1 and 54.9 respectively). Robson stage 3 was the most common followed by stage 1, 2, and 4. There was a significant difference between the amount of patients in each Robson stage $P=0.00003$, and also in each Fuhrman grade $P<0.000001$ (see table 3.2.7).

C-2578A	Clear cell RCC % (n=156)	Normal controls % (n=142)
^a VEGF Genotype		
C A	69.2 (108)	62.0 (88)
C C	20.5 (32)	21.1 (30)
A A	10.3 (16)	16.9 (24)
^b Allele		
C	55.1 (172)	52.1 (148)
A	44.9 (140)	47.9 (136)

Table 3.2.8 VEGF 18 nucleotide insert C-2578A polymorphism genotype and allele frequencies for clear cell RCC patients.

This table shows the distribution of the genotypes and alleles for the C-2578A polymorphism in clear cell RCC and control patients (the number of subjects is given in parentheses). ^a P =VEGF genotypes patients vs. controls, P =N/S, ^b P =VEGF alleles patients vs. controls, P =N/S.

The most common genotype was CA for both the patient and control groups (69.2% vs. 62.0% respectively), followed by the CC genotype (20.5% vs. 21.1%) and the AA genotype was the least prevalent with only 10.3% for the patients and 16.9% for the controls.

There were no significant differences between the patient and control groups for any of the genotypes. The C allele was the most common for both patient and control groups with 55.1% and 52.1% respectively, although there was no statistical difference between the patient and control groups for either allele (see table 3.2.8).

Fuhrman grade				
^aGenotype	1 (n=3)	2 (n=49)	3 (n=87)	4 (n=11)
CC	33.3 (1)	24.5 (12)	16.1 (14)	27.3 (3)
CA	66.7 (2)	65.3 (32)	74.7 (65)	63.6 (7)
AA	0.0 (0)	10.2 (5)	9.2 (8)	9.1 (1)
^bAlleles	(n=6)	(n=98)	(n=174)	(n=22)
C	66.7 (4)	57.1 (56)	53.4 (93)	59.1 (13)
A	33.3 (2)	42.9 (42)	46.5 (81)	40.9 (9)

Table 3.2.9 Distribution of the C-2578A alleles for clear cell RCC patients according to Fuhrman grade.

This table shows the frequency of alleles (C-2578A) according to Fuhrman grade for clear cell RCC patients. The number of subjects is given in parentheses. ^a*P*= Genotypes vs. Fuhrman grade, *P*=N/S. ^b*P*=Alleles vs. Fuhrman grade, *P*=N/S.

^a Genotype	Robson stage			
	1 (n=46)	2 (n=36)	3 (n=55)	4 (n=20)
CC	26.1 (12)	19.4 (7)	16.4 (9)	20.0 (4)
CA	65.2 (30)	63.9 (23)	74.5 (41)	75.0 (15)
AA	8.7 (4)	16.7 (6)	9.1 (5)	5.0 (1)
^b Alleles	(n=92)	(n=72)	(n=110)	(n=40)
C	58.7 (54)	51.4 (37)	53.6 (59)	57.5 (23)
A	41.3 (38)	48.6 (35)	46.4 (51)	42.5 (17)

Table 3.2.10 Distribution of the C-2578A alleles for clear cell RCC patients according to Robson stage.

This table shows the frequency of alleles (C-2578A) according to Robson stage for clear cell RCC patients. The number of subjects is given in parentheses. ^a*P*= Genotypes vs. Robson stage, *P*=N/S. ^b*P*=Alleles vs. Robson stage, *P*=N/S.

Fuhrman grade 3 was the most common for the C-2578A genotype for clear cell RCC patients. The CA genotype was the most common for all Fuhrman grades, followed by the CC genotype with the AA genotype being the most rare. There were no significant difference between the Fuhrman grades and the three genotypes. The C allele was the most common for all the Fuhrman grades, although this was not significant (see table 3.2.9). Robson stage 3 was the most common for the C-2578A genotype for clear cell RCC patients. The CA genotype was the most common for all Robson stages, followed by the CC genotype with the AA genotype being the most rare. There were no significant differences between the Robson stages and the three genotypes. The C allele was the most common for all the Robson stages, although this was not statistically significant (see table 3.2.10).

VEGF C-2578A	Age (years at operation)			
	^a Genotypes	<50 %(n=15)	51-70 %(n=91)	71> %(n=50)
CC		20.0 (3)	22.0 (20)	18.0 (9)
CA		60.0 (9)	65.9 (60)	76.0 (38)
AA		20.0 (3)	12.1 (11)	6.0 (3)
^b Alleles		(n=30)	(n=182)	(n=100)
C		50.0 (15)	54.9 (100)	56.0 (56)
A		50.0 (15)	45.1 (82)	44.0 (44)

Table 3.2.11 Distribution of C-2578A alleles with age at operation.

This table shows the frequencies of the C-2578A genotypes with respect to the age of patient at operation. ^a P = Genotypes vs. age, P =N/S. ^b P =Alleles vs. age, P =N/S.

Most of the population were in the 51-70 age group followed by the 71> and then the <50. The frequency of the AA genotype decreased as the age increased, from 20.0% in the <50 age group to 6.0% in 71> age group. The frequency of the CA genotype increased slightly with age (60.0 vs. 65.9 vs. 76.0) although these increases were not significant. The C allele was the most prevalent for the 51-70 and 71> age groups, while the <50 age group had an equal distribution of the alleles, these differences were not significant (see table 3.2.11).

VEGF isoform		
^a VEGF C-2578A genotype	VEGF 165	VEGF 189
CA (16)	3.02 ± 0.41	2.56 ± 0.42
CC (6)	^b 4.90 ± 1.68	^c 3.60 ± 1.22
AA (6)	1.78 ± 0.53	1.31 ± 0.52

Table 3.2.12 Expression of VEGF isoforms in comparison with the C-2578A polymorphism for clear cell RCC patients.

This table shows the expression levels of the VEGF isoforms 165 and 189 and the VEGF polymorphism in the promoter region (C-2578A). Values are expressed as means ± SE

^a*P*=VEGF C-2578A CA vs. CC vs. AA, *P*=N/S, ^b*P*=VEGF165 C-2578A CC vs AA, *P*=N/S, ^c*P*=VEGF189 C-2578A CC vs. AA, *P*=N/S.

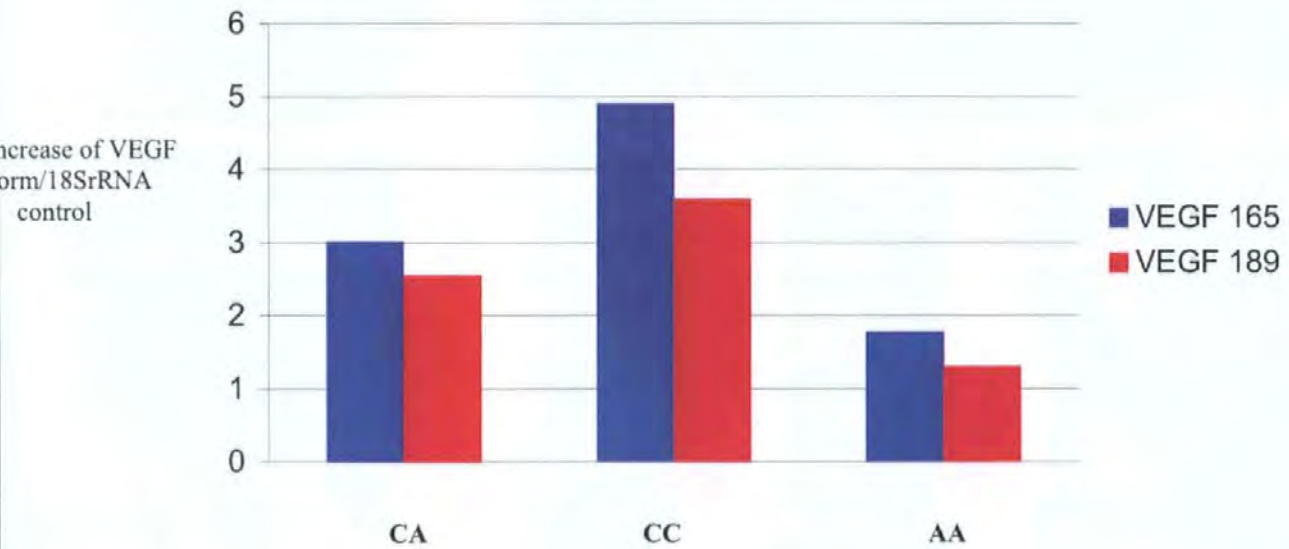


Fig 3.8 Expression of VEGF isoforms 165 and 189 for the VEGF polymorphism C-2578A.

This fig shows the expression of VEGF 165 and VEGF 189 according to VEGF C-2578A genotype in clear cell RCC patients.

Expression of VEGF isoforms with the VEGF polymorphism C-2578A shows that the CC genotype had the highest expression levels for both VEGF 165 and VEGF 189 (4.9 and 3.6 respectively). The AA genotype had the lowest expression for VEGF 165 and VEGF 189 (1.78 and 1.31 accordingly). The value of the CA genotype fell between these two values for both VEGF 165 and VEGF 189 (3.02 and 2.56 respectively).

Although differences were seen for the three genotypes there was no significant differences for VEGF 165 CA vs. CC vs. AA, or VEGF 189 CA vs. CC vs. AA. Comparisons between CC vs. AA for VEGF 165 was not significant, along with VEGF 189 CC vs. AA (see table 3.2.12 and Fig 3.6).

3.3 Polymorphisms in hypoxia-inducible factor-1 α in patients with renal cell carcinoma and normal controls

Genotyping for polymorphisms in the HIF-1 α gene began with a C to T single nucleotide polymorphism (SNP) at position C1772T, the control population was typed for this polymorphism with the results shown in table 3.2.13. Genotyping for polymorphisms in the HIF-1 α gene also included a G to A single nucleotide polymorphism (SNP) at position G1790A.

Variable	Patients		Controls	
	G1790A	C1772T	G1790A	C1772T
	Aci I %(n=146)	Hph I %(n=160)	Aci I %(n=288)	Hph I %(n=162)
Mean Age	64	64		
Range	35-96	29-89		
Gender				
Male	69.2 (101)	71.3 (114)	47.2 (136)	46.9 (76)
Female	30.8 (45)	28.7 (46)	52.8 (152)	53.1 (86)
Robson Stage				
I	31.7 (46)	34.4 (54)		
II	22.8 (33)	23.6 (37)		
III	33.8 (49)	30.6 (48)		
IV	11.7 (17)	11.4 (18)		
Histology				
Clear Cell	90.4 (132)	90.0 (144)		
Papillary	5.5 (8)	5.6 (9)		
Others	4.1 (6)	4.4 (7)		
Fuhrman Grade				
G1	1.5 (2)	2.1 (3)		
G2	35.3 (47)	33.8 (49)		
G3	57.9 (77)	56.5 (82)		
G4	5.3 (7)	7.6 (11)		

Table 3.2.13 Characteristics of patients with renal cell carcinoma and healthy controls for HIF-1 α polymorphisms.

This table shows the frequency of gender, Robson stage, Fuhrman grade, and histology of RCC patient and control groups for the HIF-1 α polymorphisms C1772T (Hph I) and G1790A (Aci I). The actual number of subjects are shown in parenthesis.

Subject characteristics

The mean age of the patients for both the G1790A and C1772T polymorphisms was 64 with a range of 35-96 and 29-89 years respectively. The G1790A consisted of 69.2% male with 30.8% female (the controls were 47.2% male and 52.8% female). The distribution of gender for the C1772T was 71.3% male and 28.7% female (controls being 46.9% male and 53.1% female).

The most common Robson stage for the G1790A was stage 3 followed by stages 1, 2, and 4. The pattern for C1772T was slightly different with Robson stage 1 being the most common, followed by stages 3, 2, and 4.

The clear cell histology was the highest for both G1790A and C1772T with 90.4% and 90.0% of the patients.

Fuhrman grade was the most abundant with 57.9% for G1790A and 56.5% for C1772T, followed by grades 2, 4, and finally 1.

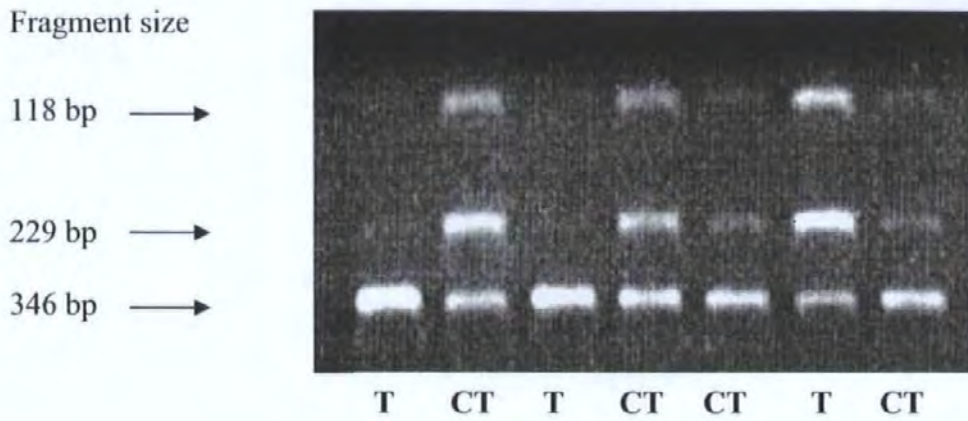


Fig 3.9 HIF-1 α C1772T polymorphism restriction endonuclease digest pattern

This fig shows the alleles for the C1772T HIF-1 α polymorphism after digestion with the restriction endonuclease Hph I

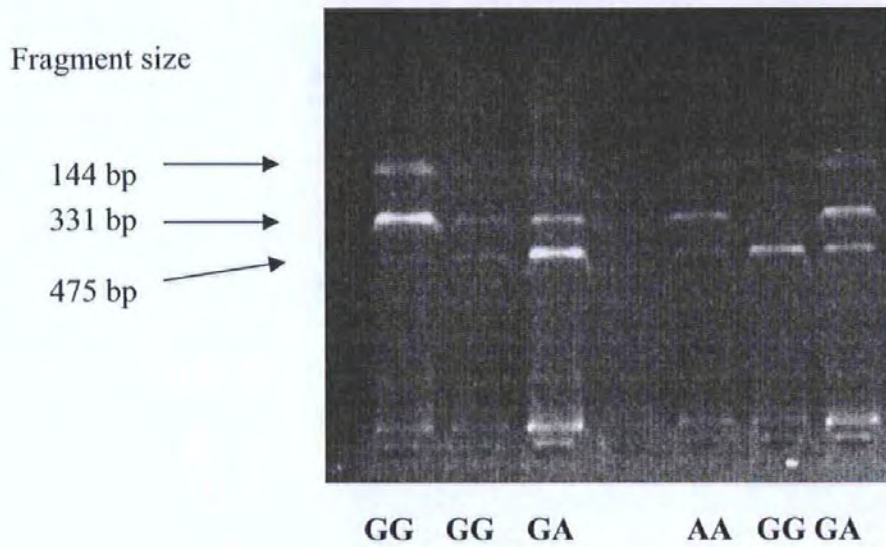


Fig 3.10 HIF-1 α G1790A polymorphism restriction endonuclease digest pattern

This fig shows the alleles for the G1790A HIF-1 α polymorphism after digestion with the restriction endonuclease Aci I

A	RCC	Controls
C1772T	% (n=160)	% (n=162)
HIF-1α (Hph I) Genotype		
^a C T	33.8 (54)	55.5 (90)
^b C C	10 (16)	0.7 (1)
^c T T	56.2 (90)	43.8 (71)
Allele		
C	26.9 (86)	28.4 (92)
T	73.1 (234)	71.6 (232)
B	RCC	Controls
G1790A	% (n=146)	% (n=288)
HIF-1α (Aci I) Genotype		
^d G A	45.9 (67)	13.5 (39)
^e G G	44.5 (65)	83 (239)
^f A A	9.6 (14)	3.5 (10)
Allele		
G	67.5 (197)	89.8 (517)
A	32.5 (95)	10.2 (59)

Table 3.2.14 Frequency of HIF-1 α polymorphisms in patients and controls

This table shows the frequency of C1772T (A) and the frequency of G1790A (B) alleles and genotypes in patients with renal cell carcinoma and normal healthy controls (The number of subjects is given in parentheses). ^a P =HIF-1 α CT patients vs. controls, P =0.0001, ^b P =HIF-1 α CC patients vs. controls, P =0.0004, ^c P =HIF-1 α TT patients vs. controls P =0.03, ^d P =HIF-1 α GA patients vs. controls, P <0.00001, ^e P =HIF-1 α GG patients vs. controls, P <0.00001, ^f P =HIF-1 α AA patients vs. controls, P =0.01, ^g P =HIF-1 α G and A alleles patient vs. controls, P <0.00001.

HIF-1 α polymorphisms in RCC and control subjects

Digestion of the amplification products with the restriction endonuclease Hph-I yielded fragments of 229 and 118 bp for the wild type C allele and 346 bp for the T allele.

Digestion with the Aci I restriction endonuclease produced fragments of 331 and 144 bp (wt) for the G allele and 475 bp for the A allele. The frequencies of the HIF-1 α C1772T and G1790A in the patients and control groups are shown in table 3.2.14.

There was an increase in both the GA (45.9% vs. 13.5%, $P < 0.00001$) and CC (10% vs. 0.7%, $P = 0.00002$) genotype in the patients with RCC, and a decrease in the GG (44.5% vs. 83%, $P < 0.00001$) and CT (33.8% vs. 55.5%, $P = 0.00002$) genotypes respectively. There was a slight increase in the TT genotype (56.2% vs. 43.8%, $P = 0.03$) and AA genotype (9.6% vs. 3.5%, $P = 0.01$). A significant difference between the G and A alleles was found with an increase in the A allele (32.5% vs. 10.2%) in the patients and decrease in the G allele (67.5% vs. 89.8%, $P < 0.000001$) no significant differences between the C and T alleles were found.

Haplotype	Patients % (n=206)	Control % (n=284)
T-G	53.4 (110) ^a	65.1 (185)
T-A	22.8 (47) ^b	9.5 (27)
C-G	18.9 (39)	24.3 (69)
C-A	4.9 (10) ^c	1.1 (3)

Table 3.2.15 Haplotype analysis of HIF-1 α polymorphisms.

This table shows the frequency of haplotypes (C1772T and G1790A) in patients with renal cell carcinoma and normal healthy controls (The number of subjects is given in parentheses). Haplotypes were assigned in those subjects who were homozygous at one or both loci. ^a P =HIF-1 α T-G haplotype patients vs. controls, P =0.01, P_c =0.03. ^b P =HIF-1 α T-A haplotype patients vs. controls, P =0.00008, P_c =0.0002. ^c P =HIF-1 α C-A haplotype patients vs. controls P =0.02, P_c =N/S.

Haplotype analysis revealed there to be an increase in the T-A haplotype (22.8% vs. 9.5%, P =0.00008, corrected P value P_c =0.002), and a decrease in the T-G haplotype (53.4% vs. 65.1%, P =0.01, P_c =0.03), and a slight increase for the C-A haplotype (4.9% vs. 1.1%, P =0.02, P_c =N/S). No differences were seen for the C-G haplotype (18.9% vs. 24.3%) respectively (see table 3.2.15).

Combined genotype	Patients (N=127)	Controls (N=149)
TT / GA	19.7 (25) ^a	5.4 (8)
TT / GG	25.2 (32)	35.6 (53)
TT / AA	7.1 (9)	4.0 (6)
TC / GA	23.6 (30) ^b	12.1 (18)
TC / GG	11.0 (14) ^c	40.3 (60)
TC / AA	3.1 (4)	2.0 (3)
CC / GA	1.6 (2)	0 (0)
CC / GG	7.9 (10) ^d	0.6 (1)
CC / AA	0.8 (1)	0 (0)

Table 3.2.16 Combined genotypes for HIF-1 α polymorphisms C1772T and G1790A

This table shows the combined genotypes for the HIF-1 α polymorphisms C1772T and G1790A. P = RCC patients vs. Controls. ^a P =HIF-1 α combined genotype TT/GA patients vs. controls, $P=0.0005$, $P_c=0.004$. ^b P =HIF-1 α combined genotype TC/GA patients vs. controls, $P=0.02$, $P_c=N/S$. ^c P =HIF-1 α combined genotype TC/GG patients vs. controls, $P=0.0000001$, $P_c=0.0000008$. ^d P =HIF-1 α combined genotype GG/AA patients vs. controls $P=0.006$, $P_c=0.05$.

For the combined genotype of the HIF-1 α G1790A and C1772T a number of statistically significant differences were found. The TT/GA combined genotype was increased in patients vs. controls (19.7 vs. 5.4% $P=0.0005$, $P_c=0.004$), as was the TC/GA (23.6 vs. 12.1% $P=0.02$, $P_c=N/S$). The TC/GG combined genotype was significantly decreased in the patients vs. controls (11.0 vs. 40.3% $P=0.0000001$, $P_c=0.0000008$). The CC/GG combined genotype was significantly increased in patients vs. controls (7.9 vs. 0.6% $P=0.006$, $P_c=0.05$) see table 3.2.16.

Genotype	Robson Stage			
	1 (n=54)	2 (n=37)	3 (n=49)	4 (n=18)
CT	33.3 (18)	45.9 (17)	26.5 (13)	22.2 (4)
CC	1.9 (1)	2.7 (1)	6.1 (3)	61.1 (11) ^a
TT	64.8 (35)	51.4 (19)	67.4 (33)	16.7 (3) ^b
^c Alleles	(n=108)	(n=74)	(n=98)	(n=36)
C	18.5 (20)	25.7 (19)	19.4 (19)	72.2 (26)
T	81.5 (88)	74.3 (55)	80.6 (79)	27.8 (10)

Table 3.2.17 Distribution of the HIF-1 α polymorphism C1772T alleles according to Robson stage.

This table shows the frequency of C1772T alleles according to Robson stage. The number of subjects is given in parentheses. ^a P =HIF-1 α CC vs. Robson stage, P <0.00000001, P_c =0.0000003. ^b P =HIF-1 α TT v. Robson stage, P =0.001, P_c =0.003. ^c P =HIF-1 α C and T alleles in Robson stage 4 vs. Robson stages 1, 2, and 3, P <0.0000001, P_c =0.0000003.

^a Genotype	Robson Stage			
	1 (n=46)	2 (n=33)	3 (n=49)	4 (n=17)
GA	39.1 (18)	45.5 (15)	55.1 (27)	41.2 (7)
GG	52.2 (24)	42.4 (14)	34.7 (17)	52.9 (9)
AA	8.7 (4)	12.1 (4)	10.2 (5)	5.9 (1)
^b Alleles	(n=92)	(n=66)	(n=98)	(n=34)
G	71.7 (66)	65.2 (43)	62.2 (61)	73.5 (25)
A	28.3 (26)	34.8 (23)	37.8 (37)	26.5 (9)

Table 3.2.18 Distribution of the HIF-1 α G1790A alleles according to Robson stage.

This table shows the frequency of G1790A alleles according to Robson stage. The number of subjects is given in parentheses. ^a P =Genotypes vs. Robson stage, P =N/S. ^b P =Allele vs. Robson stage, P =N/S.

HIF-1 α polymorphism and tumour status

The RCC group was classified into the Robson stages (I, II, III and IV) and the genotype frequencies of each polymorphism in groups were compared (see tables 3.2.17 and 3.2.18). Robson stage 1 was the most common for C1772T with 54 patients, followed by stages 3, 2, and 4.

There was a significant decrease in the TT genotype for C1772T ($P=0.001$, $P_c=0.003$) in Robson stage 4 in comparison with the other Robson stages. A highly significant increase was seen for the CC genotype for C1772T also in the Robson stage 4 patients ($P<0.000001$, $P_c=0.000003$) in comparison with the other stages. A statistically significant increase was seen for the C allele and a significant decrease for the T allele for Robson stage 4 vs. Robson stages 1, 2 and 3, $P<0.0000001$, $P_c=0.0000003$ (see table 3.2.17).

The most common Robson stage for G1790A was stage 3 followed by stages 1, 2, and 4. No significant differences between the G1790A polymorphism and Robson stage were found $P=N/S$. There were no significant differences for either the G or A allele in any of the Robson stages (see table 3.2.18).

^a Genotype	Fuhrman grade			
	1 (n=3)	2 (n=50)	3 (n=81)	4 (n=11)
CT	0.0 (0)	46.0 (23)	29.6 (24)	36.4 (4)
CC	0.0 (0)	4.0 (2)	11.1 (9)	18.2 (2)
TT	100 (3)	50.0 (25)	59.3 (48)	45.4 (5)
^b Alleles	(n=6)	(n=100)	(n=162)	(n=22)
C	0.0 (0)	27.0 (27)	25.9 (42)	36.4 (8)
T	100 (6)	73.0 (73)	74.1 (120)	63.6 (14)

Table 3.2.19 Distribution of the HIF-1 α C1772T alleles according to Fuhrman grade.

This table shows the frequency of C1772T alleles according to Fuhrman grade. The number of subjects is given in parentheses. ^a P =Genotypes vs. Fuhrman grade, P =N/S. ^b P =Allele vs. Fuhrman grade, P =N/S.

^a Genotype	Fuhrman grade			
	1 (n=2)	2 (n=47)	3 (n=77)	4 (n=7)
GA	0 (0)	51.0 (24)	48.0 (37)	57.1 (4)
GG	50.0 (1)	44.7 (21)	39.0 (30)	14.3 (1)
AA	50.0 (1)	4.3 (2)	13.0 (10)	28.6 (2)
^b Alleles	(n=4)	(n=94)	(n=154)	(n=14)
G	50.0 (2)	70.2 (66)	63.0 (97)	42.9 (6)
A	50.0 (2)	29.8 (28)	37.0 (57)	57.1 (8)

Table 3.2.20 Distribution of the HIF-1 α G1790A alleles according to Fuhrman grade.

This table shows the frequency of G1790A alleles according to Fuhrman grade (The number of subjects is given in parentheses). ^a P =Genotypes vs. Fuhrman grade, P =N/S. ^b P =Allele vs. Fuhrman grade, P =N/S.

HIF-1 α polymorphism and tumour grade

Tumours were classified into Fuhrman grades and analysed respectively. No significant differences were seen between the four Fuhrman grades and distribution of the C1772T and G1790A genotypes respectively (see tables 3.2.19 and 3.2.20 respectively).

^a Genotype	Age (years at operation)		
	<50 %(n=16)	51-70 %(n=90)	71> %(n=52)
CT	43.8 (7)	37.8 (34)	25.0 (13)
CC	6.2 (1)	11.1 (10)	7.7 (4)
TT	50.0 (8)	51.1 (46)	67.3 (35)
^b Alleles	(n=32)	(n=180)	(n=104)
C	28.1 (9)	30.0 (54)	20.2 (21)
T	71.9 (23)	70.0 (126)	79.8 (83)

Table 3.2.21 Distribution of C1772T alleles with age at operation.

This table shows the HIF-1 α polymorphism C1772T according to age. Patient numbers are shown in parenthesis. ^a*P*=Genotypes vs. age, *P*=N/S. ^b*P*=Allele vs. age, *P*=N/S.

^a Genotype	Age (years at operation)		
	<50 %(n=15)	51-70 %(n=83)	71> %(n=46)
GA	46.7 (7)	49.4 (41)	41.3 (19)
GG	46.7 (7)	41.0 (34)	47.8 (22)
AA	6.6 (1)	9.6 (8)	10.9 (5)
^b Alleles	(n=30)	(n=166)	(n=92)
G	70.0 (21)	65.7 (109)	68.5 (63)
A	30.0 (9)	34.3 (57)	31.5 (29)

Table 3.2.22 Distribution of HIF-1 α polymorphism G1790A alleles with age at operation.

This table shows the distribution of the HIF-1 α polymorphism G1790A according to age. Patient numbers are shown in parenthesis. ^a P =Genotypes vs. age, P =N/S. ^b P =Allele vs. age, P =N/S.

Most of the patients for the C1772T and G1790A polymorphisms were in the 51-70 age group followed by the 71> and then the <50 (see tables 3.2.21 and 3.2.22). The frequency of the CT genotype decreased as the age increased, from 43.8 in the <50 age group to 25.0 in 71> age group. The frequency of the TT genotype increased slightly with age (50.0 vs. 51.1 vs. 67.3) although these increases were not significant.

Variable	Patients		Controls	
	G1790A	C1772T	G1790A	C1772T
	Aci I %(n=132)	Hph I %(n=144)	Aci I %(n=288)	Hph I %(n=162)
Age (years)	64	64		
Range	33-96	39-87		
Gender				
Male	65.9 (87)	71.5 (103)	47.2 (136)	46.9 (76)
Female	34.1 (45)	28.5 (41)	52.8 (152)	53.1 (86)
Robson Stage				
I	28.8 (38)	30.6 (44)		
II	22.7 (30)	23.6 (34)		
III	35.6 (47)	33.3 (48)		
IV	12.8 (17)	12.5 (18)		
Fuhrman Grade				
G1	1.6 (2)	2.2 (3)		
G2	33.8 (43)	33.1 (46)		
G3	59.1 (75)	56.8 (79)		
G4	5.5 (7)	7.9 (11)		

Table 3.2.23 Characteristics of patients with clear cell renal cell carcinoma and healthy controls for HIF-1 α polymorphisms.

This table shows the frequency of gender, Robson stage, and Fuhrman grade for clear cell RCC patients and control groups for the HIF-1 α polymorphisms C1772T and G1790A. The actual number of subjects is shown in parenthesis.

Clear cell RCC patient characteristics

The mean age of the clear cell RCC patients for both the G1790A and C1772T polymorphisms was 64 with a range of 33-96 and 39-87 years respectively. The G1790A consisted of 65.9% male with 34.1% female (the controls were 47.2% male and 52.8% female). The distribution of gender for the C1772T was 71.5% male and 28.5% female (controls being 46.9% male and 53.1% female).

The most common Robson stage for the G1790A and C1772T was stage 3 followed by stages 1, 2, and 4. Fuhrman grade 1 was the most abundant with 59.1% for G1790A and 56.8% for C1772T, followed by grades 2, 4, and finally 1 (see table 3.2.23).

A	RCC	Controls
C1772T	% (n=144)	% (n=162)
HIF-1α (Hph I) Genotype		
^a C T	34.0 (49)	55.5 (90)
^b C C	9.7 (14)	0.7 (1)
^c T T	56.3 (81)	43.8 (71)
Allele		
C	26.7 (77)	28.4 (92)
T	73.3 (211)	71.6 (232)
B	RCC	Controls
G1790A	% (n=132)	% (n=288)
HIF-1α (Aci I) Genotype		
^d G A	47.0 (62)	13.5 (39)
^e G G	42.4 (56)	83 (239)
^f A A	10.6 (14)	3.5 (10)
^g Allele		
G	65.9 (174)	89.8 (517)
A	34.1 (90)	10.2 (59)

Table 3.2.24 Frequency of HIF-1 α polymorphisms in clear cell RCC patients and controls

This table shows the frequency of C1772T (A) and G1790A (B) alleles and genotypes in patients with clear cell RCC and normal healthy controls (The number of subjects is given in parentheses). ^a P =HIF-1 α CT patients vs. controls, P =0.002, ^b P =HIF-1 α CC patients vs. controls, P =0.0006, ^c P =HIF-1 α TT patients vs. controls P =0.04, ^d P =HIF-1 α GA patients vs. controls, P <0.00001, ^e P =HIF-1 α GG patients vs. controls, P <0.00001, ^f P =HIF-1 α AA patients vs. controls, P =0.007, ^g P =HIF-1 α G and A alleles patient vs. controls, P <0.00001.

HIF-1 α polymorphisms in clear cell RCC and control subjects

The frequencies of the HIF-1 α C1772T and G1790A in the clear cell patients and control groups are shown in table 3.2.24.

We found an increase in both the GA (47.0% vs. 13.5%, $P<0.00001$) and CC (9.7% vs. 0.7%, $P=0.0006$) genotype in the patients with renal cell carcinoma, and a decrease in the GG (42.4% vs. 83%, $P<0.00001$) and CT (34.0% vs. 55.5%, $P=0.002$) genotypes respectively. There was a slight increase in the TT genotype (56.3% vs. 43.8%, $P=0.04$) and AA genotype (10.6% vs. 3.5%, $P=0.007$). A significant difference between the G and A alleles was found with an increase in the A allele (34.1% vs. 10.2%) in the patients and decrease in the G allele (65.9% vs. 89.8%, $P<0.00001$) no significant differences between the C and T alleles were found.

Haplotype	Patients % (n=178)	Control % (n=284)
^a T-G	52.8 (94)	65.1 (185)
^b T-A	25.3 (45)	9.5 (27)
C-G	18.0 (32)	24.3 (69)
C-A	3.9 (7)	1.1 (3)

Table 3.2.25 Haplotype analysis of HIF-1 α polymorphisms.

This table shows the frequency of haplotypes (G1790A and C1772T) in patients with clear cell renal cell carcinoma and normal healthy controls. The number of subjects is given in parentheses. Haplotypes were assigned in those subjects who were homozygous at one or both loci. ^a P =HIF-1 α T-G haplotype patients vs. controls, $P=0.01$, $P_c=0.03$. ^b P =HIF-1 α T-A haplotype patients vs. controls, $P=0.00001$, $P_c=0.00003$.

Haplotype analysis revealed there to be an increase in the T-A haplotype (25.3% vs. 9.5%, $P=0.00001$, $P_c=0.00003$), and a decrease in the T-G haplotype (52.8% vs. 65.1%, $P=0.01$, $P_c=0.03$), and a slight increase for the C-A haplotype although it was not significant (3.9% vs. 1.1%, $P=N/S$). No differences were seen for the C-G haplotype (18.0% vs. 24.3%) respectively (see table 3.2.25).

Combined genotype	Patients (N=119)	Controls (N=149)
^a TT / GA	19.3 (23)	5.4 (8)
TT / GG	24.4 (29)	35.6 (53)
TT / AA	7.6 (9)	4.0 (6)
TC / GA	25.2 (30)	12.1 (18)
^b TC / GG	10.9 (13)	40.3 (60)
TC / AA	3.4 (4)	2.0 (3)
CC / GA	0.8 (1)	0 (0)
CC / GG	7.6 (9)	0.6 (1)
CC / AA	0.8 (1)	0 (0)

Table 3.2.26 Combined genotypes for HIF-1 α polymorphisms C1772T and G1790A

This table shows the combined genotypes for HIF-1 α polymorphisms C1772T and G1790A in clear cell RCC patients. ^a P =HIF-1 α combined genotype TT/GA patients vs. controls, P_c =0.006. ^b P =HIF-1 α combined genotype TC/GG patients vs. controls, P_c =0.000002.

For the clear cell RCC patients combined genotype of the HIF-1 α G1790A and C1772T a number of significant differences were found. The TT/GA combined genotype was increased in patients vs. controls (19.3% vs. 5.4%, P_c =0.006), as was the TC/GA (25.2% vs. 12.1%, although not significant when corrected for the number of groups P_c =N/S). The TC/GG combined genotype was significantly decreased in the patients vs. controls (10.9% vs. 40.3%, P_c =0.000002). The CC/GG combined genotype was increased in patients vs. controls (7.6% vs. 0.6%, although not statistically significant when corrected for the number of groups P_c =N/S) (see table 3.2.26).

Genotype	Robson Stage			
	1 (n=44)	2 (n=34)	3 (n=48)	4 (n=18)
CT	36.4 (16)	47.1 (16)	27.1 (13)	22.2 (4)
CC	0.0 (0)	0.0 (0)	6.2 (3)	^a 61.1 (11)
TT	63.6 (28)	52.9 (18)	66.7 (32)	^b 16.7 (3)
^c Alleles	(n=88)	(n=680)	(n=96)	(n=36)
C	18.2 (16)	23.5 (16)	19.8 (19)	72.2 (26)
T	81.8 (72)	76.5 (52)	80.2 (77)	27.8 (10)

Table 3.2.27 Distribution of the C1772T alleles according to Robson stage.

This table shows the frequency of HIF-1 α C1772T alleles according to Robson stage. The number of subjects is given in parentheses. ^a P =HIF-1 α CC vs. Robson stage, $P < 0.0000001$, $P_c = 0.0000003$. ^b P =HIF-1 α TT vs. Robson stage, $P = 0.002$, $P_c = 0.02$. ^c P =HIF-1 α C and T alleles in Robson stage 4 vs. Robson stages 1, 2, and 3, $P < 0.0000001$, $P_c = 0.0000003$.

^a Genotype	Robson Stage			
	1 (n=38)	2 (n=30)	3 (n=47)	4 (n=17)
GA	42.1 (16)	43.3 (13)	55.3 (26)	41.2 (7)
GG	47.4 (18)	43.3 (13)	34.1 (16)	52.9 (9)
AA	10.5 (4)	13.4 (4)	10.6 (5)	5.9 (1)
^b Alleles	(n=76)	(n=60)	(n=94)	(n=34)
G	47.4 (36)	65.0 (39)	61.7 (58)	73.5 (25)
A	31.6 (24)	35.0 (4)	38.3 (36)	26.5 (9)

Table 3.2.28 Distribution of the G1790A alleles according to Robson stage.

This table shows the frequency of HIF-1 α G1790A alleles according to Robson stage. The number of subjects is given in parentheses. ^a P =Genotype vs. Robson stage, P =N/S. ^b P =Allele vs. Robson stage, P =N/S.

HIF-1 α polymorphism and tumour status

The clear cell RCC group was classified into the Robson stages (I, II, III and IV) and the genotype frequencies of C1772T and G1790A were compared.

There was a significant decrease in the TT genotype for C1772T ($P=0.002$, $P_c=0.02$) in Robson stage 4 in comparison with the other Robson stages. A highly significant increase was seen for the CC genotype for C1772T also in the Robson stage 4 patients ($P<0.0000001$, $P_c=0.0000003$) in comparison with the other stages (see table 3.2.27). No significant differences between the G1790A polymorphism and Robson stage were found (see table 3.2.28).

^a Genotype	Fuhrman grade			
	1 (n=3)	2 (n=46)	3 (n=79)	4 (n=11)
CT	0.0 (0)	45.7 (21)	29.1 (23)	36.4 (4)
CC	0.0 (0)	4.3 (2)	11.4 (9)	18.2 (2)
TT	100 (3)	50.0 (23)	59.5 (47)	45.4 (5)
^b Alleles	(n=6)	(n=92)	(n=158)	(n=22)
C	0.0 (0)	27.2 (25)	25.9 (41)	36.4 (8)
T	100 (6)	72.8 (67)	74.1 (117)	63.8 (14)

Table 3.2.29 Distribution of the C1772T alleles according to Fuhrman grade.

This table shows the frequency of HIF-1 α C1772T alleles according to Fuhrman grade. The number of subjects is given in parentheses. ^a P =Genotype vs. Fuhrman grade, P =N/S. ^b P =Allele vs. Fuhrman grade, P =N/S.

^a Genotype	Fuhrman grade			
	1 (n=2)	2 (n=43)	3 (n=75)	4 (n=7)
GA	50.0 (1)	51.2 (22)	46.7 (35)	57.1 (4)
GG	50.0 (1)	44.2 (19)	40.0 (30)	14.3 (1)
AA	0.0 (0)	4.6 (2)	13.3 (10)	28.6 (2)
^b Alleles	(n=4)	(n=86)	(n=150)	(n=14)
G	75.0 (3)	69.8 (60)	63.3 (95)	42.9 (6)
A	25.0 (1)	30.2 (26)	36.7 (55)	57.1 (8)

Table 3.2.30 Distribution of the G1790A alleles according to Fuhrman grade.

This table shows the frequency of HIF-1 α G1790A alleles according to Fuhrman grade. The number of subjects is given in parentheses. ^a*P*=Genotype vs. Fuhrman grade, *P*=N/S. ^b*P*=Allele vs. Fuhrman grade, *P*=N/S.

HIF-1 α polymorphism and tumour grade

The clear cell RCC group was classified into the Fuhrman grades (I, II, III and IV) and the genotype frequencies of C1772T and G1790A were compared. No significant differences were seen between the four Fuhrman grades and distribution of the C1772T and G1790A genotypes respectively (see tables 3.2.29 and 3.2.30).

^b Genotype	Age (years at operation)		
	<50 %(n=13)	51-70 %(n=84)	71> %(n=47)
CT	46.2 (6)	36.9 (31)	^a 23.4 (11)
CC	7.6 (1)	9.5 (8)	10.6 (5)
TT	46.2 (6)	53.6 (45)	66.0 (31)
^c Alleles	(n=26)	(n=168)	(n=94)
C	30.8 (8)	28.0 (47)	22.3 (21)
T	69.2 (18)	72.0 (121)	77.7 (73)

Table 3.2.31 Distribution of HIF-1 α C1772T alleles with age at operation.

This table shows the distribution of HIF-1 α C1772T polymorphism according to age.

^a P =HIF-1 α CT vs. age, $P=0.02$, $P_c=0.04$. ^b P = Genotypes TT and CC vs. age, $P=N/S$.

^c P =Allele vs. age, $P=N/S$.

^a Genotype	Age (years at operation)		
	<50 %(n=13)	51-70 %(n=77)	71> %(n=42)
GA	53.8 (7)	49.3 (38)	40.5 (17)
GG	38.5 (5)	40.3 (31)	47.6 (20)
AA	7.7 (1)	10.4 (8)	11.9 (5)
^b Alleles	(n=26)	(n=154)	(n=84)
G	65.4 (17)	64.9 (100)	67.9 (57)
A	34.6 (9)	35.1 (54)	32.1 (27)

Table 3.2.32 Distribution of G1790A alleles with age at operation.

This table shows the distribution of HIF-1 α G1790A polymorphism according to age.

^a P =Genotype vs. age, P =N/S. ^b P =Allele vs. age, P =N/S.

Most of the clear cell RCC patients for the C1772T and G1790A polymorphisms were in the 51-70 age group followed by the 71> and then the <50 (see tables 3.2.31 and 3.2.32). The frequency of the CT genotype decreased as the age increased, from 46.2 in the <50 age group to 23.4 in 71> age group. The frequency of the TT genotype increased slightly with age (46.2 vs. 53.6 vs. 66.0) although these increases were not significant.

4.0 DISCUSSION

4.1 RPA DATA DISCUSSION

The expression of angiogenic factors has been poorly assessed in RCC. In this study the angiogenic factor VEGF, as well as its upstream transcription factor HIF-1 α and the HIF-1 α regulated glucose transporter gene GLUT-1 were investigated.

The increase of highly angiogenic peptides and receptors in RCC has been analysed by a number of groups [Nicol *et al* 1997, Tomisawa *et al* 1999, Thelen *et al* 1999, Takahashi *et al* 1999], with only one so far using the quantitative method of ribonuclease protection assay (RPA) [Gunningham *et al* 2001]. Increased expression of the angiogenic phenotype is now regarded as an accepted part of the tumour architecture.

The findings of this thesis showed expression of VEGF throughout all pathological stages and no correlation between higher stage and level of expression was seen. The relationship between VEGF 165 and 189 did not change with respect to either the tumour or normal tissue, indicating a similar expression pattern in both the tumour tissue and surrounding non-neoplastic tissue.

VEGF isoform expression was significantly increased for both VEGF 165 and VEGF 189 in the tumour tissue of RCC patients in comparison with adjacent normal tissue. Increases of VEGF 165 may be regarded as a replication of findings from other who have looked at mRNA expression of VEGF isoforms groups [Tomisawa *et al* 1999, Nicol *et al* 1997]. The demonstration that VEGF 189 is also increased may be of importance in the phenotype of the disease. It has been hypothesised that matrix bound VEGF may be an important source of VEGF after cleavage from the matrix [Park *et al*, 1993].

There was no significant difference between expression of VEGF 165 and VEGF189 in both tumour and normal tissue. When divided into histological subtypes it was only the clear cell population that showed a significant increase in the tumour vs. normal tissue for

VEGF 165 and VEGF 189. Increases were seen for the other subtypes but due to small numbers were not significant. Increases in VEGF 165 and VEGF 189 were seen for the three Fuhrman grades found, with significant increases seen for grades 2 and 3. Fuhrman grade 4 was not significant, probably due to small group size yet showed increases for both isoforms. The highest levels of expression were in the lower grade 2 for both isoforms, showing that increases in VEGF mRNA may correlate with the lower pathological grade. This data is in contrast to Tomisawa *et al* (1999) who have shown that pathological stage/grade correlates with increased VEGF expression. They analysed the expression of VEGF isoforms using RT-PCR and correlated the pattern of expression with clinical details, finding that tumours with pathological stage pT3-4 showed expression of VEGF121 + 165 + 189 at a significantly higher incidence than those with pT0-2. Their observations may suggest that the VEGF 189 isoform is closely associated with angiogenesis and results in growth of RCC. No preferential expression of either VEGF isoform was seen for any stage or grade with the highest numbers being in Robson 1 and 4 for the total population and clear cell (CCRCC) population. Meaning that using VEGF 165 or VEGF 189 mRNA expression levels alone does not provide any significantly useful prognosis information for RCC patients. Although circulating serum protein levels and levels measured by IHC have been used as a prognostic marker [Dosquet *et al* 1997, Paradis *et al* 2000, Jacobsen *et al* 2000, Jacobsen *et al* 2002]. There are a number of possible reasons for lack of correlation found between the RCC patients' expression of VEGF and tumour grade and stage. The techniques that have been employed were different from other studies who have demonstrated a positive correlation (RPA vs. RT-PCR) [Nicol *et al* 1997, Thelen *et al* 1999]. Measurement of the VEGF protein in comparison with the mRNA levels may also produce differences in the results [Sato *et al* 1999, Jacobsen *et al* 2000]. By measuring protein VEGF (by ELISA and immunohistochemical studies) a pan VEGF antibody has been used for the studies [Sato *et al* 1999, Jacobsen *et al* 2000, Paradis *et al* 2000, Lee *et al* 2001]. This may have provided a correlation between increasing stage

and increasing expression but does not take into account differences between isoform expression. It would therefore be important to distinguish between the expression of the various VEGF isoforms, and with the identification of a new inhibitory VEGF isoforms may complicate the picture further [Bates *et al* 2002]. Measurement of the VEGF protein may therefore also include the levels of these inhibitory isoforms, providing an inaccurate biological picture of true VEGF expression in the tumour and normal tissue.

A number of these studies were in a Japanese population and there may be ethnic differences in the expression of these factors in comparison with a Caucasian population [Tomisawa *et al* 1999, Takahashi *et al* 1999]. Significant increases for both male and female VEGF 165 and VEGF 189 tumour vs. normal were seen.

Methods used to stage and grade RCC have been analysed in conjunction with the expression of VEGF 165 and VEGF 189. Levels of VEGF 165 and VEGF 189 in tumour vs. normal were significant for both Fuhrman grades 2 and 3, although there was no relationship between Fuhrman grade and these expression levels. Indicating that the expression of VEGF is not altered according to the higher grading of the tumour. The positive correlation between HIF-1 α and VEGF 165 and VEGF 189 mRNA expression demonstrates that increasing levels of HIF-1 α correlate with increasing levels of VEGF. It has been shown in normal cells that HIF-1 α levels are regulated at the protein, not the mRNA level [Maxwell *et al* 1999]. The majority of clear cell carcinomas have mutations in the VHL gene, which in turn regulates the levels HIF-1 α protein by the attachment of ubiquitin molecules that direct it to the proteasome for destruction [Clifford *et al* 2001b].

Increases in HIF-1 α expression were seen in the tumour tissue in comparison with the normal tissue. After division into histological subtypes, the clear cell population was the only one with significant increases in mRNA expression of HIF-1 α . The expression of GLUT-1 was also increased in the tumour tissue in comparison with the normal. It was

demonstrated that the expression of HIF-1 α correlated with an increase in expression of GLUT-1 in both tumour and normal tissue. It would be expected that increases in GLUT-1 are seen in the tumour tissue in comparison with the normal tissue due to the increased metabolic activity of the tumour cells [Kunkel *et al* 2003]. Significant increases were only seen for GLUT-1 in the Fuhrman grade 3 patients and may relate to the more aggressive nature of the Fuhrman grade 3 tumours, due to their requirement for increased glucose and subsequently increased numbers of glucose transporters. In comparison Levels of HIF-1 α are regulated by hydroxylation of proline residues 402 and 564 and asparagine residue 803 (under normoxic conditions) in the protein and therefore only slight increases were seen at the mRNA level in the tumour tissue.

The pattern of expression of both HIF-1 α and GLUT-1 for Robson stage was not conclusive of any relationship between either species. Significant increases were seen in the whole RCC population for Robson stage 3 HIF-1 α tumour vs. normal. The CC RCC population had increases in Robson stage 3 HIF-1 α tumour vs. normal and Robson stage 1 GLUT-1 tumour vs. normal tissue, yet there was no particular trend in the data. There was no evidence that increased expression of HIF-1 α , and GLUT-1 may correlate with the Robson stage. The failure to find a correlation may be due to the small numbers in the study, heterogeneity of the tumour samples or the late stage of disease that the tumour was in.

HIF-1 α and GLUT-1 both showed significant increases in the male population, mirroring a similar pattern for the VEGF isoforms. With only HIF-1 α and GLUT-1 being significantly increased in the males this may point to another confounding factor as to why RCC in males is more prevalent.

In RCC, mutation or hypermethylation of the VHL gene may disrupt the binding of HIF-1 α to pVHL, subsequently leading to increases in HIF-1 α and the target genes of this transcription factor [Maxwell *et al* 1999]. GLUT-1 is one of target genes of HIF-1 α ,

consequently, if there is an unregulated increase in HIF-1 α protein the knock on effect will be increased expression of its targets including GLUT-1.

Although increased expression of HIF-1 α was shown in tumour tissue in comparison with the adjacent normal tissue it will not necessarily lead to increases in HIF-1 α protein. It has been shown that regulation of HIF-1 α occurs at the protein and not the RNA level [Maxwell *et al*, 1999].

These differences between the studies could be accounted for by the technique employed, and differences in the ethnic makeup of the population, differences in genetics between the Caucasian and Japanese populations. In this study the highly sensitive and quantitative RPA was used, rather than relying on a DNA amplification technique such as RT-PCR [Nicol *et al* 1997, Tomisawa *et al* 1999].

Increasing the sample numbers may provide a statistical significance and warrants further study, as mRNA levels appeared to increase with increasing stage. It is possible therefore that if patients with a high baseline expression of HIF-1 α and GLUT-1 develop RCC, the tumours may be of a more aggressive nature. This may reflect the effect of different patients' expression pattern on the control of angiogenesis in renal cell carcinoma.

4.2 VEGF polymorphisms discussion

The VEGF gene is highly polymorphic with a large number of genetic changes associated with the gene. More and more SNPs are being discovered and analysed in regard to disease and production of the protein. Polymorphisms in the VEGF gene have been shown to be implicated in diseases ranging from diabetic nephropathy/retinopathy to allograft rejection, skin and prostate cancer [Awata *et al* 2002, Shahbazi *et al* 2002, Howell *et al* 2002, Lin *et al* 2003, Yang *et al* 2003]. Given the importance of the role VEGF plays in angiogenesis, increases in expression or protein levels may play a significant role in the disease process, if the resulting formation of new blood vessels is a contributing factor to the disease.

Susceptibility to diabetic nephropathy in association with the C-2578A allele may be explained by the enhanced rate of transcription in comparison with the A allele. Yang *et al* (2003) also showed significant interaction between VEGF and the ALR2 loci, and it has previously been shown that polymorphisms in the ALR2 loci are associated with susceptibility to diabetic microvascular complications [Demaine *et al* 2000, Heesom *et al* 1997].

VEGF polymorphisms have also been recently linked to calcium oxalate stone disease and steroid sensitive nephrotic syndrome [Chen *et al* 2003, Holt *et al* 2003]. It has also been shown the various polymorphisms have a direct effect on the levels of the VEGF protein [Renner *et al* 2000, Watson *et al* 2000] and therefore may implicate these polymorphisms in the disease process.

Haplotypes of the VEGF gene containing the -460C/+405G were recently analysed and it was discovered that there were only two haplotypes containing these alleles [Stevens *et al* 2003]. The only difference between them was one allele contained the -160C to T, and the other contained the -116G to A. These two haplotypes were compared against the wild type (wt) promoter, and the haplotype containing the -116A did not differ from the wt. The

haplotype containing the -160T showed increased VEGF promoter activity compared to the one containing -116A or wt. It is yet uncertain as to whether it is loss of the -116A or gain of the -160T polymorphism which is related to expression levels.

VEGF polymorphisms C702T, C936T and G1612A have recently been demonstrated that they are not linked to RCC within a Japanese population [Abe *et al* 2002], and may corroborate the findings of this thesis. Of these polymorphisms the C936T has been shown to be linked with VEGF plasma levels, yet it does not appear to be of importance in RCC [Renner *et al* 2000].

No significant differences between patients and controls were found for any of the VEGF C-2578A polymorphism in this cohort of Caucasian RCC patients. Although this polymorphism shows no relationship with the disease, and is not linked to the Robson or Fuhrman stage/grade. It has been previously been shown to be associated with levels of mRNA expression and diabetic nephropathy [Yang *et al* 2003]. Although not significant, it was shown that alleles did appear to be related to levels of mRNA expression of VEGF 165 and VEGF 189, this is consistent with the previously published data about this polymorphism [Shahbazi *et al* 2002, Yang *et al* 2003]. The C allele was linked to higher production of VEGF mRNA and the A allele to lower mRNA levels.

When segregated into just the clear cell population, no significant differences were found for age, gender, Robson stage, or Fuhrman grade. This may indicate that this particular polymorphism does not appear to play a role in the aetiology of RCC in a Caucasian population.

Although the role of VEGF protein in the angiogenesis of RCC is not in any doubt the relationship between RCC and polymorphisms in the VEGF gene have yet to be confirmed and fully elucidated. The controlling factors surrounding the genetic analysis of the disease will require more comprehensive analysis before a complete picture of the role VEGF polymorphisms play in RCC progression, along with differing levels of the protein.

There are a number of other polymorphisms in the VEGF gene and many have been shown to be linked with protein production and serum levels [Renner *et al* 2000, Watson *et al* 2000].

Changes in the promoter region of the VEGF gene caused by polymorphisms (in this case C-2578A and an 18 nucleotide insert) affect the levels of expression of the gene. Given the highly polymorphic nature of the gene there may be SNP's that have yet to have been analysed in enough detail, which may be linked to RCC. The role in which this polymorphism plays in RCC maybe more subtle, and require elaborate measurement of patients VEGF levels to link the different genotype to a particular disease state. Even though no positive correlation between C-2578A and RCC could be found the trend between C-2578A and mRNA levels warrants further evaluation, in conjunction with polymorphisms that may also have a role in the susceptibility or onset of disease. Patients may therefore segregate into high or low expressers of the protein depending on their genotype. The importance of VEGF polymorphisms in disease is an important area of research that requires more comprehensive analysis, and in conjunction with genomic and proteomic data correlation's between these polymorphisms and disease may be elucidated.

4.3 HIF-1 α polymorphisms discussion

The role of HIF-1 α in RCC has increased recently by the elucidation of the structure function relationship between HIF-1 α and VHL. Description of the role of that PHDs play in the hypoxic regulation of the HIF-1 α has led to the importance of proline and asparagine residues in the HIF-1 α molecule [Epstein *et al* 2001, Ivan *et al* 2001, Jaakkola *et al* 2001, McNeil *et al* 2002].

It has been demonstrated that proline 564 (P564) and proline 402 (P402) are key amino acids in the oxygen dependent destruction of the HIF-1 α subunit by the 26S proteasome [Epstein *et al* 2001, Elkins *et al* 2003]. Amino acid changes within the ODD of the subunit (Proline to Serine at position 582 and Alanine to Threonine at position 588) have been described in relation to RCC and head and neck squamous cell carcinoma (HNSCC) [Clifford *et al* 2001a, Tanimoto *et al* 2003]. The single nucleotide polymorphisms (C1772T and G1790A) studied resulted in the change from a proline to serine and alanine to threonine (at codons 582 and 588 respectively) within the ODD of the HIF-1 α protein [Clifford *et al* 2001a].

Patients with HNSCC who had the rare alleles (T and G alleles) that encode the variant HIF-1 α proteins had significantly increased numbers of microvessels compared to those with the wild type alleles [Tanimoto *et al* 2003]. They also found the activity of the variant protein to be stronger than that of the wild type under hypoxic conditions. The proximity of these polymorphisms to the NTAD and ODD may mean that they affect the stability of the protein or its ability to bind to any accessory proteins involved in the response to hypoxia such as CBP/p300. Other residues in HIF-1 α may also be subject to O₂-dependent hydroxylation. The transactivation domain function is also O₂-regulated by mechanisms that remain incompletely understood. Theoretical modelling of the HIF-1 α protein has shown that transactivation activity can be affected by changes in amino acids in the

TAD/ODD. Therefore these polymorphisms may provide important differences between a diseased population, or, they may just be markers of other changes in the HIF-1 α gene that are related to disease.

These differences may be due to functional changes in the HIF-1 α protein and therefore affect the properties of the protein giving it an increased or decreased ability to transactivate and cause transcription of its target genes.

Although the polymorphisms studied have not been shown to be hydroxylated, we hypothesized that if it plays any part in the disease process then differences between the RCC and control populations will be found.

Significant differences were found between the patient and control populations with increases in the GA and CC genotypes in the patients, along with a decrease in the GG and CT genotypes. Increases in the TT and AA genotypes (for C1772T and G1790A respectively) for the whole RCC population may indicate the importance of the variant alleles in RCC, and the clear cell RCC population who also showed a similar increase of the alleles.

The T-A haplotype (mutant-mutant) was significantly increased in the patient population and could according to Tanimoto *et al* [2003] have the strongest transcriptional activation under both normoxic and hypoxic conditions. Demonstrating that these polymorphisms may have a role in the molecular genetic phenotype of RCC irrespective of the conditions that the cell is under. Increases were also seen for the C-A haplotype, and slight decrease in the T-G haplotype. The combined genotype TC/GG was decreased for both the whole and clear cell RCC populations and only contained one variant allele and three wt alleles indicating the importance of the variant alleles in RCC. The combined genotype TT/GA was increased in the patients and provides further evidence that the variant alleles (three variant and one wt in this genotype) may be increased in RCC, and cause some of the features of the disease via increased transcription of the genes targeted by HIF-1 α .

Patients in Robson stage 4 (for the C1772T) had significantly increased frequency of the CC genotype and a significantly decreased frequency of the TT genotype. There were only 18 patients in the Robson stage 4 group, and therefore there is likelihood that these numbers may not be representative of the Robson stage 4 group. Robson stages 1, 2 and 3 all have similar patterns of C1772T genotype frequencies and may provide a more accurate representation of the distribution of the C1772T polymorphism in relation to Robson stage. These polymorphisms play an important role in not only the transactivational activity of the protein but ultimately may be involved in the RCC disease process. This is in accordance with the positive role that HIF-1 α has been shown to play in cancer [Zhong *et al* 1999, Bos *et al* 2001, koukourakis *et al* 2001].

These changes may affect the function of the HIF-1 α protein, and we are currently investigating these changes to measure differences in the expression levels of HIF-1 α .

If segregation into clear cell RCC does not provide any new differences between the patient and normal control groups could it be assumed that these polymorphisms may be involved in RCC but not exclusively to clear cell RCC and therefore have a wider role to play in the disease. It will be interesting to see if these polymorphisms are found in varying levels in other diseases that may be affected by aberrant O₂ sensing given the ubiquitous role of HIF-1 α in O₂ sensing.

The importance of these polymorphisms as markers of disease may in the future become more and more important as we enter the post-genomic era. A wide variety of SNP databases are being developed and provide vast amounts of information for any researcher interested in the genetic basis of disease, and include the human SNP database at www-genome.wi.mit.edu/snp/human/ and also the SNP consortium at snp.cshl.org/.

Completion of the sequencing of the human genome has been described as great importance in the treatment and elucidation of disease. Currently the true promise of the data is yet to be fully realised and may take many years before its true worth can be

measured. There is also a genome browser which can be viewed at genome.ucsc.edu/ and provides useful information regarding the genome sequence so far. Susceptibility to cancer has more recently been linked to numerous low penetrance polymorphisms acting together to disrupt a cells regulatory controls. If this is the case then the differences between variant and wt polymorphisms in RCC may be just a part of these low penetrance polymorphisms in this disease, indicating their importance in the surveillance of RCC.

After diagnosis of RCC it will be important to screen the patients using SNP's or expression and serum levels of proteins such as VEGF to determine whether they fall into any particular group of increased risk patients. If for instance a patient has a HIF-1 α genotype that suggests increased transcriptional activity of HIF-1 α , and also has a high level of VEGF in their serum it may be linked to either having or the possibility to develop a more aggressive tumour. Treatment can then be tailored accordingly to suit the nature of the genetic phenotype of the patient. This would save time and money in the treatment of the patient and provide a customised approach to the diagnosis and treatment of disease. This approach may not be currently possible but as the data from the elucidation of the genome, proteome and transcriptome becomes available new possibilities like this will be ever more achievable.

4.4 Conclusions

- In conclusion;
- Increases in VEGF 165, VEGF 189, HIF-1 α , and GLUT-1 were seen in mRNA in the tumour tissue of RCC patients in comparison with the normal tissue.
- No relationship between tumour stage and grade for either VEGF or HIF-1 α and GLUT-1 was seen.
- VEGF polymorphism in the 5' promoter region (C-2578A) was not associated with RCC or progression of the disease.
- HIF-1 α C1772T polymorphism showed decreased numbers of the CT genotype and increases in the CC and TT genotypes.
- HIF-1 α G1790A polymorphism showed decreased numbers of the GG genotype and increases in the GA and AA genotypes.
- The HIF-1 α polymorphism (C1772T) CC genotype was increased in patients at the higher Robson stage, while the TT genotype was decreased, with the C allele being increased and the T allele being decreased.
- Combined genotype analysis demonstrated that TC/GG was decreased and may have a protective role by providing reduced transcriptional activity under normoxic and hypoxic conditions.
- Haplotype analysis showed that T-A was increased and may be linked to increased transcriptional activity under hypoxia and normoxia.

4.5 Future work

Given the importance of the various genes in the VEGF family, it is intended that the whole spectrum of these genes be analysed in both the tumour and normal tissue of RCC patients. This includes VEGF-B, C, and D, along with their associated receptors VEGFR-1, 2, and 3

Expression of VEGF₁₄₈ could be analysed in the RCC tumours and normal tissue by RT-PCR, to confirm whether this isoform is expressed in tumours as well as normal tissue. The predicted product of VEGF₁₄₈ is a truncated form of VEGF₁₆₅ but may have no biological activity and thus act in an inhibitory fashion. Although at present the complex nature of VEGF isoform expression has yet to be fully elucidated.

Expression of the HIF-1 α protein could be measured in the tumour and normal tissue by EMSA. Oligos designed to be able to bind to the protein and thus separate the transcription factor would be used in conjunction with antibodies to the HIF- α protein. Is there any correlation between the protein and downstream products of the transcription factor (VEGF, GLUT-1)?

Other polymorphisms in the VEGF gene could be examined (including G-1190A, G-1154A, C-634G, C-460T, G+405C and C+936T) which have been linked to serum levels or promoter activity of VEGF, and will the higher expressing genotypes correlate with higher Robson stage or Fuhrman grade. Polymorphisms in the VEGF receptor have been identified. Will these changes lead to functional differences, or affect the ability of the receptor to bind its ligand?

Growing out the samples of tumour that have been collected from patients undergoing radical nephrectomy. Use these cells to determine their expression of various proteins and mRNA species that may be involved in the development of RCC under *in vivo* hypoxic conditions.

Use of siRNA to block the expression of HIF-1 α or VEGF in a RCC cell line or primary cell culture from cells grown from patients undergoing radical nephrectomy for RCC. Will the silenced HIF-1 α reduced the expression of its downstream genes, and could this provide a useful tool to treat RCC, by reducing the angiogenic potential of the tumour? It has been suggested that using siRNA to sequentially target the key proteins involved in cancer development it may be possible to arrest the tumour growth.

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