Involvement of the *WT1* and *p16* Genes in Wilms' Tumour and Human Malignant Mesothelioma

Kathleen A. Williamson

Ph.D.
The University of Edinburgh
1996

Declaration

I declare that this thesis has been composed by myself, and that all of the work is my own unless otherwise stated.

Kathleen A. Williamson
February, 1996

Acknowledgements

I would like to thank Veronica, my MRC supervisor, and Stewart, my University supervisor, for all the help they have given to me during both the lab. based and library based phases of this project.

In the lab. Anne and Co. provided a great working environment, happy and industrious, and many people were always willing to give excellent advice and discuss results. I would particularly like to thank Melissa, for showing me the ropes of mutation analysis, Cathy, for her expertise on all aspects of PCR, and Jane, for her expertise on all aspects of HOT. During the latter stages of this project Sheila helped me find many references and Douglas drew (and drew and drew) many figures and tables for me, including all of the ones in this thesis; I would like to thank both of them for all their help.

Also, to all at the Unit who have become such good friends of mine over the last few years I would like to say a big thank you for being a great bunch of people - and start saving for those plane tickets now.

It is with these same feelings that I want to thank all of my friends who will probably not understand any of this thesis but who understand me perfectly. They have provided much love, support and stability; special thanks go to Vicky, Peter and my family.

Abstract

Normal cellular homeostasis is established and maintained by the positive and negative regulatory activities of many genes. When these genes are mutated to forms that can contribute to tumorigenesis, either by dominant, gain of function mutations in positive regulators or recessive, loss of function mutations in negative regulators, they are termed oncogenes or tumour suppressor genes, respectively. Identified human tumour suppressor genes, though still relatively few in number, are found to be active in many different cellular processes. I have studied two such genes, the *WT1* and *p16* genes, which in their wild type forms are known to contribute to development of the urogenital system and the mesothelium (*WT1*) and to control of the cell cycle (*p16*).

WT1 was identified as a gene repeatedly disrupted in Wilms' tumour, a common paediatric renal malignancy. This study is one of many which sought to confirm and understand some of the roles of *WT1* in both normal development and tumorigenesis. Mutation analysis of *WT1* in samples from patients with sporadic, bilateral and syndrome-associated Wilms' tumour has produced a pattern of results consistent with the findings of other groups. No mutations were detected in the sporadic and bilateral tumours, however exonic point mutations were detected in 7 out of 9 syndrome-associated Wilms' tumour samples that were analysed in detail. Studies examining parental *WT1* status, genomic imprinting and allele loss distal to the *WT1* locus are detailed for the syndrome-associated Wilms' tumour samples. Additionally, analysis of *WT1* in samples from patients with testicular tumours or malignant mesothelioma did not identify any mutations believed to be significant in tumorigenesis.

p16 was identified not only as a cell cycle regulator, but also as a gene repeatedly deleted in tumour cell lines. Previous studies showed that tumours of various types, including primary malignant mesothelioma, often contain deletions spanning the *p16* locus. In this study detailed deletion analysis is presented for *p16* and the adjacent related gene, *p15*, in Wilms' tumour and malignant mesothelioma samples. Both primary tumours and tumour cell lines were analysed. Deletions of *p16* were not detected in Wilms' tumour and derived tumour cell lines. Deletions of *p16* were however detected in all malignant mesothelioma cell lines analysed, and in the majority of these (14 out of 15) the deletions extended to the *p15* locus. Analysis of *p16* and *p15* in primary malignant mesothelioma samples did not identify deletions of either gene.

The contribution, resulting effects and complementary nature of *WT1* and *p16* mutations in Wilms' tumour and malignant mesothelioma is discussed.

Contents

Figures

Tables

Abbreviations

A	adenine (nucleic acid context) or alanine (protein context)
BOR	branchio-oto-renal syndrome
bp	base pair
BWS	Beckwith-Wiedemann syndrome
C	cytosine (nucleic acid context) or cysteine (protein context)
CDGE	constant denaturant gel electrophoresis
CDK	cyclin dependent kinase
cDNA	complementary DNA
^{o}C	degrees centigrade
D	aspartic acid
DDS	Denys-Drash syndrome
DEPC	diethyl pyrocarbonate
DGGE	denaturing gradient gel electrophoresis
dH_2O	distilled water
DMS	diffuse mesangial sclerosis
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleoside triphosphates
DTT	dithiothreitol
E	glutamic acid
EDTA	ethlyenediaminetetra-acetic acid
EGR1	early growth response 1 protein
EGR1	early growth response 1 gene
F	phenylalanine
G	guanine (nucleic acid context) or glycine (protein context)
GU	genitourinary
H	histidine
HA	hydroxylamine
HOT	hydroxylamine and osmium tetroxide
I	isoleucine
IGF2	insulin-like growth factor 2 gene
ILNR	intralobar nephrogenic rest
K	lysine
kb	kilobase pair
KTS	lysine, threonine and serine, *WT1* alternative splice II

L	leucine
LCL	lymphoblastoid cell line(s)
LMP	low melting point
LOH	loss of heterozygosity
M	methionine
Mb	megabase pair
$MgCl_2$	magnesium chloride
MM	malignant mesothelioma
mRNA	messenger RNA
MTS1	multiple tumour suppressor gene 1
N	asparagine
NaCl	sodium chloride
NaOAc	sodium acetate
NaOH	sodium hydroxide
OsO_4	osmium tetroxide
P	proline
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PDGF-A	platelet derived growth factor A-chain gene
PGD	partial gonadal dysgenesis
PLNR	perilobar nephrogenic rest
PNK	polynucleotide kinase
Q	glutamine
R	arginine
RB1	retinoblastoma predisposition protein
RB1	retinoblastoma predisposition gene
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNase A	ribonuclease A
RT	reverse transcription
S	serine
SDS	sodium dodecyl sulphate
SRO	smallest region overlap
SSCP	single strand conformation polymorphism
T	thymidine (DNA context) or threonine (protein context)
T_A	annealing temperature
TD	transregulatory domain
TGGE	temperature gradient gel electrophoresis

T_M	melting temperature
UV	ultra violet
V	valine
W	tryptophan
WAGR	Wilms' tumour, aniridia, genitourinary abnormalities and mental retardation
WT	Wilms' tumour
WT1	Wilms' tumour predisposition protein
WT1	Wilms' tumour predisposition gene
Wt1	murine orthologue of *WT1*
Y	tyrosine
ZF	zinc finger
17aa	17 amino acids, *WT1* alternative splice I

Chapter 1
Introduction

1.1 Contribution of tumour suppressor genes to tumorigenesis

1.1.1 Mode of action

Normal cellular homeostasis is established and maintained by the positive and negative regulatory actions of many genes. Collectively these genes can encode many different types of product, for example functional RNA molecules, cell surface and cytoplasmic proteins, and nuclear proteins that include transcription factors. This variety of products suggests that when any one (or more) of these genes is disrupted a wide spectrum of cellular processes may be affected, perhaps causing a breakdown of normal homeostasis. This in turn can result in aberrant cell growth and division, a feature which is the hallmark of all forms of tumorigenesis, including development of both benign and malignant tumours. When genes are mutated to forms that can contribute to tumorigenesis, either by dominant, gain of function mutations in positive regulators or recessive, loss of function mutations in negative regulators, they are termed oncogenes or tumour suppressor genes, respectively.

In comparison to identified human oncogenes, identified human tumour suppressor genes are still relatively few in number. The cellular processes by which the latter contribute to normal development (when wild type) and tumorigenesis, although not fully understood, are known to be varied (reviewed by Bryant 1993). Detection of loss of function mutations can be used to identify candidate tumour suppressor genes. Techniques to detect such mutations include: functional analysis of growth suppression by transfection of whole or partial chromosomes, or specific genes, into tumorigenic cells; analysis of homozygous loss of markers using cytogenetic or molecular methods; analysis of loss of heterozygosity (LOH) to reveal acquisition of homozygosity for an original mutation; and positional cloning of genes and subsequent mutation analysis (reviewed by Ponder 1988, Sager 1989). Before these techniques came into use, a statistical study of a paediatric tumour, retinoblastoma, by Knudson (1971) introduced a model detailing the requirement for two mutational events for tumorigenesis in this type of early onset malignancy. This concept was later extended to indicate that these events represent functional loss of both copies of one gene, that is, the action of a tumour suppressor gene. This type of statistical study is now applied to distinct paediatric tumour types to investigate the possible involvement of tumour suppressor genes and, similarly, the model is applied to genes that are candidate tumour suppressors. This model is most commonly referred to as the two hit model for tumorigenesis.

1.1.2 The two hit model for tumorigenesis

As mentioned in 1.1.1, the two hit model for tumorigenesis resulted from a statistical study of a paediatric malignancy, retinoblastoma (Knudson 1971). Application of this model to distinct

paediatric malignancies is reported, for example for Wilms' tumour (see 1.2), however the data detailed in the original study remains the most comprehensive reported. The major aspects of this data are as follows. Retinoblastoma is a malignancy of the eye which can present as a unilateral, bilateral or familial tumour. The majority of unilateral retinoblastoma are predicted to be sporadic, whereas all bilateral retinoblastoma are predicted to be hereditary. Moreover, for individuals with the latter form or with familial retinoblastoma, 50% of offspring develop a similar tumour, indicating the involvement of an autosomal dominant gene. A further difference is that the proportion of the total number of retinoblastoma and the mean age at presentation is lower for bilateral and familial tumours than for unilateral tumours; approximately 40% of retinoblastoma are bilateral or familial and present at a mean age of 15 months, as compared to a mean age of 32 months for unilateral tumours.

To account for these differences it was proposed that development of retinoblastoma requires two mutational events, the pattern of which alters for hereditary and sporadic tumours. More specifically, individuals with hereditary tumours suffer a first mutational event, or hit, constitutionally, thereby affecting every cell, and a second hit somatically and affecting a retinal cell, whereas individuals with sporadic tumours suffer both hits somatically and affecting the same retinal cell (Figure 1.1.1). As the incidence of two somatic hits affecting the same cell is low it will, on average, take longer to generate this form of double hit, hence explaining the later mean age at presentation for unilateral retinoblastoma.

The fact that the first and second hits affect the two copies of a single gene was subsequently reported by Comings (1973), and for retinoblastoma chromosome loss studies, involving cytogenetic analysis and assaying the activity of a closely linked marker, highlighted the involvement of a locus within the region chromosome 13q14 (Knudson *et al.* 1976, Benedict *et al.* 1983). This in turn facilitated the positional cloning from this region of the actual retinoblastoma gene, *RB1*, (Friend *et al.* 1986, Lee *et al.* 1987) and numerous reports of mutation analysis of this gene have identified a pattern of mutations that are consistent with the two hit model for tumorigenesis (reviewed by Cowell and Hogg 1992). The product of this gene is a nuclear protein, RB1, which, by suppressing the activity of the E2F group of transcription factors, performs a critical role as a negative regulator of the cell cycle (reviewed by Weinberg 1995).

Furthermore, although the rate of transmission of bilateral and familial retinoblastoma indicated involvement of an autosomal dominant gene, the requirement for loss of both functional copies of *RB1* for tumorigenesis indicates that, at the cellular level, this gene is actually recessive. This apparent ambiguity can be explained by the fact that the first hit, although simply causing predisposition for tumorigenesis, can appear to be dominant when present constitutionally as a second hit, functionally inactivating the remaining wild type copy of *RB1* in a retinal cell, will occur in virtually all cases.

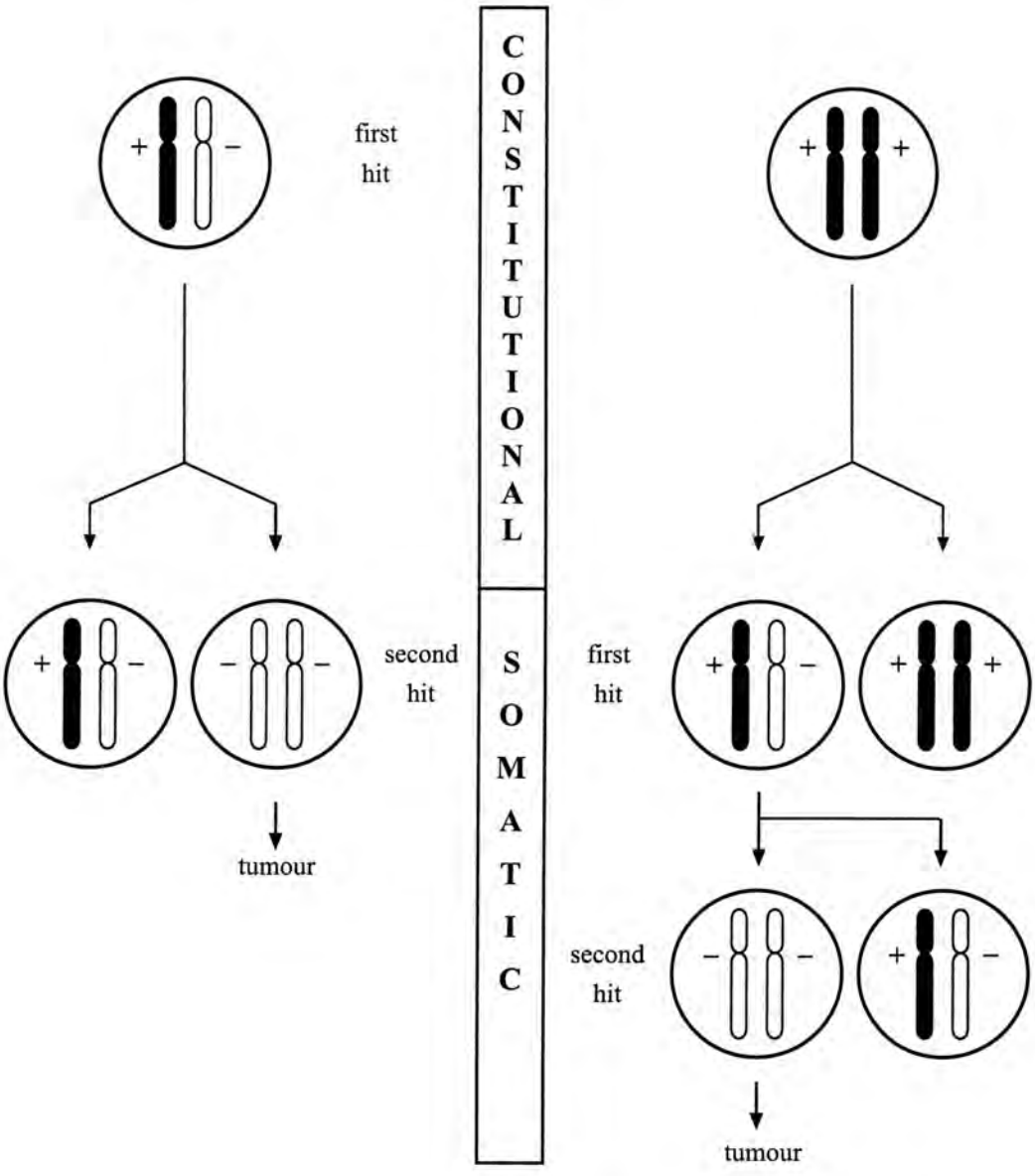


Figure 1.1.1 The two hit model for tumorigenesis. On the left of the figure the pattern of mutations predicted for hereditary tumours is illustrated; the first hit is present constitutionally and the second hit present somatically. On the right of the figure the pattern predicted for sporadic tumours is illustrated; both the first and second hits present somatically, and within the same cell.

1.1.3 Paediatric and adult tumours

The involvement of mutations in only one or very few critical genes is predicted for paediatric tumours, such as retinoblastoma, to a greater extent than for adult tumours. One reason for this difference may be that the progenitor cells of paediatric tumours have had less time than adult cells to accumulate many mutations, and are therefore less likely to display a multigene and multistep series of mutations. The proportion of adult tumours which display complexities such as the latter rather than relatively few mutations is not known.

In this study the involvement of tumour suppressor genes in paediatric and adult tumours has been investigated; analysis is focused on the paediatric renal malignancy, Wilms' tumour, and the predominantly adult tumour, malignant mesothelioma, and on two specific genes, the *WT1* and *p16* genes, which in their wild type forms contribute to distinct cellular processes. A detailed description of each of these tumour types and genes, and the reasons for their selection, is presented in the following sections.

1.2 Wilms' tumour

1.2.1 Histopathology of the kidney and of Wilms' tumour (WT)

1.2.1.1 Structure and function of the kidney

In humans the two kidneys are abdominal visceral organs, located laterally, one on either side of the vertebral column. They differ from most of the other abdominal organs as they lie out with the peritoneal cavity, adjacent to the dorsal wall of the abdomen. This retroperitoneal position is vulnerable to forces of impact and the kidneys are therefore embedded in adipose tissue for protection. Each kidney is directly enveloped by a renal capsule and is composed of three major segments; the outer renal cortex, the central renal medulla and the inner renal pelvis (Figure 1.2.1a) (van de Graaff 1995). The cortex and the medulla are comprised of nephrons, of which there are approximately one million in a mature human kidney (Lawler 1991). The nephron is the functional unit of the kidney and has a specific orientation within it (Figure 1.2.1b). The glomerulus, glomerular capsule (Bowman's capsule), proximal convoluted tubule and distal convoluted tubule structures of the nephron lie within the renal cortex, whereas the descending and ascending tubules of the nephron loop (loop of Henle) lie within the renal medulla. The distal end (relative to the glomerulus) is fused to a collecting duct, which extends through the renal cortex and renal medulla to the renal pelvis, where it opens to permit passage of contents to the ureter (van de Graaff 1995).

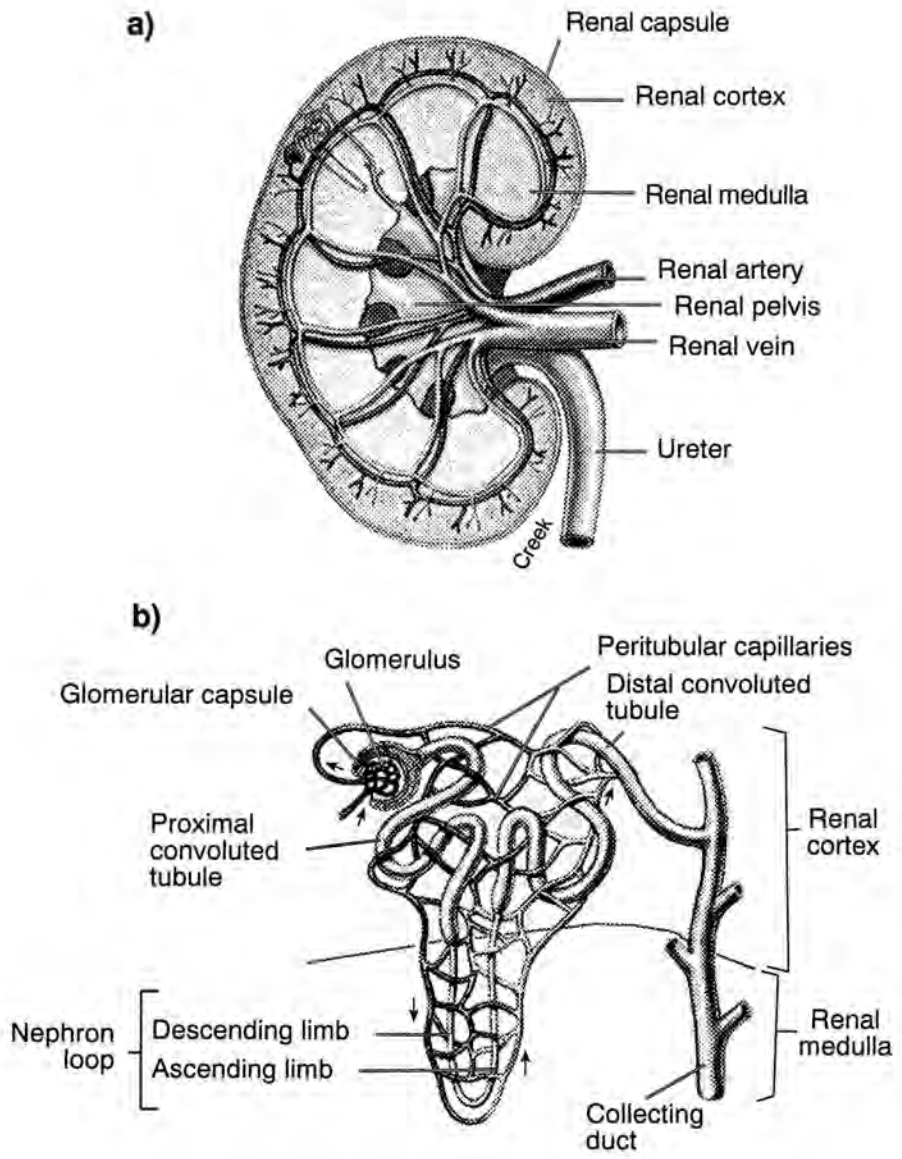


Figure 1.1.2 Structure of the human kidney and nephron. **a)** gross structure of the kidney illustrating the three major segments of this tissue; the renal cortex, medulla and pelvis. **b)** the nephron, which comprises a glomerulus and a proximal and distal convoluted tubule within the renal cortex, and a nephron loop within the renal medulla. Both drawings reproduced from van de Graaff (1995).

The functions of the kidney are to remove waste products from the blood and to maintain the fluid, electrolyte and acid-base levels of the body. Waste products are removed by ultrafiltration. Arterial blood is pumped in to the capillary network of the glomerulus and the resulting filtrate removed by the glomerular capsule to the nephron tubules. Waste products contained in the filtrate pass from here to the collecting system which transports them, via the ureter, to the urinary bladder for storage and subsequent voiding from the body. Components of the filtrate that are to be retained, such as salts, water, amino acids and glucose, are actively reabsorbed in to the blood from the proximal convoluted tubule and ascending limb of the nephron loop (van de Graaff 1995).

1.2.1.2 Development of the kidney

The mammalian kidney develops from intraembryonic mesoderm in three consecutive and overlapping stages; the pronephric, mesonephric and metanephric stages. In humans formation of pronephric structures commences during the fourth week of embryogenesis. Pronephric ducts form within the nephrogenic ridge region of the intermediate mesoderm and extend in a caudal direction to open in to the cloaca. Rudimentary kidney structures, pronephroi, join the pronephric ducts. In humans pronephroi are non-functional and they degenerate by the sixth week of embryogenesis. Their apparently unnecessary formation is considered an evolutionary remnant. The pronephric ducts do not degenerate but persist to the following stage, when mesonephroi form. These intermediate kidney structures form caudal to the pronephroi and each consists of a mesonephric tubule fused to the persistent duct, now termed the mesonephric or Wolffian duct. The free end of the mesonephric tubule forms a simple glomerular capsule and this surrounds a primitive glomerulus. Unlike the pronephroi, the mesonephroi are functional (Moore and Persaud 1993).

The progressively caudal nature of nephrogenesis is maintained in the final stage, the metanephric stage. Metanephroi commence development during the fifth week of embryogenesis and form from two distinct domains of mesoderm. The first of these is the mesonephric duct, which, at its most caudal end, develops an outgrowth, the primitive ureteric bud. This extends in a cranial direction to the second mesodermal domain, the metanephric mesenchyme which, in common with the pronephric duct, derives from the nephrogenic ridge region of the intermediate mesoderm. Reciprocal inductive interactions between these two mesodermal structures result in dichotomous branching of the ureteric bud and condensation of the metanephric mesenchyme at the tip of each branch. Successive branching of the ureteric bud forms the renal pelvis and the network of renal collecting ducts. Simultaneously, the condensed mesenchyme, termed metanephric blastema, proliferates and differentiates to form immature epithelial renal vesicles which, in turn, form comma- and S- shaped bodies, and glomeruloid bodies (Figure 1.2.2). These structures are the precursors of the more complex epithelial tubules and glomerular capsule of the mature nephron. In contrast to the proliferative nature of metanephric blastema, many cells within the uncondensed mesenchyme are eliminated during nephrogenesis by apoptosis, a form of programmed cell death which appears to be

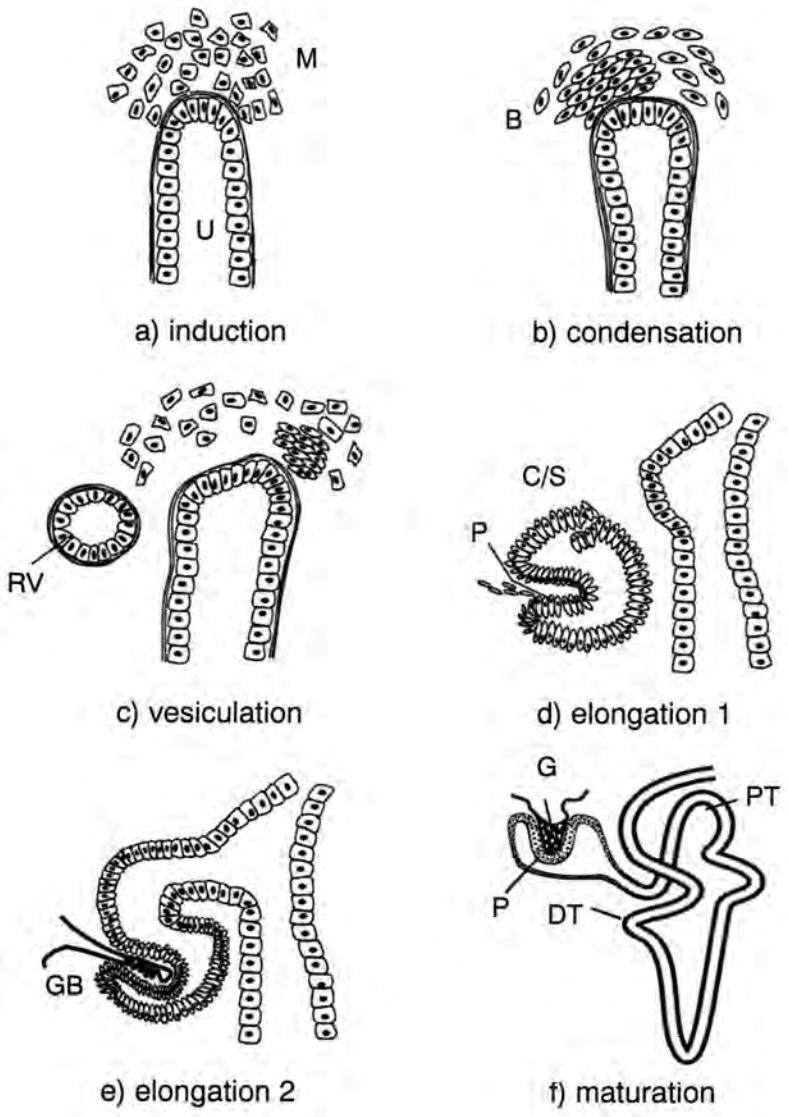


Figure 1.2.2 Development of the metanephros. **a)** reciprocal induction of the ureteric bud (U) and metanephric mesenchyme (M). **b)** condensation of mesenchyme forms metanephric blastema (B) **c)** differentiation of the latter forms renal vesicles (RV) **d)** these elongate and differentiate further to form comma- and S-shaped bodies (C/S) and podocytes (P) **e)** elongation and differentiation continues, with formation of glomeruloid bodies (GB) **f)** the mature nephron is formed, with a glomerulus (G) and a proximal and distal convoluted tubule (PT and DT).

critical for normal development of this tissue (Chandler *et al.* 1994, Koseki *et al.* 1992 [rat study]). During weeks five to nine of embryogenesis a sufficient number of mature nephrons are formed to permit the metanephroi to replace the mesonephroi as the functioning kidneys. The metanephroi continue to mature and form the permanent kidneys, which, by the end of the foetal period, contain the full complement of nephrons. Functional maturation of these nephrons is achieved postnatally (Moore and Persaud 1993).

The vascular and mesangial cells of the glomerulus differ from the other components of the nephron as they do not derive from the nephrogenic ridge region of the intermediate mesoderm but from the cardiogenic region of the lateral mesoderm (Moore and Persaud 1993). Mature glomeruli are composed of a network of capillaries that are intimately associated with two types of specialised cells, mesangial cells and podocytes, both of which are critical for the correct functioning of the glomerulus (Figure 1.2.3). Mesangial cells are interspersed within the capillary network and secrete mesangial matrix. By varying the volume of matrix these cells are thought to assist in regulating the pressure within the glomerulus (Lawler 1991). Podocytes are part of the glomerular capsule. This structure has an outer, parietal layer and an inner, visceral layer, between which is the glomerular space. The podocytes are the epithelial cells of the visceral layer, which forms a sheath over the glomerulus. These cells have numerous cytoplasmic foot processes, termed pedicels, which interdigitate to surround individual capillaries. Slits are present between adjacent pedicels and filtrate has to pass through these to enter the glomerular space. Podocytes are therefore thought to assist in regulating the flow of filtrate and, like mesangial cells, perhaps also the pressure within the glomerulus (van de Graaff 1995).

1.2.1.3 Contribution to gonadal development

Although redundant as excretory organs by the end of the embryonic period (week nine), the mesonephric structures have by this stage contributed to the developing gonadal system. This association between the renal and gonadal systems is not only temporal but also spatial. Gonads develop from the gonadal ridge, a mesodermal region of thickened mesenchyme and mesothelium, which forms during the fifth week of embryogenesis. The gonadal ridge is positioned adjacent to the mesonephroi and is therefore contiguous with the nephrogenic ridge. Both ridges are often collectively termed the urogenital ridge. The initial development of the gonadal ridge is indifferent between the sexes. Cells from degenerating mesonephric structures migrate to the latter and associate with the mesothelial component (germinal epithelium), permitting formation of tubules, the primary sex cords, within the mesenchyme (Buehr *et al.* 1993). During the sixth week of embryogenesis a second cell type migrates to the gonadal ridge, primordial germ cells. These cells, which derive from endoderm, are destined for the primary sex cords. It is a further week until the gonad is distinguishable as either male or female (Moore and Persaud 1993).

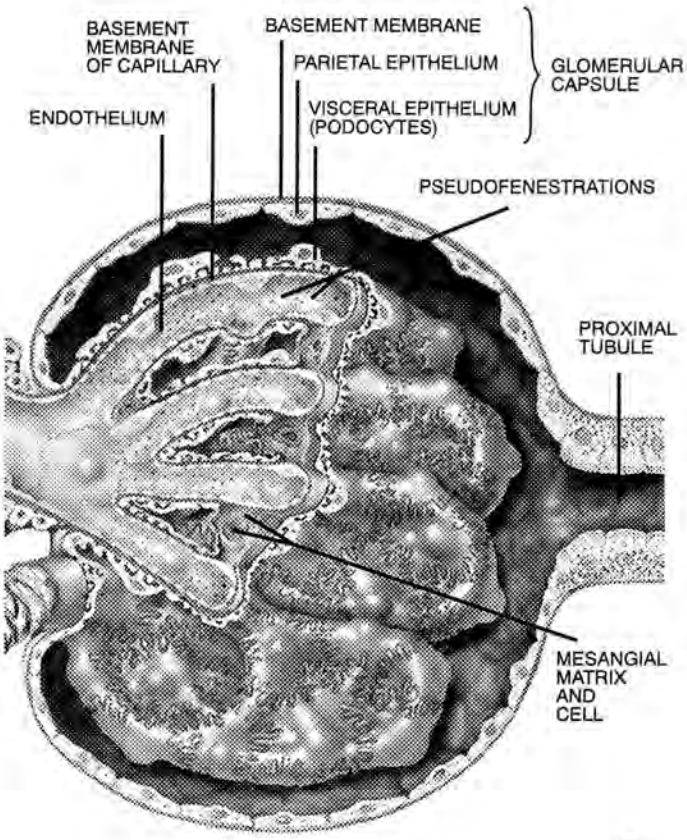


Figure 1.2.3 Structure of the glomerulus. The arrangement and gross structure of the cell types that comprise a glomerulus is illustrated. These specialised cell types include podocytes and mesangial cells, which are intimately associated with the capillary network. Drawing reproduced from Netter (1979).

If the gonad is male, development of the Mullerian duct is inhibited and the adjacent mesonephric structures are incorporated in to the developing testis and the associated genital tissues, most obviously the Wolffian duct forms the epididymis, vas deferens and seminal vesicle (Figure 1.2.4). Of vital importance at this stage are the male-specific somatic cells within the testis, of which there are two types, Sertoli cells and Leydig cells. Sertoli cells derive from and are present within the primary sex cords. These cells are the support cells of the spermatocytes/spermatids and they secrete Mullerian-inhibiting substance (MIS), which blocks development of the Mullerian duct. Leydig cells are thought to derive from mesonephric tubule epithelium and are present in the interstitium of the testis (Buehr *et al.* 1993). The main function of these cells is to produce and secrete testosterone, which is necessary for maintenance of the Wolffian duct derived structures of the genital system (Moore and Persaud 1993).

If the gonad is female, the opposite pattern to that seen in males is observed. Firstly, development of the Mullerian duct is not inhibited, as there are no Sertoli cells, and it forms the fallopian tubes, uterus and upper vagina. Secondly, the adjacent mesonephric structures are not incorporated into the ovary or the associated genital tissues (Figure 1.2.4). Some non-functional remnants of mesonephric tubules may persist in the ovary but, in general, these and the Wolffian duct degenerate because there are no testosterone secreting Leydig cells to maintain them. Female-specific somatic cells within the ovary include granulosa cells, which are thought to derive from the primary sex cords. These cells are the support cells of the oocyte and therefore their function, and origin, is similar to that of the male-specific Sertoli cells. It is evident though that by this stage of development the renal and gonadal systems are far more independent of each other in females than in males (Moore and Persaud 1993).

1.2.1.4 Structure of WT

WT is human nephroblastoma, a malignant embryonal tumour of the kidney. Embryonal tumours develop postnatally but contain structures normally present only during embryogenesis. Such tumours can therefore contain structures appropriate for the tissue of origin but wholly inappropriate for the developmental stage of that tissue, a feature which illustrates clearly the extent to which normal development and tumorigenesis are interrelated. The embryonal precursors in nephrogenesis comprise metanephric blastema and the immature epithelial structures which differentiate from it; renal vesicles, comma- and S-shaped bodies, and glomeruloid bodies. It is these normally transitory structures that present in WT. The cells of origin in this tumour are thought to be abnormally persistent nephrogenic cells, perhaps metanephric blastema. These nephrogenic stem cells may lie dormant during early postnatal life until stimulated to proliferate. Their arrest at the embryonal stage in tumorigenesis is thought to result from an inability to respond correctly to signals for differentiation and maturation and, hence, in WT precursor structures accumulate as their further differentiation to form mature nephrons is blocked.

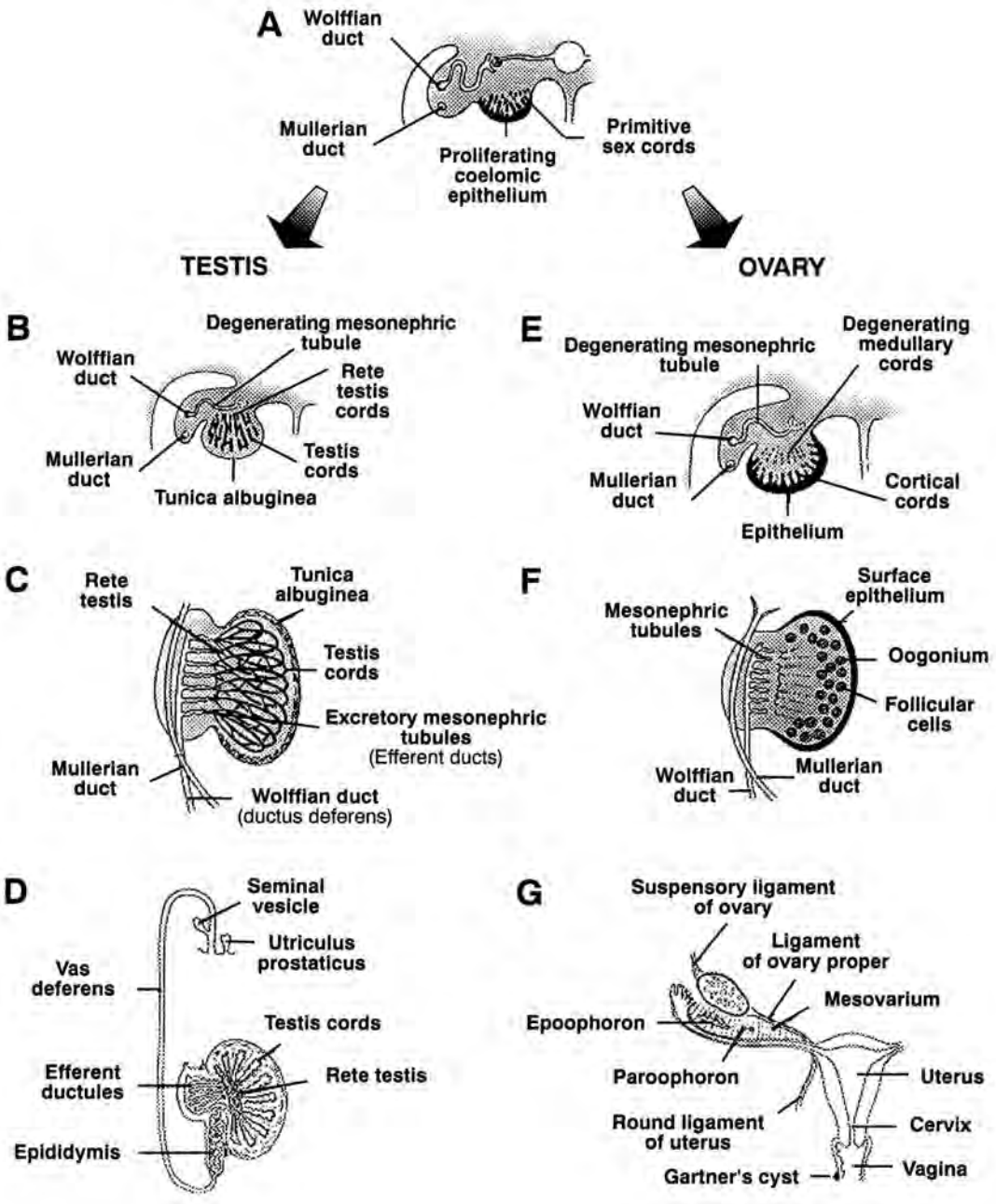


Figure 1.2.4 Mesonephric contribution to gonadal development. **a)** the indifferent gonad. The mesonephric (Wolffian) duct and tubules contribute to the sex cords. **b) c)** and **d)** if the gonad is male the Wolffian duct persists and mesonephric structures contribute to the efferent ducts and ductules, epididymis, vas deferens and seminal vesicle components of the testis. **e) f)** and **g)** if the gonad is female the Wolffian duct degenerates. If mesonephric structures persist they are present as only non-functional remnants within the epoophoron, paroophoron and Gartner's cyst components of the ovary.

In addition to metanephric blastema and immature epithelium, WT also contain mesenchyme (stroma). This is not thought to be original uncondensed metanephric mesenchyme but rather is thought to derive, in common with the epithelial component, from differentiation of metanephric blastema (Pritchard-Jones and Fleming 1991). The majority of WT contain approximately equal proportions of blastema, epithelium and stroma and are said to display triphasic or classic histology. In WT that display a different histological pattern the proportions of each of these elements can vary, and any one of these can predominate. Furthermore, a minority of WT contain elements of heterotopic cells, such as mesodermally derived smooth and striated muscle cells, chondrocytes and adipocytes, and occasionally neuroblasts and ganglia (Sotelo-Avila 1990). These inappropriate cell types are thought to derive from aberrant differentiation of the nephrogenic stem cells along non renal pathways. This implies that these cells can be pluripotent, a feature masked during normal development when only nephrogenic signals are responded to. Only when normal development is disrupted in WT are other differentiation pathways realised.

1.2.2 Epidemiology

1.2.2.1 Incidence

The first comprehensive description of human nephroblastoma was published in 1899 by Max Wilms (reference not consulted), after whom the tumour is named. Approximately 1 per 10 000 children develop WT, making it one of the most common paediatric solid tumours and the most common paediatric renal malignancy. The majority of WT develop in the first decade of life, with a median age at presentation of 3 years for males and 3 years, 6 months for females (Breslow *et al.* 1988). The reason for the difference between the sexes is not clear. Only rarely does this tumour present in adults or at extrarenal sites, with less than 200 and 34 cases, respectively, reported worldwide (Mehta *et al.* 1991). It has been suggested that these rare forms of WT develop from remnants of mesonephric structures that have failed to degenerate (Akhtar 1977, Aterman 1989). The existence of congenital WT is debatable as many pathologists consider all congenital renal tumours, which are extremely rare, to be the histologically distinct tumour, benign mesoblastic nephroma (reviewed by Olsen 1984).

The incidence of WT is uniform for all but one of the parameters studied, geographical location; with figures approximately 40% lower than and 30% higher than average in Japan and the Scandinavian countries, respectively (Breslow and Langholz 1983). This variation may be an effect of the different gene pools of these populations.

1.2.2.2 Unilateral, bilateral and familial WT

The majority of WT are sporadic and unilateral (Knudson and Strong 1972, Breslow and Beckwith 1982) but between 5% and 10% are bilateral (Coppes *et al.* 1989). Individuals with the

latter form present at a median age that is lower by 1 year, that is at 2 years and 2 years, 6 months for males and females, respectively, in comparison to the majority (Breslow *et al.* 1988). The statistical study of retinoblastoma that produced the classic two hit model for tumorigenesis (Knudson 1971) (see 1.1.2) has similarly been applied to WT (Knudson and Strong 1972). Many aspects of WT aetiology are found to differ from the classic model (see below), however the earlier presentation of bilateral tumours does correlate. Similarly to the extended model for retinoblastoma, individuals with bilateral WT are predicted to have a constitutional first hit affecting a predisposition gene and a somatic second hit affecting the remaining copy of this gene. Individuals with sporadic, unilateral WT will most often have suffered both hits somatically and therefore these tumours present at a later age, again concordant with retinoblastoma statistics. These data therefore provided evidence for involvement of a tumour suppressor gene in WT development. In contrast to the retinoblastoma statistics however, the predicted frequency of bilateral WT is approximately four times that of the observed frequency, and a further discrepancy is evident when familial WT is considered; only approximately 1% of WT present in this form. It is however important to note that, as described by Knudson (1993), comparisons between retinoblastoma and WT may not always be appropriate as the former is known to be a monogenic disorder whereas the complete genetic basis of the latter remains unknown. Nonetheless, the paucity of inherited WT (bilateral and familial) does indicate that, in general, the genetic factors involved in development of this tumour are either non-transmissible or are deleterious to development soon after transmission. In agreement with this proposal, from a total of 270 offspring of unilateral WT individuals (a proportion of whom will have constitutional mutations), only one child developed a similar tumour (reviewed by Pritchard-Jones and Hastie 1990). Moreover, the observation that the incidence of genital abnormalities in WT individuals is higher than average (see 1.2.2.3) suggested that the fertility of these individuals is compromised, implicating non-transmission of a predisposing mutation (van Heyningen 1990).

1.2.2.3 Associated developmental abnormalities

Approximately 8% of WT individuals have associated developmental abnormalities (Clericuzio 1993), the most common of which are aniridia (iris hypoplasia), genitourinary (GU) abnormalities and hemihypertrophy (asymmetrical overgrowth). The range of GU abnormalities observed includes those most frequently affecting the genital system; hypospadias (misplaced urethral orifice, usually of the penis) and cryptorchidism (undescended testes), which together constitute 75% of WT associated GU abnormalities (Breslow *et al.* 1988), and those most frequently affecting the urinary system; horseshoe kidney (fusion of the superior or, more commonly, inferior poles), duplex kidney and bifid ureters (division of one or both ureters).

The frequency of these abnormalities is considerably higher than average in WT individuals; 0.8% (children with WT) versus 0.001% (children without WT) for aniridia, similarly 1.8% versus 0.9% for hypospadias, 2.8% versus 1.4% for cryptorchidism and 2.5% versus 0.003% for hemihypertrophy (Clericuzio 1993). These figures indicated that common genetic defects may

contribute to development of both WT and the associated abnormalities. Subsequent studies have identified such defects, these are described in 1.2.4.

1.2.2.4 Prognosis

Prognosis for WT is, in general, good, with overall survival rates currently approaching 90%. This figure can however vary greatly, depending on the stage and histology of the tumour. Individuals with stage I or II tumours (least aggressive) with favourable histology have a two year survival rate of 97%. This rate decreases for stage III, IV and V tumours (most aggressive, metastases) and those of unfavourable, anaplastic histology. The two year survival rate for these tumours can be as low as 52% (D'Angio *et al.* 1989). These rates are however improving as a result of continued advances made in surgical and post operative regimes. Furthermore, deaths among individuals with syndrome-associated WT (see 1.2.4) are more frequently attributable to the associated abnormalities than to the tumour and therefore overall prognosis for these individuals can be poor.

1.2.3 Precursor lesions

Nephrogenic rests are foci of nephrogenic cells which persist in the kidney postnatally but have only a weak propensity for malignancy. They are classified into two major categories, intralobar nephrogenic rests (ILNR) and perilobar nephrogenic rests (PLNR). Both ILNR and PLNR derive from metanephric blastema but each has a distinct histology and is not classified solely on lobar position. ILNR are predominantly stromal and frequently contain structures from the early stages of nephrogenesis. In addition they can also develop heterotopic elements. In contrast, PLNR are predominantly blastemal and epithelial, frequently contain structures from the later stages of nephrogenesis and rarely develop heterotopic elements. ILNR and PLNR can solely or jointly associate with metanephric blastema and other cell types derived from it to form nephroblastomatous lesions, often termed nephroblastomatosis. Extrapolation of infant autopsy data indicates the presence of PLNR-type lesions in approximately 1% of the general population (Beckwith *et al.* 1990). As the incidence of WT in the paediatric population is approximately one hundredth of this figure, it would appear that the majority of these lesions are benign and that only a minority may progress to malignancy. Evidence that progression to malignancy does occur was reported by Beckwith *et al.* (1990), with the observation that approximately 40% of kidneys with unilateral WT and virtually 100% with bilateral WT contain nephrogenic rests. Additionally, tumour histology matched that of the associated nephrogenic rest. These data indicated that, in these cases, nephrogenic rests were precursor lesions for WT, concordant with their befitting the description of WT stem cells. Furthermore, a recent study has identified an association between nephrogenic rests and WT at the molecular level (Park *et al.* 1993a). In this study specific mutations in the *WT1* gene (described in detail in 1.3) were found to be not present in normal kidney, but present homozygously in an ILNR

and associated WT, and heterozygously in a PLNR and associated WT, indicating that these tumours were clonally derived from the rests.

1.2.4 Syndrome-associated WT

1.2.4.1 Common features

A minority of WT present as a component of specific clinical syndromes (reviewed by Clericuzio 1993), the most prominent of which are listed in Table 1.2.1. Many of the abnormalities that WT individuals are predisposed to, as described in 1.2.2.3, are also observed in these syndromes. This feature, together with the degree of overlap between these syndromes, again suggests the involvement of common or interacting genetic defects in the development of WT and associated abnormalities. The WAGR, Denys-Drash and Beckwith-Wiedemann syndromes are described in more detail below.

1.2.4.2 WAGR syndrome

WAGR is an acronym derived from the components of this syndrome, WT, aniridia, GU abnormalities and mental retardation. An association between these phenotypes was first described by Miller *et al.* (1964). Subsequent cytogenetic studies showed that these individuals have constitutional, heterozygous interstitial deletions of chromosome 11p13 (Francke *et al.* 1979). The critical loci deleted are described in 1.3.1. Not all WAGR syndrome individuals develop the full spectrum of abnormalities, many have aniridia and WT in the absence of GU abnormalities and mental retardation. Virtually all cases are sporadic, with only a single example of inherited 11p13 deletion reported (Fantes *et al.* 1992). Both individuals in this report had partial spectrum WAGR syndrome, the mother had aniridia and her son aniridia, WT and horseshoe kidney. Aniridia is fully penetrant in WAGR syndrome as it is an autosomal dominant trait. In contrast, WT, and to a greater extent, GU abnormalities and mental retardation, display moderate penetrance, suggesting that constitutional heterozygous deletions of 11p13 are predisposing for the development of these traits but that additional genetic factors are involved. WAGR-WT, which present at an incidence of approximately 30% (Coppes *et al.* 1994), are most frequently unilateral, can contain ILNR-type nephroblastomatous lesions and are, accordingly, predominantly stromal (Beckwith *et al.* 1990). The predominance of this type of lesion, containing relatively uncommitted metanephric blastema, may reflect the timing of the effect that WAGR deletions have on development of these cells. Perhaps related to this, these tumours have a lower median age at presentation in comparison to those not associated with ILNR (Breslow *et al.* 1988). GU abnormalities and mental retardation also display variable expressivity. As with sporadic unilateral and bilateral WT individuals, the most common forms of GU abnormality in WAGR syndrome are hypospadias and cryptorchidism, and accordingly there is a paucity of GU abnormalities in females with this syndrome in contrast to presentation in an estimated 80% of such

Syndrome	components	references
WAGR syndrome	WT, aniridia, GU abnormalities and mental retardation	Miller *et al.* 1964
Denys-Drash syndrome	WT, nephropathy and partial gonadal dysgenesis	Denys *et al.* 1967, Drash *et al.* 1970
Beckwith-Wiedemann syndrome	WT or other embryonic tumours, gigantism, organomegaly, hemihypertrophy, exomphalos	Sotelo-Avila 1980
Perlman syndrome	WT, nephroblastomatosis, gigantism, bilateral nephromegaly, GU abnormalities	Perlman *et al.* 1975
Sotos syndrome	WT and other tumours, cerebral gigantism, developmental delay	Sotos *et al.* 1964
Bloom syndrome	WT and other tumours, growth failure, facial telangiectasia, immunity defects	Cairney *et al.* 1987

Table 1.2.1 Clinical syndromes associated with WT

males. This increased sensitivity in males is perhaps a direct result of the greater association between the developing renal and genital systems in this sex.

1.2.4.3 Denys-Drash syndrome (DDS)

DDS comprises WT, nephropathy and partial gonadal dysgenesis (PGD). An association between these phenotypes was first described by Denys *et al.* (1967) and Drash *et al.* (1970), after whom the syndrome is named. Subsequent cytogenetic studies have failed to identify gross chromosomal defects in virtually all DDS individuals investigated. Interestingly, the two exceptional individuals identified (one XY individual and one XX female) both had constitutional heterozygous deletions at chromosome 11p13 and a mixed WAGR syndrome and DDS phenotype (Jadresic *et al.* 1991, Baird *et al.* 1992b, Henry *et al.* 1993). This chromosomal and phenotypic overlap suggested a common genetic basis, involving loci at 11p13, in the development of both syndromes (described in 1.3.6). In DDS the nephropathy appears to be fully penetrant, although this may result from ascertainment bias, and is of a specific type, diffuse mesangial sclerosis (DMS) (Habib *et al.* 1985). Histological studies of DMS have identified the mesangial cells, interspersed within the glomerular capillary network, as frequently hypercellular and secreting excess matrix, which destroys the normal structure and functioning of the glomeruli and the associated podocytes (Habib *et al.* 1985, Lawler 1991). In this syndrome WT and PGD are both highly penetrant, the incidence of the former is approximately 90% (Coppes *et al.* 1994). Individuals presenting with only one of these features together with DMS must therefore be considered as DDS patients and be closely monitored as they are

at high risk for having or developing full spectrum DDS (Habib *et al.* 1985, Friedman and Finlay 1987, Jadresic *et al.* 1990, Melocoton *et al.* 1991). DDS-WT are similar to WAGR-WT in that they are associated with ILNR, are consequently predominantly stromal (Beckwith *et al.* 1990) and have a presentation age lower than the median value (Breslow *et al.* 1988). The GU abnormalities that present in this syndrome are, in contrast to those in WAGR syndrome, severe. The abnormalities observed, encompassed by the term PGD, range from streak gonad, where development of the gonadal ridge is minimal, to XY pseudohermaphroditism, where the individual is phenotypically female. Internal examination of these latter individuals, and of XX females, does however frequently reveal abnormalities such as ovotestes and persistent Wolffian duct structures. Similar to the pattern in WAGR syndrome, DDS-GU abnormalities are more prevalent in males, or XY individuals, than in females. The severity of these abnormalities in both sexes, together with the high frequency of paediatric death, often from end stage renal failure (ESRF), dictates that all cases of full spectrum DDS arise sporadically.

1.2.4.4 Beckwith-Wiedemann syndrome (BWS)

BWS is an overgrowth syndrome, with both complete and incomplete forms described (Sotelo-Avila 1980). In a proportion of affected individuals constitutional rearrangements that disrupt loci at chromosome 11p15 have been identified (Grundy *et al.* 1991, Henry *et al.* 1991). These disruptions always favour overrepresentation of the paternally derived alleles and indicated the involvement of imprinted genes at this region (see 1.2.6). In addition to sporadic presentation, familial BWS is also observed and is similarly associated with 11p15 (Koufos *et al.* 1989). BWS overgrowth can affect the whole or part of one side of the body, causing hemihypertrophy, or specific organs and tissues such as the tongue and umbilicus, causing macroglossia and exomphalos, respectively. Perhaps as an extension of this general feature, the development of WT and other embryonal tumours is moderately penetrant in this syndrome, the incidence of the former is approximately 5% (Coppes *et al.* 1994). BWS-WT differ from WAGR-WT and DDS-WT in a number of features. Firstly, and most obviously, loci at 11p15 rather than 11p13 are implicated in tumour predisposition. Secondly, when nephroblastomatous lesions are present they are of PLNR-type and, accordingly, BWS-WT are predominantly bastemal and epithelial (Beckwith *et al.* 1990). This suggests that the nature of the constitutional defects in BWS affects metanephric blastema at a later stage of development, when it is more committed. Thirdly, and perhaps a reflection of the previous feature, the median age at presentation of these tumours is unremarkable (Breslow *et al.* 1988).

1.2.5 11p13

The involvement of 11p13 loci in WT development was initially highlighted by the constitutional heterozygous deletions of this region in WAGR syndrome individuals. Subsequent

studies have showed that between approximately 30% and 40% of WT display LOH at 11p, further implicating this region (Schroeder *et al.* 1987, Williams *et al.* 1989, Pal *et al.* 1990). As one consequence of LOH is loss of both functional copies of a gene, these studies also suggested a requirement for such loss of at least one gene in this region. In WAGR-WT loci at 11p13 are hemizygous and therefore only one additional event could, similar to LOH, cause loss of both functional copies of genes in this region. These data provided strong evidence that the putative tumour suppressor gene involved in WT development, suggested by Knudson's two hit model for tumorigenesis, was allelic with the putative WT predisposition gene at 11p13. Interestingly, one study has identified LOH at 11p in normal tissues of WT individuals, indicating that this feature is compatible with normal development (Chao *et al.* 1993).

LOH can be achieved by a number of mechanisms, most frequently identified are mitotic recombination, loss and reduplication, and deletion. These events can constitute the first and/or second hit, as can independent mutation of a locus. This last mechanism differs from the others in that heterozygosity is retained. All of these mechanisms have been observed at 11p13 in WT (Figure 1.2.5). A further aspect of WT aetiology identified by these studies is that the 11p alleles lost are almost exclusively those derived maternally, highlighting the possibility that the putative WT predisposition gene at 11p13 may be imprinted, that is expressed only from either the paternal or maternal allele. A gene from 11p13 has been isolated and characterised and is recognised as the sole WT predisposition gene within this region. This gene, the *WT1* gene, is described in detail in 1.3.

1.2.6 11p15 and imprinting

More detailed studies of LOH at 11p identified that in approximately 50% of informative WT with this feature loss does not involve the p13 region but is confined to the most distal region, p15.5 (Figure 1.2.5) (Mannens *et al.* 1988, Henry *et al.* 1989, Koufos *et al.* 1989, Reeve *et al.* 1989, Wadey *et al.* 1990). Furthermore, these studies showed that, as at p13, the alleles lost at the distal region are almost exclusively those derived maternally. This again implicated the involvement of imprinting in WT development and suggested that a second WT predisposition gene, speculatively named *WT2*, is located at 11p15.5, concordant with the chromosomal rearrangements identified in BWS individuals. It is not known if the WT and BWS putative genes at 11p15.5 are allelic. Genes in this region include *p57*, a CDK inhibitor gene (Matsuoke *et al.* 1995) (see Table 1.5.1), *H19*, a putative tumour suppressor gene, and insulin-like growth factor 2 (*IGF2*). The latter two genes are known to be imprinted in humans; *H19* is exclusively maternally expressed (Zhang and Tycko 1992) and *IGF2* exclusively paternally expressed (Ohlssen *et al.* 1993, Giannoukakis *et al.* 1993). Consequently, in WT displaying LOH at p15.5 expression of the negative regulator of growth, *H19*, is virtually or completely abolished (Steenman *et al.* 1994). In contrast, expression of the positive regulator of growth, *IGF2*, is increased in many WT (Reeve *et al.* 1985, Scott *et al.* 1985). Furthermore, aberrant

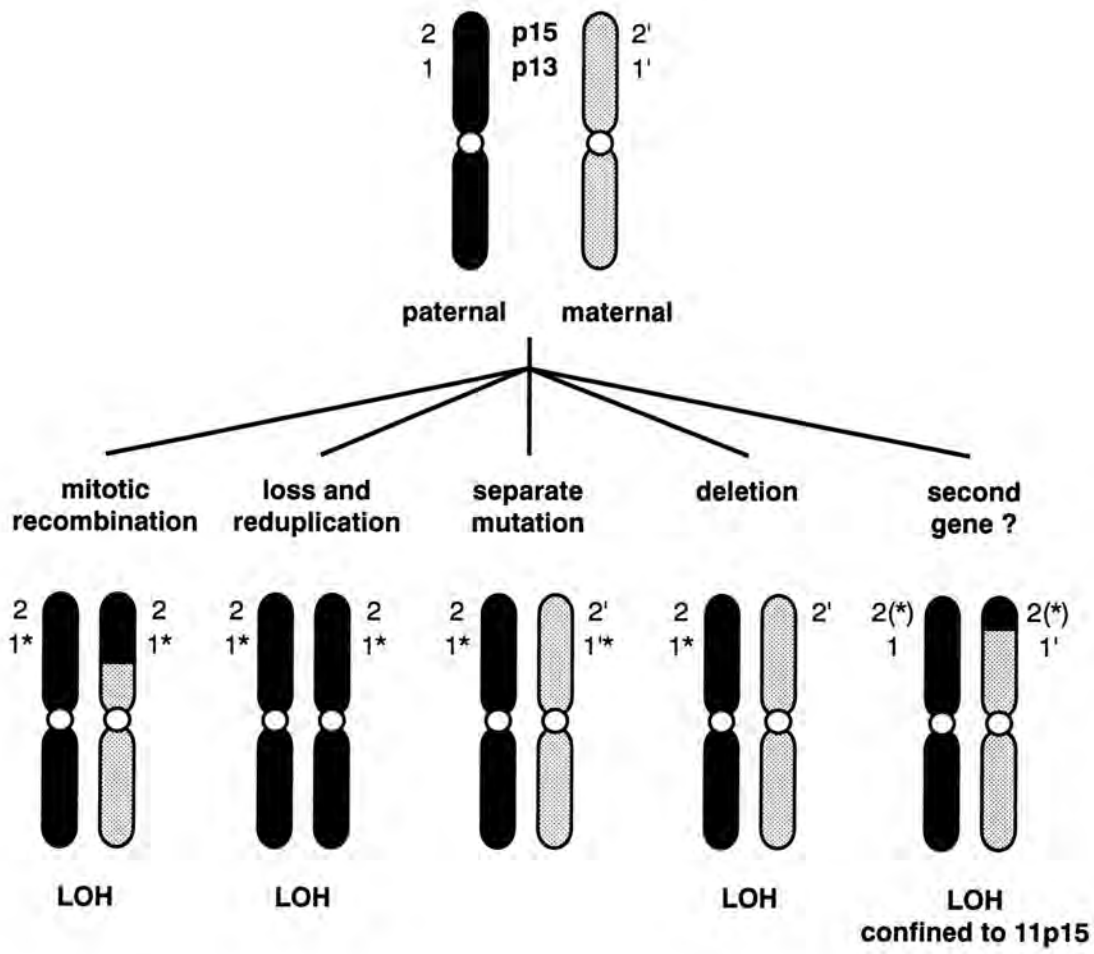


Figure 1.2.5 Mechanisms for functional loss of both copies of a chromosome 11p gene in WT development. When LOH presents, the alleles lost are almost exclusively those derived maternally, and loss can be confined to 11p15. 1 - locus at 11p13; 2 - locus at 11p15; primed numbers indicate the maternally derived allele; * - hit.

expression of *H19* and *IGF2* is also observed in WT that do not display LOH. In these tumours loss of imprinting (LOI) results in biallelic expression of one or both of these genes (Rainier *et al.* 1993, Ogawa *et al.* 1993a). More recent studies of this feature suggest that LOI of *IGF2* is associated with the down-regulation of *H19* expression (Moulton *et al.* 1994, Steenman *et al.* 1994). These aberrant expression patterns obviously contribute to growth promotion and the development of WT and BWS, however the basis of cause and effect remains ambiguous. Perhaps causing further ambiguity, the human homologue of the exclusively maternally expressed murine gene, *Mash2* (Guillemot *et al.* 1995) may also map to this region. However, as this gene is not expressed in the developing renal system, possible involvement of the human homologue in WT is not obvious.

1.2.7 Other loci

Chromosome 11 loci do not appear to be involved in development of virtually any familial and in many other WT. It is not known whether there is just a single additional locus, *WT3*, or multiple additional loci involved. The frequently observed non-random rearrangements of four specific chromosomes, 1, 7, 12 and 16, in WT supports the latter proposal. Chromosome 1 rearrangements predominantly cause trisomy 1q (Kaneko *et al.* 1983, Douglass *et al.* 1985, McDowell *et al.* 1989, Golden *et al.* 1992), chromosome 7 rearrangements comprise trisomy 7q and LOH at 7p (Wang-Wuu *et al.* 1990, Wilmore *et al.* 1994) and chromosome 12 rearrangements comprise trisomy 12 (Wang-Wuu *et al.* 1990, Golden *et al.* 1992, Austruy *et al.* submitted). Chromosome 16 rearrangements cause LOH at 16q (Kaneko *et al.* 1983, Coppes *et al.* 1992a, Maw *et al.* 1992, Grundy *et al.* 1994), and this is observed in WT at a frequency second only to that for 11p (Austruy *et al.* submitted). These data indicate strongly that there is a WT predisposition gene in this region, however linkage studies have shown that 16q is not associated with familial WT (Huff *et al.* 1992).

Breakpoints in chromosome 17p have also been reported in WT at a lower frequency (reviewed by Slater and Mannens 1992). The *p53* tumour suppressor gene maps to this region, at 17p13 (Isobe *et al.* 1986), and, as the most frequently mutated known gene in tumours, it was selected for more detailed investigation. Intragenic *p53* point mutations have been identified in a total of 17 out of 168 (10%) WT and it appears that these mutations correlate with tumour progression to anaplasia (Bardeesy *et al.* 1994a, Malkin *et al.* 1994, Bardeesy *et al.* 1995). *p53* remains the only gene other than *WT1* (described in 1.3) to be found to be specifically mutated in WT.

A number of genes that are known to be expressed during nephrogenesis, for example *PAX2* and *PAX8* (reviewed by Stuart and Gruss 1995) and *RET* (reviewed by van Heyningen 1994) may also contribute to development of WT. It is however important to note that, although such genes may be overexpressed in this tumour type, this feature may simply be a consequence rather than a cause of the malignant state of specific cells within the kidney. Furthermore, subtractive hybridisation studies

using normal kidney and WT material have isolated additional sequences, although none of these are obvious candidates for involvement in WT (Austruy *et al.* 1993).

The apparent involvement of multiple loci, including those at 11p, and the potential for synergistic interactions between them may be one reason for the incomplete penetrance of WT.

1.3 The *WT1* gene

1.3.1 Isolation and identification

As mentioned previously, partial and full spectrum WAGR syndrome individuals are constitutionally deleted for loci at chromosome 11p13. These observations highlighted this region as a target for containing genes for aniridia and WT predisposition. A distinct locus for each of these putative genes was identified as a result of characterisation of a constitutional deletion present in an individual with WT alone, and positioned the WT locus proximal to that for aniridia (Davis *et al.* 1988). The aniridia gene, *AN2*, also named *PAX6*, has been identified at the more distal locus (Ton *et al.* 1991). The position and extent of the WT locus was confirmed by the discovery of a small homozygous 11p13 somatic deletion in a sporadic WT (Lewis *et al.* 1988). This deletion covered 345kb and was proposed to encompass all or part of the putative WT gene. This positional cloning approach led to the isolation of an 11p13-specific complementary DNA (cDNA) clone, WT33 (Rose *et al.* 1990). Preliminary characterisation of this clone demonstrated that it hybridises to RNA from kidney, spleen and heart (baboon and mouse), and from WT and haematopoietic cell lines. As these features are consistent with a gene involved in development of WT, WT33 was forwarded as the first cloned candidate sequence representing the WT predisposition gene at 11p13 (Call *et al.* 1990). The corresponding gene and was named *WT1* (Haber *et al.* 1990). This gene was also isolated, using chromosome jumping techniques, in an independent study (Gessler *et al.* 1990).

1.3.2 Expression pattern

1.3.2.1 In normal development

During human embryogenesis expression of *WT1* messenger RNA (mRNA) is detected in mesonephroi and metanephroi. In the latter structures expression is restricted to the metanephric blastema and its epithelial derivatives; renal vesicles, and comma- and S-shaped bodies. As these structures mature expression becomes confined to, and maximised in, the podocytes, the cells that comprise the visceral layer of the glomerular capsule (Pritchard-Jones *et al.* 1990). These cells

continue to express *WT1* postnatally, however infant and adult levels are much reduced in comparison to those observed during nephrogenesis (Pritchard-Jones *et al.* 1990, Haber *et al.* 1990).

A second site of expression during embryogenesis is the gonadal ridge and indifferent gonad, where expression is observed in the mesothelium derived sex cords. As this tissue matures expression is confined to the male-specific Sertoli cells and female-specific granulosa cells (Pritchard-Jones *et al.* 1990), the somatic supporting cells of spermatocytes/spermatids and oocytes, respectively. Similarly to podocytes, postnatal expression is observed in Sertoli cells (Armstrong *et al.* 1992) and is predicted for granulosa cells. Further sites of expression during embryogenesis are the mesothelium, spleen, brain and spinal cord (Pritchard-Jones *et al.* 1990, Armstrong *et al.* 1992).

Similar expression patterns have been observed for the orthologue of *WT1*, *Wt1*, in mouse (Buckler *et al.* 1991, Pelletier *et al.* 1991c, Armstrong *et al.* 1992) and rat (Sharma *et al.* 1992). Furthermore, creation of *Wt1* homozygous mutant mice has shown that expression of this gene is critical for murine development (Kriedberg *et al.* 1993). These mutant mice are non viable and have bilateral renal agenesis, stemming from failure of the ureteric bud to develop, and are agonadal, stemming from failure of the gonadal ridge to thicken. Additionally, the mesothelium fails to develop correctly, causing partial diaphragmatic hernia and an incomplete pleura (see 1.4.1.2). Heart abnormalities, perhaps another consequence of abnormal mesothelial development (of the pericardium), are also observed and are thought to be the cause of death, which occurs *in utero*. It is evident, therefore, that in these mutant mice all of the tissues that fail to develop or develop abnormally are those which would normally express *Wt1*.

In addition to the above mRNA expression studies, *in vitro* translation and transfection studies have shown that WT1 is expressed as an exclusively nuclear protein (Morris *et al.* 1991, Telerman *et al.* 1992). These studies involved the use of polyclonal antisera and immunofluorescence analysis, immunoprecipitation or confocal microscopy. Similar techniques have been used by Mundlos *et al.* (1993), who confirmed this subcellular expression pattern and identified WT1 *in vivo* in podocytes, presumptive granulosa cells, Sertoli cells, and in blastemal and derived epithelial cells in WT.

1.3.2.2 In WT

WT express *WT1* at levels which are, in general, comparable to those observed in foetal kidney (Haber *et al.* 1990, Pritchard-Jones *et al.* 1990). These high levels are attained because the cell types which express *WT1* during normal nephrogenesis; metanephric blastema, the derived immature epithelium and the podocytes, are those that present in WT, where, even though malignant, they continue to express this gene. Accordingly, WT with predominantly blastemal and/or epithelial histology express far higher levels of this gene than those with triphasic or predominantly stromal histology (Pritchard-Jones *et al.* 1990, Pritchard-Jones and Fleming 1991, Miwa *et al.* 1992b, Gerald *et al.* 1992, Yeger *et al.* 1992).

1.3.2.3 Imprinting

The *WT1* locus may be imprinted in a subset of expressing tissues. Jinno *et al.* (1994) have presented evidence for monoallelic expression in a proportion of placentae (5 out of 9 investigated) and in foetal brain. When the parental origin of this expression could be determined (placentae only) it was found to be maternal. The significance of this feature in placentae, which had not previously been identified as an expressing tissue, is however uncertain as no other differences between these samples were reported. In normal kidney and WT this locus has been found to be not imprinted (Little *et al.* 1992a, Zhang and Tycko 1992).

1.3.3 Gene structure

WT1 contains ten exons which span approximately 50kb in length. Each exon contains coding sequence, exons 1 to 5 encode a proline (P) and glutamine (Q) rich region, exon 6 a linker region, and exons 7 to 10 each encode a zinc finger (ZF) motif (Figure 1.3.1) (Call *et al.* 1990, Haber *et al.* 1991, Gessler *et al.* 1992, Tadokoro *et al.* 1992b). Within the 3' untranslated region of exon 10 there is a guanine and thymidine $(GT)_n$ microsatellite sequence (Call *et al.* 1990). Transcription proceeds in a centromere proximal to distal direction and, as a result of two independent alternative splices, four mRNA isoforms of 2.944kb, 2.953kb, 2.995kb and 3.004kb are produced. Both alternative splices are within the coding sequence and therefore the open reading frame alters in length accordingly, it has a maximum length of 1.347kb.

Alternative splice I involves 51bp and comprises exon 5, which encodes 17 amino acids (17aa) within the P and Q rich region. Alternative splice II involves 9bp and utilises two splice donor sites at the 3' end of exon 9. If the downstream site is used an additional three amino acids, lysine, threonine and serine (KTS), are encoded within the linker region of ZF3 and ZF4 (Figure 1.3.1). The four mRNA isoforms are designated A (−17aa −KTS, or −/−), B (+17aa −KTS, or +/−), C (−17aa +KTS, or −/+) and D (+17aa +KTS, or +/+) and in normal kidney they are always present in a ratio of approximately 1A:2.5B:4C:8D (Haber *et al.* 1991).

The promoter of *WT1* is G and cytosine (C) rich, containing putative binding sites for the transcription factor Sp1 (Hofmann *et al.* 1993). A putative enhancer element 3' of the gene has been described (Fraizer *et al.* 1994).

1.3.4 Protein structure

The protein product of *WT1*, WT1, is between 49kD and 52kD in size (Morris *et al.* 1991, Telerman *et al.* 1992) and contains between 429 and 449 amino acids, depending on protein isoform, the ratios of which has not been determined. As mentioned above, these residues present as an N-

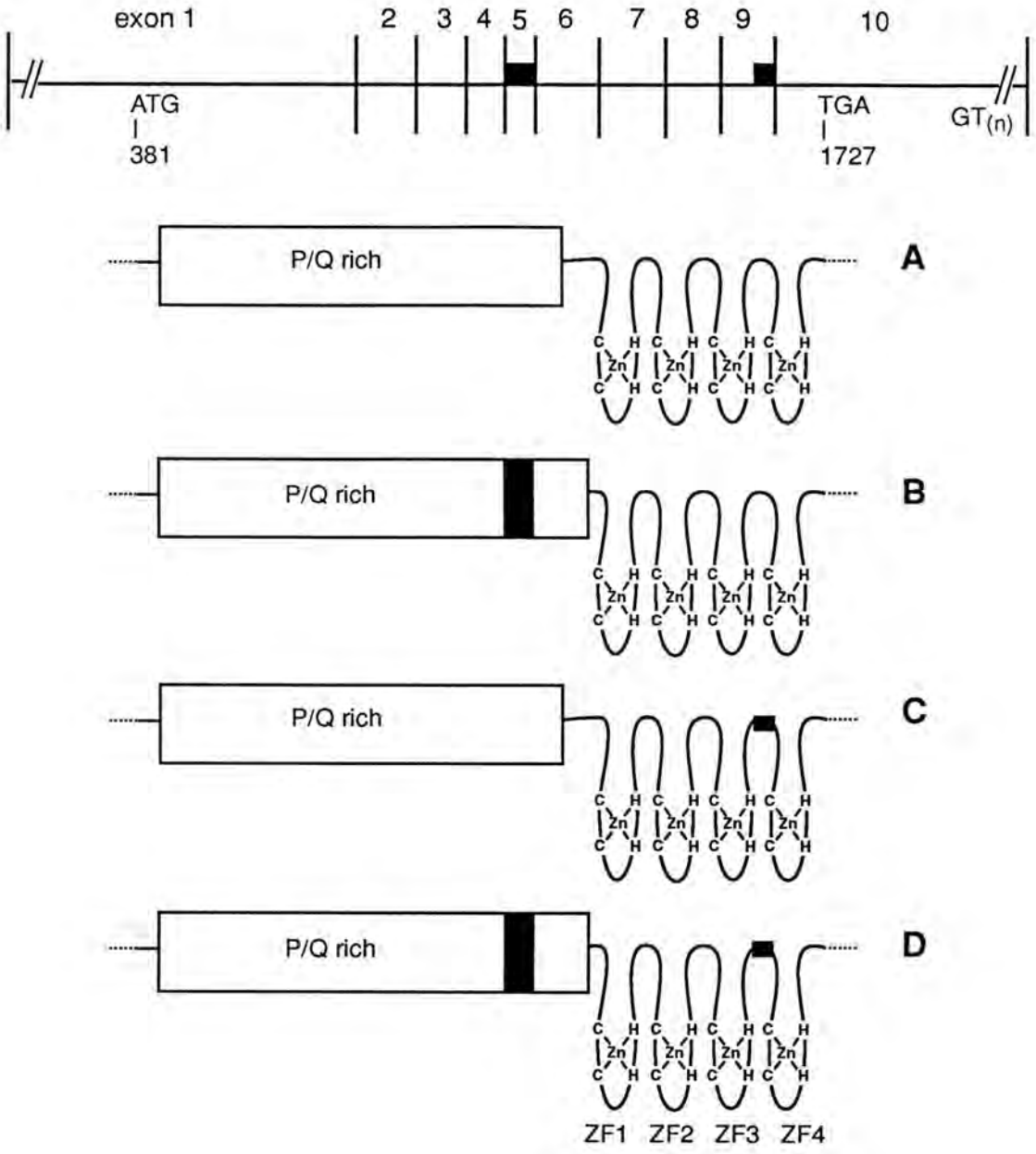


Figure 1.3.1 Structure of the *WT1* gene at the cDNA level. The relative size of each exon is illustrated along the top of the figure. Solid boxes depict the two independent alternative splices, I (17aa) and II (KTS). The four mRNA isoforms, designated A, B, C and D, are illustrated below. For each, the coding sequence is represented by a schematic illustration of the protein motifs predicted, a P/Q rich domain and four zinc fingers (ZF1-ZF4). The dotted lines depict the untranslated sequence at the junctions of the coding region.

terminal P and Q rich region, a linker region and four ZF motifs at the C-terminal. Regions rich in either P or Q residues have been identified in other proteins, such as the transcription factors CTF/NF-I (P rich) and Sp1 (Q rich). In these proteins the P or Q residues are critical components of the transregulatory domain (TD), which mediates activation or repression of transcription. The high content of these specific residues within this domain is thought to create an environment that promotes contact with other components of the transcription complex (reviewed by Mitchell and Tijan 1989). In WT1 20% of the residues within the N terminal region are P or Q. By performing domain swap experiments with the early growth response gene family member protein, EGR1 or the yeast transcription factor GAL4, both transcriptional activators, Madden *et al.* (1991) and Madden *et al.* (1993) identified this latter region of WT1 as a TD which repressed transcription. It is proposed that, similar to CTF/NF-I and Sp1, the high content of P and Q residues within the TD of WT1 permit contact with other proteins. Studies to identify such proteins, using the yeast two hybrid system, are in progress (R. Davies, personal communication). Moreover, comparison of the −17aa and +17aa WT1 isoforms has identified the latter as a stronger transcriptional repressor (Rupprecht *et al.* 1994, Wang *et al.* 1995), a feature which is thought to be mediated by the higher serine (S) content of the latter isoforms (5/17 residues encoded by alternative splice I are S) (Wang *et al.* 1995). S and threonine (T) residues are substrates for phosphorylation, a mechanism used frequently to regulate transcription (reviewed by Hunter and Karin 1992). This mechanism does not appear to be used to a great extent by WT1 (Morris *et al.* 1991), however this does not of course diminish the potential significance of any phosphorylation events that do occur. Furthermore, from DNA sequence analysis it is predicted that the most extensive region of polyP in the TD is conserved in Wt1 in mouse and rat but not in chick, and also that +17aa isoforms are not present in the latter and in alligator (Kent *et al.* 1995). This study therefore suggests that some aspects of TD function may be mammalian-specific.

A linker region, which is highly conserved, joins the TD and the four ZF motifs. Each of these motifs contains between 28 and 30 amino acids and has the consensus sequence $X_3CX_{2-4}CX_{12}HX_{3-4}HX_4$, where X is any residue, C is cysteine and H is histidine (Berg 1988). The invariant C and H residues co-ordinate a zinc atom and, as a result, a finger-like conformation is adopted (Figure 1.3.2a, schematically illustrated in Figure 1.3.1). The ZFs are the most highly conserved part of WT1, displaying 99% identity at the amino acid level in all vertebrates investigated (Kent *et al.* 1995). This suggests that any alteration to their structure is not tolerated in normal development. ZFs are present as tandem repeats in many proteins and the C_2H_2 type of WT1 are similar to those identified in the transcription factors TFIIIA and Sp1 (reviewed by Evans and Hollenberg 1988). Furthermore, WT1 ZF2, 3 and 4 are 63% identical at the amino acid level to the three ZFs of EGR1 (also named Zif268 and Krox 24) (Sukhatme *et al.* 1988). The primary function of ZFs is formation of sequence-specific deoxyribonucleic acid (DNA) binding domains. The EGR1 ZFs bind DNA at the consensus target sequence 5' GCG GGG GCG 3' (Christy and Nathans 1989) and, in this protein, specific residues at the fingertips have been identified as those that contact the target nucleotides (Pavletich and Pabo 1991). This last study also demonstrated that each ZF recognises three such nucleotides and these lie

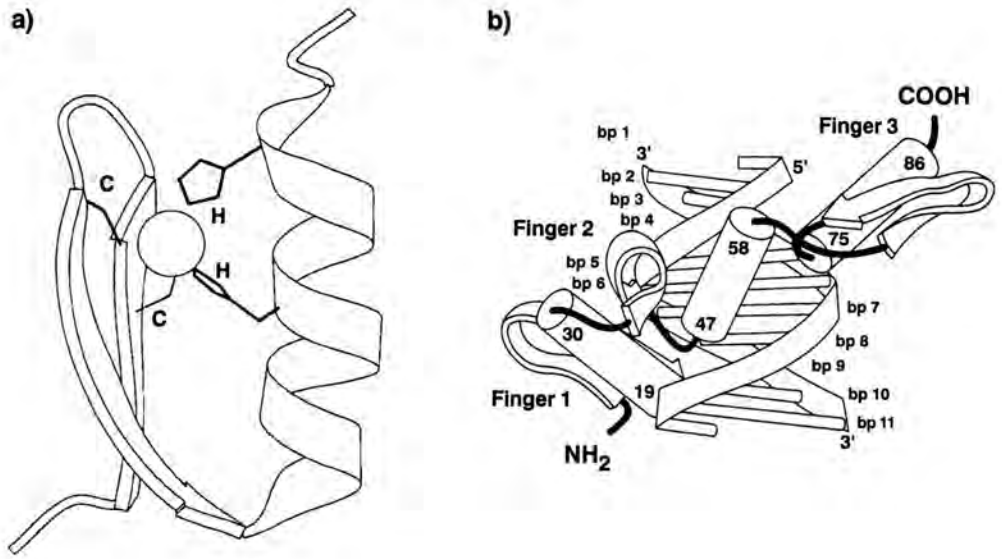


Figure 1.3.2 Conformation of zinc fingers motifs. a) schematic illustration of a zinc finger motif. The arrows represent anti-parallel β-sheet and the coil represents α-helix. The former structure contains the invariant C residues and the latter structure the invariant H residues. A zinc atom, co-ordinated by these residues, is depicted by an open circle. The junction between the β-sheet and α-helix forms the fingertip region. Drawing adapted from Berg (1988). **b)** binding of three zinc fingers to target nucleotides within the major groove. The residues that contact the target nucleotides are those at the fingertip, depicted as a solid line between arrowhead (β-sheet) and cylinder (α-helix). Each zinc finger contacts three target nucleotides. Drawing reproduced from Pavletich and Pabo (1991).

within the major groove (Figure 1.3.2b). In *in vitro* experiments WT1 −KTS isoforms, the ZFs of which are the most similar to those of EGR1, also bind this target sequence (Rauscher *et al.* 1990) and other target sequences, not exclusively G and C in composition, to which the WT1 +KTS isoforms bind (Bickmore *et al.* 1992). Recent studies have identified additional target sequences (Wang *et al.* 1993b, Rupprecht *et al.* 1994), including one to which WT1 binds with higher affinity (Nakagama *et al.* 1995), suggesting this may be a true, *in vivo* target.

Two additional features of WT1 structure have been identified more recently. Firstly, two putative leucine zipper like motifs, one discontinuous and one continuous, are encoded by exons 2, 3 and 4 (Madden *et al.* 1993). These motifs have been identified in other proteins, such as the transcription factors Fos and Jun, and function as protein-protein interaction domains (reviewed by Mitchell and Tijan 1989). If they are functional in WT1 they may permit homo- and/or heterodimerisation. Secondly, Sharma *et al.* (1994) discovered that a leucine (L) or a P residue can be present at codon position 281 (encoded by exon 6) as a result of ribonucleic (RNA) editing converting nucleotide ^{1222}uracil (U) to ^{1222}C in a proportion of mRNA. The L form may be a stronger transcriptional repressor than the P form, as demonstrated for rat WT1.

1.3.5 Protein function

The presence of a TD and a sequence specific DNA binding domain are hallmarks of transcription factors and the structure of WT1 therefore includes it as a member of this class of proteins. The previously mentioned domain swap experiments suggested that WT1 functions as a transcriptional repressor, perhaps acting on genes encoding growth promoters. This feature has been demonstrated using *in vitro* transcription assays, where WT1 −KTS isoforms bind to the promoters and repress transcription of the *EGR1* (Madden *et al.* 1991), *IGF2* (Drummond *et al.* 1992), platelet derived growth factor A chain, *PDGF-A* (Gashler *et al.* 1992, Wang *et al.* 1992), insulin-like growth factor I receptor, *IGF-IR* (Werner *et al.* 1993), colony stimulating factor I, *CSF-1* (Harrington *et al.* 1993) and retinoic acid receptor α, *RAR-αI* (Goodyer *et al.* 1995) genes. These gene promoters all contain sequences similar to the 5' GCG GGG GCG 3' consensus target sequence of EGR1, however, as this sequence is highly represented throughout the human genome, it is proposed to be present within the promoters of many other genes. There is no direct evidence that these five selected genes are true, *in vivo* targets of WT1, although *IGF2* is clearly involved in WT and is a candidate for the putative *WT2* gene (see 1.2.5). Additionally, it must be remembered that +KTS isoforms may interact with a completely different set of promoters. Recent studies have been focusing on what the true target genes may be. The observation that heterotopic elements in WT are often muscle led to experiments that indicate that myogenic genes are transcriptionally repressed by WT1 (Miyagawa *et al.* 1994). Similarly, persistent expression of *PAX2* in WT indicates that this gene may also be an *in*

vivo target (Dressler and Douglass 1992, Eccles *et al.* 1992, Ryan *et al.* 1995). A third gene identified as such a target is *WT1* itself, resulting in negative autoregulation (Rupprecht *et al.* 1994).

Additionally, it is possible that WT1 may function not only as a repressor, but also as an activator of transcription. A number of proteins are known to have such dual function (reviewed by Foulkes and Sassone-Corsi 1992) and activator regions have been identified within the WT1 TD (Wang *et al.* 1993a). This study also observed two distinct binding sites for WT1 in the promoter of the *PDGF-A* gene and noted that unless both sites were occupied, WT1 functioned as an activator. Furthermore, one study has indicated that WT1 binds p53 and switches to an activator when the latter is functionally absent (Maheswaran *et al.* 1993).

A function of WT1 distinct from that as a transcription factor has been described by Larsson *et al.* (1995). This study identified the –KTS and +KTS isoforms as partially having different subnuclear localisation, the former associating with domains of transcription factors and the latter with the snRNP components of RNA splicing complexes. Perhaps associated with this feature, it is interesting that, *in vitro*, WT1 ZFs can also bind RNA (A. Ward, personal communication). An exclusive role for +KTS isoforms in splicing is, however, unlikely because of the evidence supporting a role for them in transcription.

Overall, the utilisation of alternative splicing, RNA editing and post-translational modifications demonstrates that from one gene a considerable range of WT1 variants may be produced, and these could each have a distinct function.

1.3.6 A role in developmental abnormalities and tumorigenesis?

The fact that WT and GU abnormalities often co-develop indicated that *WT1* acts pleiotropically on the kidney and gonad (van Heyningen *et al.* 1990). Elucidation of the expression pattern of *WT1* (and *Wt1*) corroborates this proposal and suggests that mutations in this gene may contribute to developmental abnormalities and/or tumorigenesis in these tissues, and furthermore, may elicit all of the components of DDS (Pritchard-Jones *et al.* 1990). Somatic mutations alone may be sufficient for tumorigenesis, however constitutional mutations are predicted in individuals with developmental abnormalities. The loss of one copy of this gene in WAGR syndrome individuals highlighted the possibility that, at the constitutional level, having only 50% of normal levels of WT1 causes relatively mild GU abnormalities, such as hypospadias and cryptorchidism. In this situation WT1 would be described as haploinsufficient. Additionally, if *WT1* is allelic with the 11p13 tumour suppressor gene then mutation analysis should reveal functional loss of the remaining copy of *WT1* in WAGR-WT and specific functional loss of both copies in other forms of WT. This feature was suggested by the homozygous 11p13 deletion in the sporadic WT reported by Lewis *et al.* (1988), however genes additional to *WT1* may have been affected by this deletion. Furthermore, if WT1 is a transcription factor, it is obvious that constitutional or early somatic alteration of this protein could

disrupt the normal pattern of gene expression, perhaps resulting in aberrant growth and differentiation, and hence developmental abnormalities or tumorigenesis.

1.3.7 Mutation analysis in WT

Intragenic *WT1* mutations have been identified in a total of 24 sporadic unilateral and bilateral WT, representing approximately 10% of WT analysed. These mutations are detailed in Table 1.3.1 and schematically illustrated in Figure 1.3.3. With the exception of exon 5 (alternative splice I), all exons have had mutations identified within them and these can be deletions, insertions, missense or nonsense mutations. The majority of the deletions and insertions are frameshift mutations, the exceptions being one deletion that causes splicing out of exon 9 (Haber *et al.* 1990) and two deletions that remove exon 10 sequences (Cowell *et al.* 1991, Algar *et al.* 1995). At the protein level, the majority of intragenic mutations identified in these forms of WT are predicted to disrupt only the ZFs (18 out 25 mutations, 72%) or the TD and ZFs (5 out of 25 mutations, 20%). Of the 'ZF only' class, all but the four missense mutations would cause loss of at least one ZF and therefore, most likely, loss of sequence-specific DNA binding. The ZF residues altered by the missense mutations are those critical for zinc co-ordination or contact with target nucleotides and, similarly, these mutations are predicted to also cause loss of or compromise sequence-specific DNA binding. Of the 'TD and ZF' class, only residual N-terminal peptide structures are predicted, with no capacity to bind DNA directly or elicit novel phenotypes. The remaining two intragenic mutations are both exon 2 missense mutations and are therefore predicted to affect only the TD (Park *et al.* 1993a, Gessler *et al.* 1994). LOH constitutes the second hit in approximately 50% of these WT, however in four tumours heterozygosity is retained as the second hit was by independent mutation (Radice *et al.* 1993, Gessler *et al.* 1994, Varanasi *et al.* 1994). In seven WT a second hit was not identified (Little *et al.* 1992b, Park *et al.* 1993a, Gessler *et al.* 1994, Varanasi *et al.* 1994), suggesting that independent mutation within *WT1* is present but undetected or that mutations affecting other loci are also involved, perhaps in concert with a single hit at *WT1*. Alternatively, these single hits may have a dominant effect, rendering a second hit unnecessary. In the sporadic unilateral WT described by Haber *et al.* (1990) LOH and a deletion causing splicing out of exon 9 were detected, however a normal copy of *WT1* is also present, suggesting that LOH constitutes the first and not the second hit. *In vitro* studies have shown that the predicted mutant protein, named WT1-ZF3 or WTAR, has altered DNA binding (Rauscher *et al.* 1990, Drummond *et al.* 1994) and suggest that this mutant has a dominant effect (Haber *et al.* 1992, Maheswaran *et al.* 1993).

There is one report of constitutional, intragenic *WT1* mutation contributing to familial WT not associated with a clinical syndrome (Pelletier *et al.* 1991b). In this family the father, a WT survivor, transmitted a frameshift deletion within exon 6 (Table 1.3.1) to his son, who was born with GU abnormalities and developed WT. This mutation is predicted to produce a protein that lacks all four

ZFs and therefore cannot bind DNA directly. The GU abnormalities present in WAGR syndrome individuals, who are constitutionally hemizygous for *WT1*, are predicted to develop because of WT1 haploinsufficiency. As the GU abnormalities observed in this familial WT individual and in one of the bilateral WT individuals (Pelletier *et al.* 1991b) are similar to those of WAGR syndrome individuals, *WT1* mutations associated with the former individuals appear to be functionally analogous to the hemizygous *WT1* mutations present in the latter, that is they cause haploinsufficiency. This indicated that the critical aspect of mutations identified in bilateral and familial WT is complete loss of DNA binding and, if present residual N-terminal peptide structures that are non-functional. A similar nature is proposed for the majority of *WT1* mutations associated with unilateral WT, the exceptions being the two 'TD only' mutations which may contribute to tumorigenesis by a different mechanism (see below). Furthermore, similar to WAGR syndrome individuals, not all constitutional intragenic *WT1* mutations elicit GU abnormalities, as demonstrated by three of the unilateral WT individuals and two of the bilateral WT individuals (Tadokoro *et al.* 1992a, Akasaki *et al.* 1993, Coppes *et al.* 1993a, Huff *et al.* 1991, Little *et al.* 1992b).

Second hit intragenic *WT1* mutations have been identified in 8 WAGR-WT, representing the majority investigated. These mutations are detailed in Table 1.3.2 and schematically illustrated in Figure 1.3.3. Similar to the mutations detailed above, those in WAGR-WT are positioned throughout the gene and are of different types. Again, no mutations within exon 5 have been identified. All but one of the mutations create premature stop codons, through frameshift or nonsense mutations. In these cases loss of sequence-specific DNA binding is predicted, through disruption to either the ZFs only (5 out of 7 mutations), with the removal of at least two ZFs, or the TD and ZFs (2 out of 7 mutations), again resulting in residual N-terminal peptide structures that are apparently non-functional. The one exceptional mutation is an exon 3 missense mutation, predicted to affect the TD only (Park *et al.* 1993c). Functional studies suggest that this mutation, which encodes the change G201D, may results in WT1 switching from a repressor to an activator of transcription. It is possible that the two additional 'TD only' mutations described above may also elicit this effect.

The residues that are predicted to be altered most frequently in the above forms of WT are detailed in Table 1.3.3.

Forty two constitutional intragenic *WT1* mutations have been identified in DDS individuals with or without WT, representing virtually all DDS samples analysed. These mutations are detailed in Table 1.3.4 and schematically illustrated in Figure 1.3.3. The pattern of mutations in DDS differs from that observed for the other forms of WT described above. In DDS exon 9 missense mutations predominate and the residues that are predicted to be altered most frequently are detailed in Table 1.3.5. Similar mutations within exon 8 are also common. These mutations are predicted to alter ZF2 and ZF3 residues critical for zinc co-ordination or contact with target nucleotides. At the constitutional level, if such alterations resulted in complete loss of DNA binding, a phenotype of relatively mild GU abnormalities, such as those observed in WAGR syndrome individuals, would be expected. However, in DDS the GU abnormalities are severe, as is the additional feature DMS

WT	sex/ associated abnormalities	first hit	second hit	mutation	predicted disruption to TD	predicted disruption to ZFs	reference
u	nd/-	som LOH	del	25bp del in exon9-intron9		+	Haber *et al.* 1990
u	nd/-	?	LOH	70-150kb del of exon 10		+	Cowell *et al.* 1991
u	f/-	som	nd	C→T mis in exon 8,R366C		+	Little *et al.* 1992b
u	m/-	som	LOH	1bp ins in exon 10; fs		+	Coppes *et al.* 1993a
u	f/-	som	LOH	2bp del in exon 10; fs		+	
u	nd/-	som	del	asymmetrical del of central exons; fs?	+	+	Radice *et al.* 1993
u	f/-	som	nd	4bp dup in exon 2; fs	+	+	Park *et al.* 1993a
u	f/ cleft palate, down turned mouth, protub-erant eye and abdomen, asymmetrical facial hypoplasia	som	nd	T→C mis in exon 2, F154S	+		
u	nd/-	som	LOH	C→T non in exon 9, R390*		+	Varanasi *et al.* 1994
u	nd/-	som	LOH	13bp ins and 2bp del in exon 9; fs		+	
u	nd/-	som	LOH	C→T non in exon 8, R362*		+	
u	nd/-	som	ins or non	7bp ins in exon 3; fs C→T non in exon 8, R362*	+	+ +	
u	nd/-	som	nd	2bp ins and 9bp del in exon 8; fs		+	

continued/-

-/continued

WT	sex/ associated abnormalities	first hit	second hit	mutation	predicted disruption to TD	predicted disruption to ZFs	reference
u	nd/-	som	nd	C→A mis in exon 7,S338Y		+	Varanasi *et al.* 1994
u	m/-	som	mis	*WT1* del C→T mis in exon 8,H373Y		+	Gessler *et al.* 1994
u	f/-	som	del	*WT1* del 5bp del in exon 1; fs	+	+	
u	XYm/ foot anomaly	?	nd	C→T mis in exon 2, P181S	+		
u	nd/-	som	LOH	≥1kb del of exon 10		+	Algar *et al.* 1995
u	m/-	con	LOH	8kb del of exons 6 and 7; fs		+	Tadokoro *et al.* 1992a
u	f/-	con	LOH	C→T non in exon 8, R362*		+	Coppes *et al.* 1993a
u	f/-	con	LOH	C→T mis in exon9,R394W		+	Akasaki *et al.* 1993
b	f/-	con	LOH	<11kb del of exon 6; fs		+	Huff *et al.* 1991
b	m/ hypospadias, cryptorchidism	con	LOH	17bp del in exon 4; fs	+	+	Pelletier *et al.* 1991b
b	nd/-	con	LOH and nd	C→T non in exon 9, R390*		+	Little *et al.* 1992b
fa	m/ hypospadias, cryptorchidism	con	LOH	1bp del in exon 6; fs		+	Pelletier *et al.* 1991b

Table 1.3.1 Intragenic *WT1* mutations detected in unilateral, bilateral and familial WT.

u - unilateral; b - bilateral; fa - familial; m - male; f - female; nd - not determined; som - somatic; con - constitutional; fs - frameshift; del - deletion; ins - insertion; non - nonsense; mis - missense; dup - duplication; A - adenine; * - stop codon; + - present; - - absent. The standard single letter code for amino acids is used and is explained in full on page xii.

WT	sex / associated abnormalities	mutation	predicted disruption to TD	ZFs	references
u	XY/ aniridia, hypospadias, cryptorchidism and bilateral duplex kidneys	<1.4kb del of exons 2 and 3; fs	+	+	Brown *et al.* 1992 & 1993
u	nd/ aniridia, GU abnormalities and mental retardation	10bp dup in exon 7; fs		+	Baird *et al.* 1992a
u	nd/ aniridia, GU abnormalities and mental retardation	C→T non in exon 8, R362*		+	
u	XYm/ aniridia, hypospadias and cryptorchidism	1bp del in exon 7; fs		+	Gessler *et al.* 1993 & 1994
u	m/ aniridia, cryptorchidism and mental retardation	G→A mis in exon 3,G201D	+		Park *et al.* 1993c
u	f/ aniridia, nystagmus, glaucoma, gonadal agenesis, no uterine cavity and mental retardation	14bp dup in exon 7-intron7; fs		+	Santos *et al.* 1993
u	XYm/ aniridia, hypospadias, failure of scrotal fusion and persistent cloaca	C→T non in exon 9, R390*		+	Gessler *et al.* 1994
u	XYm/ unusual WAGR as 11p13 del but no aniridia; low set ears, cardiomegaly, developmental delay	5bp del in exon 2; fs	+	+	

Table 1.3.2 Second hit intragenic *WT1* mutations detected in WAGR-WT. u - unilateral; m - male; f - female; nd - not determined; fs - frameshitft; del - deletion; dup - duplication; non - nonsense; mis - missense; * - stop codon; + - present. The standard single letter code for amino acids is used and is explained in full on page xii.

WT	MS	PGD	karyo-type	second hit	mutation	predicted disruption to TD	predicted disruption to ZFs	reference
–	+	+	nd		C→T mis in exon9,R394W		+	Pelletier *et al.* 1991a
+ u[a]	+	+[b]	XY,f	LOH	"		+	
+ u	+	+	XY	nd	"		+	
+ u	+	+	XY	LOH	"		+	
+ u	+	+	XX, f	nd	"		+	
+ u	+	+	XX, f	LOH	"		+	
+ b	+	?	XX, f	nd	"		+	
+ u	+	+	XY	nd	G→A mis in exon9, D396N		+	
–[a]	+	?	XX, f		A→G mis in exon9, D396G		+	
–	+	+[b]	XY, f		G→A mis in exon8, R366H		+	
–[c]	+	+	XX		G→A mis in exon7, C330Y		+	Bruening *et al.* 1992
–[a]	+	+	XY, f		G→A in intron9, no +KTS		+	
+	+	+	XY	nd	C→T mis in exon9,R394W		+	
+	+	+	XY, f	LOH	G→C mis in exon9, R394P		+	
–[a]	+	+	XY, f		C→T mis in exon9,R394W		+	Baird *et al.* 1992b
+ u	+	+	XY	nd	"		+	
+ u	(+)	+	XY,(f)	nd[d]	"		+	
+ u[e]	+	–	XY, m	nd[d]	G→A mis in exon9, D396N		+	

continued/-

-/continued

WT	MS	PGD	karyo-type	second hit	mutation	predicted disruption to TD	predicted disruption to ZFs	reference
+ u	+	+	XY	nd	G→A mis in exon8, R366H		+	Baird *et al.* 1992b
+ b	+	+	XY	nd[d]	1bp ins in exon 6; fs		+	
–[a]	+	+	XY, m		C→T mis in exon9,R394W		+	Coppes *et al.* 1992b
+ b	+	–	XX, f	nd	〃		+	
–[a]	+	+	XY, f		C→T mis in exon8, H377Y		+	
–	+	+	nd, m		1bp ins in exon 9; fs		+	Ogawa *et al.* 1993b
+ u	+	+	XY, f	nd	C→T mis in exon9,R394W		+	Poulat *et al.* 1993
+ u	+	+	XY, m	LOH	G→C in intron 6; fs		+	Scheider *et al.* 1993
–	+	+	XY, f		G→A in intron9, no +KTS		+	Konig *et al.* 1993
–	+	+	XY, f		C→T mis in exon9,R394W		+	Sakai *et al.* 1993
+ u	+	–	XX, f	nd	〃		+	
+ u	+	–	XX, f	nd	G→A mis in exon8, C355Y		+	
–	+	+	XY, f		G→A mis in exon8, C360Y		+	Clarkson *et al.* 1993
(+ u)	+	+	XY, f	(nd)	C→T mis in exon9,R394W		+	
+ u	+	–	XX, f	nd	C→T mis in exon9, H401Y		+	Baird and Cowell 1993
–	+	+	XY, f		C→T mis in exon9,R394W		+	Tsuda *et al.* 1993

continued/-

-/continued

WT	MS	PGD	karyo-type	second hit	mutation	predicted disruption to TD	predicted disruption to ZFs	reference
+	+	–	XX, f	nd	T→C mis in exon9, L398P		+	Tsuda *et al.* 1993
–[a]	+	+	XY		C→T mis in exon9,R394W		+	Nordenskjold *et al.* 1994
+ u	+	–	XX, f	LOH	G→A mis in exon9, D396N		+	
+ u	+	+	XY	LOH	"		+	
+ u	+	+	XY	LOH	A→G mis in exon8, H377R		+	
–	+	+	XY, f		G→A in intron9, no +KTS		+	Bardeesy *et al.* 1994b
+ u	+	–	XX, f	nd	1bp del in exon 6; fs		+	
+ b	+	+	nd, m	nd	C→G non in exon 3,Y221*	+	+	
–	+	+	XY, f		G→A mis in exon8, R366H		+	Devriendt *et al.* 1995

Table 1.3.4 Constitutional intragenic *WT1* mutations detected in DDS individuals.

u - unilateral; b - bilateral; + - present; - - absent; nd - not determined; ? - status uncertain; m - male; f - female; [a] - nephrectomy performed; [b] - associated gonadal tumour; [c] - bilateral ILNRs present; [d] - wild type and mutant sequence observed (authors give contamination as reason); [e] - reported as biateral by Jadresic *et al.* 1991. * - stop codon. The standard single letter code for amino acids is used and is explained in full on page xii. Two entries have some features in parentheses (Baird *et al.* 1992 and Clarkson *et al.* 1993). These entries refer to the same individual (LB and CAM14 in references) and the features in parentheses are those described by the other set of authors.

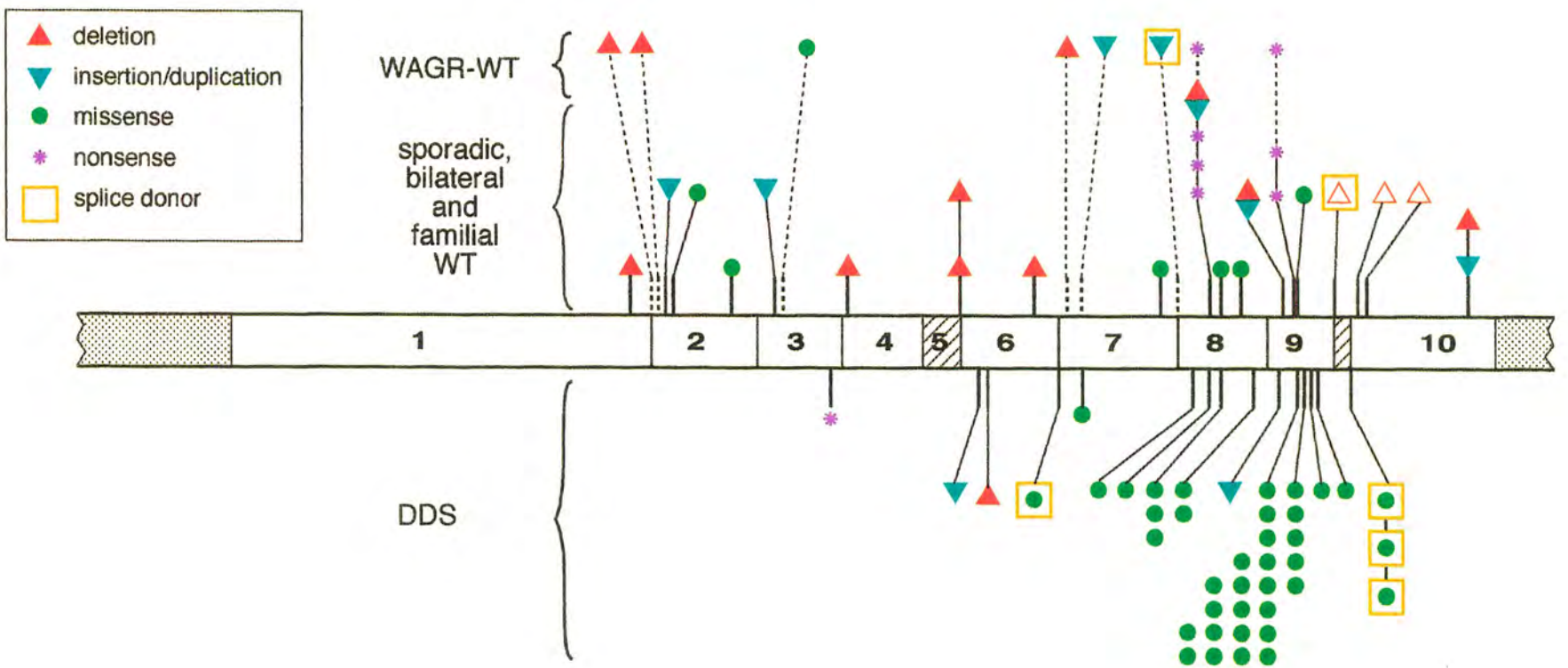


Figure 1.3.3 Spectrum of intragenic *WT1* mutations detected in unilateral, bilateral, familial and WAGR-WT, and in individuals with DDS. Each deletion and insertion mutation depicted with a solid symbol creates a frameshift.

Residue	codon position	CpG in codon	frequency of alteration
R	362	+	5/33 (15%)
R	390	+	3/33 (9%)

Table 1.3.3 Residues predicted to be altered most frequently in unilateral, bilateral, familial and WAGR WT. C to T transition mutations (and the reciprocal G to A) frequently occur at CpG dinucleotides. Within these dinucleotides the C is prone to methylation, creating 5-methyl cytosine (5-mC). This in turn is substrate for hydrolytic deamination, converting 5-mC to T (Yang *et al.* 1995). + - present.

nephropathy, indicating that these missense mutations confer a different aberrant function on WT1. It was initially proposed that these mutations elicited a dominant action, with the altered ZFs perhaps binding to novel and inappropriate DNA target sequences, or a dominant negative action (Pelletier *et al.* 1991a). Studies to which I contributed indicated that it is the latter mutational mechanism that is involved in this syndrome. These experiments are described in detail in Chapter 5. Similar to the other forms of WT, mutations within exon 5 have not been identified. In addition to missense mutations predicted to affect the ZFs, other studies have identified deletion, insertion, nonsense and splicing mutations which are predicted to create premature stop codons (Baird *et al.* 1992b, Ogawa *et al.* 1993b, Schneider *et al.* 1993, Bardeesy *et al.* 1994b). The predicted consequences of these mutations are discussed in Chapter 5. Unique to DDS are three reports of an identical mutation in intron 9, which eliminates generation of the +KTS isoforms (Bruening *et al.* 1992, Konig *et al.* 1993, Bardeesy *et al.* 1994b). The ratio of the four mRNA isoforms is highly conserved, even in WT not associated with DDS investigated (Brenner *et al.* 1992). Alteration of the ratio of the isoforms is therefore thought sufficient to elicit DDS. All of the above DDS *WT1* mutations are present heterozygously at the constitutional level. When available for analysis, WT from DDS individuals virtually always display LOH for the constitutional mutation. Additionally, one study has identified a familial case of DDS, where a phenotypically normal male transmitted the classic DDS mutation

Residue	codon position	CpG in codon	frequency of alteration
R	394	+	19/42 (45%)
D	396	+	5/42 (12%)

Table 1.3.5 Residues predicted to be altered most frequently in DDS individuals. + - present.

encoding R394W, which he carried constitutionally, to his son, who developed DDS (Coppes *et al.* 1992b). Furthermore, this mutation was identified constitutionally in a female with sporadic, unilateral WT alone (Akasaki *et al.* 1993).

These last two studies and that of inherited WT1 mutation reported by Pelletier *et al.* (1991b) illustrate that identical *WT1* mutations can elicit different phenotypes in different individuals, indicating the involvement of additional factors, such as genetic background. Providing evidence that genetic sex may influence the effect of *WT1* mutations, Henry *et al.* (1993) describe identical 11p13 deletions in two related children, an XY individual who developed WAGR syndrome and DDS and an XX female who developed only aniridia.

Finally, the many examples of functional loss of both copies of *WT1* clearly demonstrate that this gene is a tumour suppressor gene and is involved in different forms of WT. The different types of *WT1* mutation identified do not, however, correlate with specific histological types of WT.

1.3.8 Mutation analysis in other tumour types

WT1 mutations are implicated in myeloid leukaemias. The original cDNA clone, WT33, was isolated from a pre-B cell leukaemia cell line library (Call *et al.* 1990) and encodes the change F365S (Haber *et al.* 1990). This may represent a mutation rather than a polymorphism as the S residue compromises DNA binding (Bickmore *et al.* 1992, Little *et al.* 1995). Expression in primary leukaemias has been reported (Miwa *et al.* 1992a, Miyagi *et al.* 1993) and an exon 9 mutation, encoding the change C385Y, has been identified in a leukaemia that developed in a WAGR syndrome individual (Pritchard-Jones *et al.* 1994). More recently, a further five *WT1* mutations, each of which creates a premature stop codon, have been identified in this tumour type (King-Underwood, submitted).

WT1 mutations may also contribute to gonadal tumours as a bilateral juvenile granulosa cell tumour, a form of gonadoblastoma, co-developed with WT in a DDS individual carrying the classic DDS mutation encoding the change R394W (Pelletier *et al.* 1991a).

Finally, the Ewings sarcoma gene, *EWS*, can interact with *WT1*. The former gene is predicted to produce protein which, although of unknown function, has an N-terminus putative TD and a C-terminus RNA binding domain (Delattre *et al.* 1992). Translocations that juxtapose the 5' sequence to those encoding DNA binding domains are observed and are thought to create aberrant EWS protein which, with a novel but functional C-terminus, may have oncogenic potential. In desmoplastic small round cell tumour (DSRCT) translocations that create a *EWS-WT1ZF2-4* hybrid gene and mRNA have been identified (Ladanyi and Gerald 1994, Rauscher *et al.* 1994, Gerald *et al.* 1995).

1.4 Human malignant mesothelioma

1.4.1 Histopathology of the mesothelium and of mesothelioma

1.4.1.1 Structure and function of the mesothelium

The mesothelium is an epithelial membrane which lines the three major serosal cavities of the human body; the pleural, the pericardial and the peritoneal cavities. The pleural cavity houses the lungs, the pericardial the heart, and the peritoneal many of the abdominal visceral organs. Additionally, in males the mesothelium invests the testes (Jones 1987). Although a membrane of single cell thickness, the mesothelium does have a double layered structure, with an inner, visceral layer adhering to the tissues and an outer, parietal layer adhering to the wall of the serosal cavity. In healthy individuals the two layers in both the pleural and the pericardial cavities (and in males, in the testes also) are juxtaposed and hence the fluid filled space between them is obscured. In the peritoneal cavity double folds, termed mesenteries, juxtapose the visceral and parietal peritoneum (van de Graaff 1995).

The functions of the mesothelium are threefold. Firstly, it secretes serous fluid to lubricate the adjacent tissues, stopping friction between them and the appropriate cavity wall. Secondly, by surface tension of serosal fluid, it allows both the lungs and the heart to expand and contract in unison with the pleural and pericardial cavity walls, respectively. And thirdly, sections of the peritoneum form into folds, termed the lesser and the greater omentum, which store fat and cushion the abdominal visceral organs against forces of impact (van de Graaff 1995).

1.4.1.2 Development of the mesothelium

The mesothelium forms from intraembryonic mesoderm, a process which commences during the third week of human embryogenesis, coincident with somite development. Numerous small cavities, termed coelomic spaces, form within the lateral mesoderm. As mesenchymal cells migrate towards the midline these spaces fuse to form a horseshoe shaped structure, the intraembryonic coelom. This process divides the lateral mesoderm in to two layers, a dorsal layer of somatic mesoderm and a ventral layer of splanchnic mesoderm; the former gives rise to the parietal mesothelium and the latter to the visceral mesothelium. At this stage of development the mesothelium is continuous within the intraembyonic coelom. During the fourth week of embryogenesis compartmentalization of the intraembryonic coelom is initiated, forming the primitive pericardial and peritoneal cavities, which are linked together by two pericardioperitoneal canals. It is a further three weeks until the pleural cavity is fully developed from and separated from the pericardial cavity, although separation of the pleural and peritoneal cavities is completed one week earlier (week six) concurrent with formation of the diaphragm. At week seven of embryogenesis therefore, when the

three cavities are fully distinct from each other, the mesothelium is similarly isolated as pleurae, pericadium and peritoneum (Moore and Persaud 1993).

When initially formed from the somatic and splanchnic mesoderm, parietal and visceral mesothelial cells are cuboidal. A basal lamina is then established, separating these cells from the underlying mesenchyme, and, concurrently with the formation of intercellular junctions, the cuboidal form is lost. The morphology of the mature parietal and visceral mesothelium is simple, squamous epithelium (Thomas 1987).

1.4.1.3 Structure of mesothelioma

Mesothelioma is a highly malignant tumour which derives from and selectively spreads along the mesothelium. This type of tumour tends not to infiltrate the adjacent tissues directly, a feature which is clearly evident when looking at the most common site of human malignant mesothelioma (MM), the pleurae, as the lungs are rarely seeded with malignant cells (Jones *et al.* 1987). Lung function is, however, greatly affected as the tumour encapsulates and 'strangles' the tissue. Similarly, in pericardial MM, heart tissue can be free of malignancy but heart function can be severely, and sometimes fatally, impaired, because of the surrounding tumour mass. The tumour is grey-white in appearance and forms an extremely hard and tenacious structure, varying in thickness from a few millimetres to several centimetres. It is usual for the visceral and parietal layers to become fused when malignant (Jones *et al.* 1987). Additionally, it is common for MM to spread to other cavities, such as the contralateral pleural cavity or from the pleura to the pericardial cavity or , by direct spread across the diaphragm, to the peritoneal cavity. Metastases of MM are also frequent (Jones *et al.* 1987, Suzuki 1980).

Histologically, the majority of MM are epithelial predominant tumours, containing aberrant sheetlike, tubular and papillary structures. Many of these tumours also contain foci of mesenchyme, a component which is itself predominant in approximately 7% of MM. Approximately 25% of tumours contain much of both components, and are said to be biphasic or mixed in composition. Tumours of this latter histological group often contain a third cell type, intermediate in form between the epithelial and mesenchymal cells. The presence of such transitional cells reflects the inherent plasticity of mesothelial cells, having the capability to either further differentiate (epithelial cells) or to de-differentiate (mesenchymal cells) (Suzuki 1980).

1.4.2 Epidemiology

1.4.2.1 Incidence

MM in humans was described first in 1870 by Wagner (reference not consulted), who used the term endothelioma. Spontaneous MM is a rare tumour, with an incidence of approximately 1-2 cases per million individuals per year (McDonald and McDonald 1986). This figure is most probably

equivalent to all cases of MM up to the 1950's, however over the last forty five years the aetiology of this malignancy has altered dramatically. In Britain there are currently approximately 1000 deaths per year from MM, with only a maximum of one tenth of these deaths attributable to the spontaneous form of the tumour. Indeed, projected figures forecast a continuation of the increasing frequency of MM cases in Britain, culminating in a peak incidence of >3000 deaths per year by the year 2020 (Webb 1995).

1.4.2.2 Influence of asbestos

The reason why an ever increasing number of individuals are developing MM is because they, unlike their immediate ancestors, have experienced mainly occupational exposure to mineral fibres, in particular the highly carcinogenic fibre, asbestos. Shipbuilders, building industry workers, World War I and II gas mask assemblers and miners working asbestos quarries have been particularly affected by unacceptable levels of asbestos exposure, although the dangers were not recognised until 1960, when the first report of an association between asbestos and MM was published (Wagner *et al.* 1960). Alarmingly, unacceptable levels of exposure have persisted within certain sectors of employees until as recently as the early 1980's (Webb 1995). Indeed, this type of asbestos exposure, together with domestic exposure which affects employees' families (Vianna and Polan 1978, Li *et al.* 1978), is now thought to be responsible for approximately 85% of all cases of MM (Jones *et al.* 1987). Passive, environmental exposure to asbestos may also be capable of inducing this tumour (Risberg *et al.* 1980). Neither the length of exposure time nor the intensity of exposure to asbestos is correlated with the duration of the latent period or the severity of the resulting tumour, however, a threshold level of asbestos burden, required for induction of MM, may exist (Browne 1983a).

With the exception of the World War I and II workforces, the occupations associated with asbestos exposure have always been those that employ many times more men than women. It is this pattern of employment that causes MM to present predominantly in males. Also, asbestos induced MM does not, in general, develop until later life, as there is an average period of latency of between thirty to forty years after first exposure (Browne 1983a). This extended latent period accounts for the forecast peak of deaths from MM by the year 2020.

Again because of the influence of asbestos exposure, the majority of MM are pleural (approximately 57% of cases), and >90% of these are unilateral (Fishman 1988). Peritoneal MM are next most common (approximately 40% of cases), with pericardial and testicular MM both observed infrequently (Suzuki 1980).

1.4.2.3 Asbestos-independent cases

Approximately 15% of MM patients do not have a history of occupational or domestic exposure to asbestos, and represent the constantly rare and 'basal' incidence of this tumour. A minority of these patients may have unknowingly suffered environmental asbestos exposure, however the majority do appear to have developed MM in an asbestos-independent manner.

The term spontaneous MM was used earlier (1.4.2.1) to encompass all asbestos-independent tumours. Two reports (Ilgren and Wagner 1991, Peterson *et al.* 1984) detail the subdivision of asbestos-independent MM into eight separate categories, although many of these can be overlapping. The categories are as follows, 1- spontaneous cases, particularly applicable to pericardial and testicular MM where intercavity spread is not apparent; 2- cases with a latent period of <10 years; 3- childhood cases, post-mortem studies confirm this form is not associated with asbestos (Browne 1983b); 4- familial cases; 5- cases prior to 1900, when asbestos was not an industrial material; 6- mineralogically negative, histologically positive cases; 7- cases involving non-asbestos fibrous agents, such as fibreglass and 8- cases involving non-fibrous agents, such as radiation, chemicals and chronic inflamation.

Neither report details the possible involvement of specific genetic defects in the development of this form of MM (see 1.4.4.3), a component which may explain why these patients have a younger age at presentation, by an average of over seven years, in comparison to asbestos induced MM patients (Hirsch *et al.* 1982). A second contrasting feature is that this form of the tumour affects men and women at an equal frequency (Law *et al.* 1983, Hirsch *et al.* 1982). The lack of a sex bias reflects the lack of involvement of asbestos, and, consequently, the proportion of MM individuals that are female is far higher for the asbestos-independent form than for the asbestos induced form of the tumour.

1.4.2.4 Prognosis

Prognosis for MM is poor for two main reasons; firstly, the tumour is unresponsive to both radiotherapy and chemotherapy and secondly, it tends to be well established before any symptoms manifest. Hence the median survival time post-presentation is only one year (Fishman 1988). When patients with asbestos-independent MM are considered separately, survival time is extended by between seven and twelve months (Law *et al.* 1983, Hirsch *et al.* 1982). Death usually results from respiratory failure, bronchopneumonia or pericardial haemorrhage (Browne 1994). MM has not been reported as a component of any clinical syndromes.

1.4.3 Precursor lesions

Pleural plaques are dense masses present on the pleural mesothelium. They are most often multiple, bilateral and acellular, and are composed of collagen bundles that form white or yellow thickenings on the parietal surface. Well defined margins keep plaques distinct from the adjacent and underlying mesothelium, which does not display signs of reactive change. The presence of pleural plaques is an indicator for previous exposure to asbestos, with immunological reactions apparently causing collagen deposition around the inhaled fibres subsequent to their transportation to the parietal pleurae. The mesothelial cells do not appear to be involved in these reactions and pleural plaques are therefore thought of as submesothelial structures. Hence, although their presence has a feature in

common with mesothelioma, that is, exposure to asbestos, the distinct nature of pleural plaques abolishes their consideration as precursor lesions for this malignancy (Jones *et al.* 1987).

1.4.4 Genetic component

1.4.4.1 Asbestos induces gross genetic change

Features of both asbestos induced and asbestos-independent MM indicate the involvement of genetic defects in the development of this tumour. Early studies highlighted numerical and structural chromosome abnormalities observed when asbestos fibres were introduced into Chinese hamster ovary cell cultures (Sincock and Seabright 1975) and human mesothelial cell cultures (Lechner *et al.* 1985). More recently, experiments have observed that phagocytosed asbestos fibres have affinity for the cellular matrix rather than for chromosomes (Ault *et al.* 1995). It is believed that if the fibres are sufficiently long they extend into and interfere with the mitotic spindle, causing the types of gross genetic change mentioned above.

1.4.4.2 Predisposition for asbestos insult?

The idea that genetic change may precede asbestos insult has also been examined. Browne (1983a) proposed that mesothelial cells may be initiated for asbestos insult prior to exposure, and the fibres then act solely as the promoter of tumorigenesis. The mechanism of initiation is not specified but could represent genetic predisposition. The fact that many asbestos exposed individuals never develop MM, even after a suitable latent period, also supports the idea that an individual's genetic background can influence whether the mesothelium remains normal or adopts malignancy in response to asbestos insult (Hammar *et al.* 1989). An extension of this proposal is that, subsequent to promotion, initiated mesothelial cells may still have to accumulate additional genetic changes, such as discrete mutations, before they can become fully transformed, a model comparable to the multigene model proposed for colorectal tumorigenesis (Fearon and Vogelstein 1990). The length of time required to accumulate the minimum amount of genetic change for MM tumourigenesis is perhaps the factor which dictates the precise length of the extended latent period (Gabrielson *et al.* 1992).

Further evidence for genetic predisposition comes from five independent studies where domestic exposure to asbestos, though apparent, is thought insufficient to explain all aspects of particular cases. Firstly, Vianna and Polan (1978) showed that the parents of asbestos exposed females have a higher incidence of cancer (though never MM) in comparison to control cohorts. Secondly, Browne (1983a) showed that familial cases of MM occur, in general, as parent-child or sibling-sibling associations, and rarely develop in genetically distinct partners. And finally, Li *et al.* (1978), Risberg *et al.* (1980) and Hammar *et al.* (1989) detail families where some members are believed to have experienced only background levels of asbestos exposure and yet have developed MM, often of a histological type identical to that of their relative(s) with confirmed asbestos exposure.

1.4.4.3 Can genetic defects act alone?

Cases of MM presenting after a latent period of <10 years and/or in childhood are known to develop in an asbestos-independent manner (see 1.4.2.3). It is possible that, because of the rapid progression and/or paediatric nature of these particular tumours, other environmental factors have also not had sufficient time to contribute significantly to tumorigenesis. In these cases, therefore, genetic defects may constitute both the initiator and the promoter of tumorigenesis. This mechanism may also apply to other forms of spontaneous MM (see 1.4.2.3), such as the familial form. With respect to the latter, the report by Hirsch *et al.* (1982) of asbestos-independent MM presenting in two siblings (both in their fifth decade of life) further indicates that genetic defects alone may be sufficient for MM tumorigenesis.

Certain genetic defects may contribute to other types of malignancy together with MM. Kane *et al.* (1990) report an individual with asbestos-independent MM and familial granular cell myoblastoma, who subsequently developed renal oncocytoma, malignant melanoma and adenocarcinoma of the bladder. No form of genetic analysis is detailed, but it is possible that this individual was genetically predisposed for tumorigenesis, similar to individuals with Li-Fraumeni syndrome (Li *et al.* 1988), who have constitutional mutations affecting the *p53* gene (Malkin *et al.* 1990).

1.4.4.4 Specific cytogenetic and genetic defects

To date, there are no known genetic defects which are unique, and therefore diagnostic, for human MM. Numerous chromosomal abnormalities are however a consistent feature of this tumour type and many are observed as non-random events. These comprise both genetic gain, affecting chromosomes 5, 7, 11 and 12, and genetic loss, affecting chromosomes 1, 3, 6, 9 and 22. The majority of MM samples analysed for specific genetic defects are found to contain more than one of these gains and/or losses, further indicating a multigene mechanism for tumorigenesis, perhaps involving both oncogenes and tumour suppressor genes. This type of model has been outlined for MM tumorigenesis by Meloni *et al.* (1992).

The most frequently observed gain involves chromosome 7 and is confined to 7p (Tiainen *et al.* 1988, Tiainen *et al.* 1989, Hansteen *et al.* 1993). The *PDGF-A* gene maps to this region, at 7p21-p22 (Bonthron *et al.* 1988), and may be significant as the growth factor it encodes has been implicated in the malignant transformation of mesothelial cells (Gerwin *et al.* 1987, Versnel *et al.* 1988). The reasons for the gains of chromosomes 5 (Tiainen *et al.* 1989), 11 (Tiainen *et al.* 1988, Tiainen *et al.* 1989, Hansteen *et al.* 1993) and 12 (Tiainen *et al.* 1988, Tiainen *et al.* 1989) also remain unknown.

The most frequently observed loss is monosomy of chromosome 22 (Tiainen *et al.* 1988, Tiainen *et al.* 1989, Flejter *et al.* 1989, Hansteen *et al.* 1993, Taguchi *et al.* 1993). The *PDGF-B* gene (Dalla-Favera *et al.* 1982, Swan *et al.* 1982) and the neurofibromatosis tumour suppressor gene, *NF2* (Rouleau *et al.* 1993, Trofatter *et al.* 1993), both map to this chromosome and may contribue to MM tumorigenesis. *PDGF-B* is implicated through interaction with *PDGF-A*. The potential involvement

of *NF2* is more apparent, though based on a single study. This study identified deletions and nonsense mutations affecting *NF2* in 7 out of 17 (41%) MM samples, including one primary tumour (Sekido *et al.* 1995). Relatively few mutations in only one other tumour suppressor gene, *p53* (chromosome 17p13), have been reported for MM, although these data are based exclusively on the analysis of tumour cell lines (Cote *et al.* 1991, Metcalf *et al.* 1992).

Loss of chromosome 1 appears to be confined to 1p (Gibas *et al.* 1986, Popescu *et al.* 1988, Tiainen *et al.* 1988, Flejter *et al.* 1989, Tiainen *et al.* 1989, Hansteen *et al.* 1993, Taguchi *et al.* 1993), of chromosome 3 to 3p (Gibas *et al.* 1986, Popescu *et al.* 1988, Flejter *et al.* 1989, Tiainen *et al.* 1989, Hansteen *et al.* 1993, Taguchi *et al.* 1993, Lu *et al.* 1994, Zeiger *et al.* 1994) and of chromosome 6 to 6q (Gibas *et al.* 1986, Meloni *et al.* 1992, Taguchi *et al.* 1993).

For chromosome 9, loss is confined to the short arm (Tiainen *et al.* 1988, Tiainen *et al.* 1989, Center *et al.* 1993, Hansteen *et al.* 1993) and, more specifically, to the region 9p21-22 (Cheng *et al.* 1993, Taguchi *et al.* 1993). Subsequent to the identification of the *p16* gene at 9p21 (Kamb *et al.* 1994a, Nobori *et al.* 1994), this locus has been studied extensively in relation to tumorigenesis (see 1.5).

1.5 The *p16* gene

1.5.1 Isolation and identification

The human multiple tumour suppressor gene, *MTS1*, was isolated from chromosome 9p21 by positional cloning and presented as a candidate tumour suppressor gene by Kamb *et al.* (1994a). This study employed detailed deletion analysis of an approximately 120kb region of 9p21 in cell lines derived from twelve different tumour types, including melanoma, lung cancer, glioma and leukaemia. Earlier studies had implicated this locus in malignant melanoma (Cannon-Albright *et al.* 1992, Fountain *et al.* 1992), non-small cell lung carcinoma (Center *et al.* 1993, Olopade *et al.* 1993), malignant glioma (Ichimura *et al.* 1994), leukaemia (Diaz *et al.* 1990, Einhorn *et al.* 1990) and, as mentioned above, MM (Cheng *et al.* 1993, Taguchi *et al.* 1993). Of all the different tumour cell lines analysed, only those derived from colon carcinoma and neuroblastoma failed to show repeated homozygous deletion of *MTS1* or the flanking region.

Within the putative exonic segments of *MTS1* complete identity to a previously known cDNA sequence was demonstrated. This latter sequence had been deduced from a protein isolated through its ability to complex with cyclin dependent kinase 4 (CDK4), an enzyme critical in cell cycle regulation (Serrano *et al.* 1993). The protein was termed $p16^{INK4}$ (inhibitor of kinase 4) and was recognised as

the product encoded by *MTS1*. An independent study using similar techniques also isolated this gene (Nobori *et al.* 1994). They named it *CDK4I*, as, again, the connection with $p16^{INK4}$ was recognised.

To date, there are five names for this gene; *MTS1*, *CDK4I*, *CDKN2* (the official name), $p16^{INK4}$ and, most simply and now most commonly, *p16*. The protein is now also referred to most commonly as p16.

1.5.2 Expression pattern

As a cell cycle regulator p16 may be a critical protein, and it is therefore feasible that it is required by all cells for correct growth and division. This would necessitate ubiquitous expression of *p16*, making it, in this respect, comparable to the genes that encode some of the other CDK inhibitors. One study has addressed this topic by analysing p16 levels in nine distinct and non-tumorigenic human cell types (Tam *et al.* 1994). T lymphocytes were the only cell type found to lack p16. The other mesoderm derived cell types analysed, all of which expressed p16, consisted of endothelial cells, osteocytes, fibroblasts and smooth muscle cells. Additionally, transformed epithelial cells from breast and kidney (EMBK cells) were found to contain high levels of p16 (also reported by Aagaard *et al.* 1995).

Within the limits of a single cell cycle levels of p16 vary, with a peak of expression observed during the G1-S transition phase (Tam *et al.* 1994).

1.5.3 Gene structure

p16 contains three exons which span approximately 10kb in length. Each exon contains coding sequence, 66% (307bp out of 468bp) of which is contained in exon 2. Both exons 1 and 2 encode ankyrin repeat motifs (Figure 1.5.1a). Transcription proceeds in the proximal to distal direction, as described by Kamb *et al.* (1994a) and produces a mRNA of at least 966bp (see Appendix A, Part II). No alternative splice variants have been identified. The structure of the promoter has not been elucidated.

1.5.4 Protein structure

p16, named because it is 16kD in size, contains 156 amino acids (Serrano *et al.* 1993, revised by Hannon and Beach 1994). As mentioned above, the majority of the primary amino acid sequence can be aligned as four contiguous repeats, each displaying homology to the consensus amino acid sequence of the ankyrin repeat motif (Figure 1.5.1b). Ankyrin repeats have been identified in a

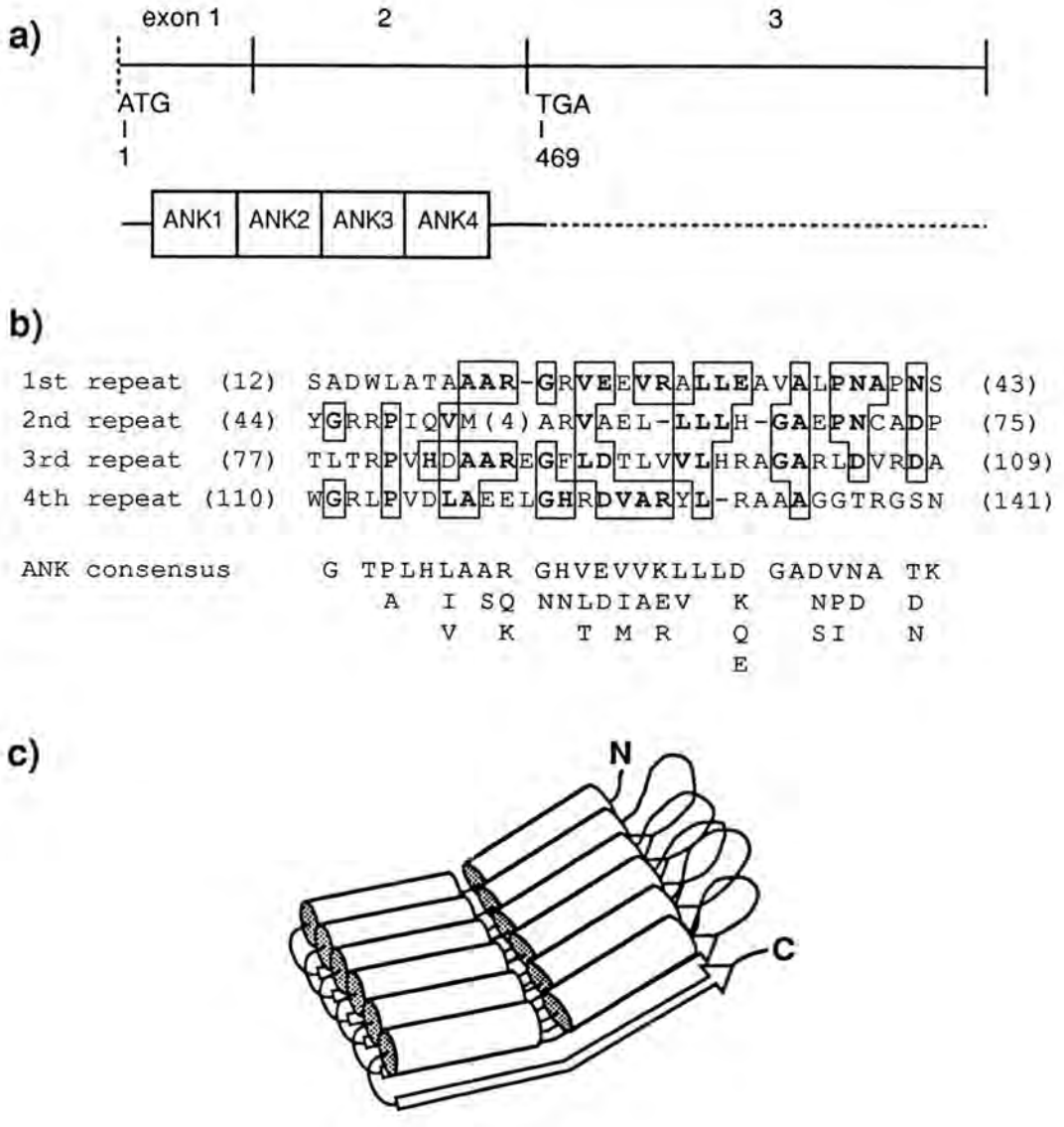


Figure 1.5.1 Structure of the *p16* gene at the cDNA level and of the predicted protein motifs. a) the relative size of each exon is illustrated. The full extent of exon 1 is not known, but this cDNA is predicted to contain the complete coding sequence. The latter is represented by a schematic illustration of the protein motifs predicted, four ankyrin (ANK) repeats. The dotted line depicts the 3' untranslated sequence. **b)** alignment of the primary amino acid sequence of p16 demonstrates the potential for formation of ankyrin repeat motifs. Drawing adapted from Serrano *et al.* (1993). **c)** predicted conformation of ankyrin repeat motifs, six repeats are present. Drawing reproduced from Michaely and Bennett (1992).

number of diverse proteins (reviewed by Michaely and Bennett 1992) and are thought to function exclusively as protein-protein interaction domains (Figure 1.5.1c). No additional motifs have been identified.

1.5.5 Protein function

As mentioned above, p16 is a CDK inhibitor, originally isolated through its association with CDK4 (Serrano *et al.* 1993). Subsequently, a CDK4-related molecule, CDK6, has also been identified as a target for p16 (Hannon and Beach 1994). It is believed that the four ankyrin repeats which comprise p16 mediate the association with both CDK4 and CDK6. p16 targets these CDK molecules at a specific stage of the cell cycle, the G1-S transition phase, when maximum levels of p16 are observed. By inhibiting kinase activity at this stage, p16 antagonises entry in to S phase and, in doing so, stalls cell proliferation. The function of p16 in the cell cycle is therefore that of a negative regulator, blocking premature DNA synthesis and cell division.

1.5.6 A role in tumorigenesis?

The positive regulators of the cell cycle are the CDKs and their cyclin molecule partners, and the negative regulators are, as mentioned above, the CDK inhibitors. These components interact with each other in many different combinations, with the exact composition depending on the phase of the cell cycle together with the nature of the substrate complex (reviewed by Morgan 1995). At a basic level, the genes encoding CDK and cyclin molecules have the potential to be oncogenes and conversely, those encoding CDK inhibitors have the potential to be tumour suppressor genes. There are currently six identified CDK inhibitors (Table 1.5.1) and all of them have been put forward as candidate tumour suppressors. Much of the work that is required to confirm or refute these proposals is still preliminary, as these genes are only recently cloned. Additionally, it is believed that the tumorigenic potential of many of the cell cycle regulators will be masked because of the considerable number and complexity of cell cycle interactions, which may result in functional redundancy.

There are a number of reasons for p16 remaining the strongest candidate, among the CDK inhibitors, for being a true tumour suppressor. Firstly, as p16 does not appear to be expressed in a stringent, tissue specific pattern, many different types of tissue should be affected if the gene is mutated. This aspect is reflected by the observed repeated disruption of the *p16* locus in tumours derived from many different tissue types (see 1.5.1). Secondly, studies suggest that loss of p16 may substitute for loss of RB1, as mutations in the corresponding genes appear to be mutually exclusive (Li *et al.* 1994, Otterson *et al.* 1994, Aagaard *et al.* 1995). Associated with this, RB1 may

CDK inhibitor	gene locus	expression	targets	references
p15	9p21	nd	CDK4, CDK6	Kamb *et al.* 1994a, Hannon and Beach 1994.
p16	9p21	ub?	CDK4, CDK6	Serrano *et al.* 1993, Kamb *et al.* 1994a, Nobori *et al.* 1994.
p18	1p32	ts	CDK4, CDK6	Guan *et al.* 1994.
p21	6p21	ub	CDK2, CDK4, CDK6	Xiong *et al.* 1993.
p27	12p12-13	ub	CDK2, CDK4, CDK6	Ponce-Castaneda *et al.* 1995.
p57	11p15.5	ts	CDK2, CDK3, CDK4	Lee *et al.* 1995, Matsuoka *et al.* 1995.

Table 1.5.1 Features of the six identified CDK inhibitors. nd - not determined; ub - ubiquitous and ts - tissue specific.

transcriptionally repress *p16* (Li *et al.* 1994). Thirdly, recent *in vitro* studies indicate that p16, together with p21, may contribute to myoblast differentiation by eliminating the inhibitory effect an active specific CDK complex has on myogenesis (Skapek *et al.* 1995, reviewed by Marx 1995). Mutations affecting *p16* may therefore disrupt normal differentiation pathways and hence contribute to tumorigenesis.

1.5.7 Mutation analysis in MM

Only one study has identified *p16* mutations in MM samples (Cheng *et al.* 1994). The majority of these mutations are homozygous deletions, present in 34 out of 40 (85%) cell lines and 5 (matched) out of 23 (22%) primary tumours. None of these deletions were however demonstrated to be intragenic and were classified in to two main categories; those affecting proximal loci and/or *p16* and those affecting distal loci and/or *p16*. In six of the homozygously deleted cell lines both the proximal and distal markers flanking *p16* were retained, but as these markers are approximately 1Mb apart genes additional to *p16* may be disrupted (see 1.5.10). One intragenic mutation was detected, an A to G transition in intron 2. This mutation, identified in one cell line, is predicted to eliminate splicing in

of exon 2, and would consequently create a frameshift. At the protein level it is predicted that the three most C-terminal ankyrin repeat motifs would be destroyed, most likely causing loss of binding to CDK4 and CDK6. In four additional cell lines *p16* mRNA was present at much reduced levels, perhaps implicating a further mutational mechanism.

Finally, approximately 60% of the samples in this study derived from individuals known to have suffered asbestos exposure. A correlation between *p16* mutation and MM aetiology or histology could not, however, be demonstrated.

1.5.8 Mutation analysis in other tumour types

Intragenic *p16* mutations have been identified in cell lines derived from head and neck tumours, lung carcinoma and melanoma at frequencies of 11%, 14% and 33%, respectively (Zhang *et al.* 1994, Okamoto *et al.* 1995, Ohta *et al.* 1994). One recent study has calculated an overall frequency of 18% for this type of mutation in tumour cell lines (Liu *et al.* 1995). In this type of sample homozygous deletions of *p16* are, in general, observed at a higher frequency, ranging from 27%, for melanoma cell lines, to 40%, for breast carcinoma cell lines (Ohta *et al.* 1994, Xu *et al.* 1994). Inclusion of the figures reported by Kamb *et al.* (1994a) and Nobori *et al.* (1994) extends this range from 25%, for lung carcinoma and leukaemia cell lines, to 87%, for glioma cell lines. This apparent predominance of homozygous deletions in comparison to point mutations may, however, result from experimental bias and not reflect a preferred mutational mechanism of *p16*.

In primary tumours only a minority have been found to contain intragenic *p16* point mutations. For example, studies on melanoma and nasopharangeal carcinoma identified no such mutations in 30 and 42 tumours, respectively (Ohta *et al.* 1994, Sun *et al.* 1995), whereas only 3% of bladder carcinoma and astrocytoma (Spruck *et al.* 1994, Ueki *et al.* 1994) and 9% of lung carcinoma (Okamoto *et al.* 1995) were mutated in this way. Head and neck tumours and oesophogeal carcinoma contain point mutations at a higher frequency, 16% and 52%, respectively (Zhang *et al.* 1994, Mori *et al.* 1994), suggesting that *p16* may be more widely involved in tumorigenesis in these tissues.

Furthermore, in primary tumours, as in cell lines, homozygous deletions appear to be more prevalent than point mutations. Lung carcinoma, bladder carcinoma, leukaemia, anaplastic astrocytoma and glioblastoma have been found to contain homozygous deletions at frequencies of 18%,19%, 35%, 52% and 59%, respectively (Okamoto *et al.* 1995, Spruck *et al.* 1994, Otsuki *et al.* 1995, Walker *et al.* 1995). Additionally, xenograft studies have demonstrated that 40% of pancreatic adenocarcinomas are homozygously deleted and 85% display LOH for *p16* (Caldas *et al.* 1994).

Again, these studies suggest that *p16* mutations do contribute to tumorigenesis. Further evidence for this is provided by studies on familial melanoma. One of the putative familial melanoma genes, *MLM*, has been mapped to 9p21 by linkage studies and, as previously mentioned, by LOH studies (Cannon-Albright *et al.* 1992, Fountain *et al.* 1992). *p16* was considered a candidate for *MLM*

and, in support of this, one study has detected constitutional *p16* point mutations in individuals from 13 out of 18 9p-linked families and in 92% of associated melanomas (Hussussian *et al.* 1994). Somewhat in contrast however, Kamb *et al.* (1994b) identified similar mutations in only 2 out of 13 9p-linked families. It is not possible to decide from these data whether *p16* is *MLM* and mutations remain undetected in some families or whether an additional gene at 9p21 is involved.

The intragenic deletions and insertions, splicing and nonsense mutations identified in *p16* are most likely loss of function mutations. These mutations are predicted to cause disruption, including loss, of the ankyrin repeat motifs in the protein and, therefore, perhaps affect binding to CDK4 and CDK6. The effect that missense mutations have at the protein level is more difficult to predict, especially as a proportion of these mutations have been shown to be polymorphic (Spruck *et al.* 1994, Cairns *et al.* 1994, Ranade *et al.* 1995). Clearly, functional studies are required. Such studies have been performed for missense mutations identified in some of the 9p-linked melanoma families and demonstrate that inhibition of CDK's is compromised (Ranade *et al.* 1995). Recently an additional mutational mechanism has been implicated for *p16*. Merlo *et al.* (1995) have demonstrated that methylation of 5' sequences of *p16* blocked gene expression in primary lung carcinoma, head and neck tumours and gliomas. Furthermore, in this latter tumour type and in associated cell lines amplification of *CDK4*, rather than loss of *p16*, has been detected (He *et al.* 1994). Similar observations have been made for other tumour types (Maelandsmo *et al.* 1995), suggesting that correct stoichiometry between inhibitor and target proteins is essential for normal cell cycle control.

1.5.9 Cell lines versus primary tumours

When the frequencies of homozygous deletions in tumour cell lines and primary tumours are compared it is evident that, in general, the former far exceeds the latter. For example, 27% (cell line) versus 0% (primary) for melanoma, similarly 33% versus 0% for head and neck tumours, 40% versus 0% for breast carcinoma, 32% versus 18% for lung carcinoma and 38% versus 19% for bladder carcinoma (Ohta *et al.* 1994, Zhang *et al.* 1994, Xu *et al.* 1994, Okamoto *et al.* 1995, Spruck *et al.* 1994). These data suggest that loss of *p16* offers a growth advantage to tumour cells *in vitro* more readily than to tumour cells *in vivo*. This in turn suggests that for tumours displaying LOH at 9p21 the critical event involves a locus distinct from *p16*. In support of this, from a total of 90 tumours with LOH at 9p21 only 3 (3.3%) were identified as containing *p16* mutations (Cairns *et al.* 1994, Ueki *et al.* 1994). Additionally, the proposal that there is at least one other gene at 9p21 involved in tumorigenesis is also indicated by some 9p-linked melanoma families, as discussed in 1.5.8.

1.5.10 *p15* and *p18*

The *p15* gene maps approximately 25kb proximal and adjacent to *p16*. Part of the genomic sequence was identified by Kamb *et al.* (1994a) and was initially named *MTS2*. This partial sequence displays 93% identity to *MTS1* (*p16*) and is structurally very similar to this gene, implicating the occurrence of gene duplication and limited sequence divergence. Hannon and Beach (1994) isolated the corresponding cDNA, 837bp in length, and the encoded protein, p15, which contains 137 amino acids. The overlapping sequences of p15 and p16 display 76% identity, however in the region which forms the three most C-terminal ankyrin repeat motifs identity reaches 97%. The similarity of protein structure is reflected at the functional level as p15 is also an inhibitor of CDK4 and CDK6. However, unlike *p16*, *p15* expression is induced by transforming growth factor-β (TGF-β).

One other p16-like protein, p18, has also been isolated (Guan *et al.* 1994). Like p15 and p16, p18 is a CDK inhibitor, and targets CDK4 (weakly) and CDK6 (strongly). The primary amino acid sequence of this protein displays moderate identity to p15 and p16 (less than 40% for each) but is most similar to that of the Notch proteins. Initial studies suggest that the genomic structure of *p18* is similar to that of *p15* and *p16*, again implicating a common ancestral gene. *p18* is not, however, linked to *p15* and *p16*; it maps to chromosome 1p32.

1.6 Do both genes contribute to both tumour types?

1.6.1 Association of urogenital and mesothelial tumours

1.6.1.1 Developmental association

As described previously, the kidney, gonad and mesothelium all derive from intraembryonic mesoderm. More specifically, the kidney forms from intermediate and lateral mesoderm, whereas the mesothelium forms from the latter only. Both the mesonephros and the mesothelium contribute to gonadal development, hence this tissue also forms from intermediate and lateral mesoderm. A further similarity between these tissues is that they all undergo a mesenchyme to epithelial cell type transition. This pattern of regulated conversion during development is rare, and has been identified in only two other tissues; trophectoderm in preimplantation embryos and endothelium (Ekblom 1989). The capillary network of the glomerulus incorporates this last tissue type with the developing kidney. The developmental origins of the kidney, gonad and mesothelium are therefore intimately associated, suggesting that constitutional factors causing disruption to any one of these tissues may similarly disrupt the others.

1.6.1.2 Genetic association

So far I have discussed in detail the *WT1* and *p16* genes and the reasons why they are believed to contribute to development of WT and human MM, respectively. These studies also reveal a number of features, outlined and expanded below, supportive of the reciprocal arrangement, that is, contribution of *WT1* to MM and of *p16* to WT. Indeed, a number of years prior to the identification of these genes, evidence was gathering for a common genetic defect, predisposing for tumours of both the urogenital system and the mesothelium. Antman *et al.* (1984), Anderson *et al.* (1985) and Austin *et al.* (1986) describe a total of four individuals who each developed WT and MM. Further, Stock *et al.* (1979) describe a case of MM developing subsequent to treatment for seminoma, which, despite the influence of radiotherapy as emphasised by the authors, again provided early evidence for a genetic link in these tumour types.

1.6.2 Involvement of *WT1* in MM?

Analysis of the expression pattern of *WT1* and the expression pattern and homozygous mutants of *Wt1* indicated a requirement for this gene in development of the mesothelium (see 1.3.2). Additionally, *WT1* is expressed in a spatial and temporal pattern concordant with involvement in regulating mesenchyme to epithelial cell type transitions, further implicating this gene in mesothelial development. Evidence that constitutional *WT1* mutations can disrupt development of the mesothelium is presented by Devriendt *et al.* (1995), who describe an individual with a constitutional *WT1* missense mutation (Table 1.3.4), DDS (no WT, neonatal death) and an abnormal mesothelium in the form of diaphragmatic hernia, a feature also observed in *Wt1* homozygous mutant mice. One study has jointly analysed *WT1* and the mesothelium from a tumorigenic perspective (Park *et al.* 1993b). *WT1* mutation analysis was performed on 32 MM and 1 mesothelial lesion. The latter sample was the only one in which a *WT1* mutation was identified; a missense mutation in exon 6, which was found to be not present constitutionally. Hence, although not contributing to MM tumorigenesis, this somatic mutation may be a second example of *WT1* mutation contributing to abnormal development of the mesothelium.

1.6.3 Involvement of *p16* in WT?

As previously stated the majority of sporadic unilateral, bilateral and familial WT, together with a minority of syndrome-associated WT, do not appear to contain mutations in *WT1* and are therefore presumed to contain mutations which affect other critical genes. In addition to *p53* and the potential WT predisposition genes described earlier (1.2.6 and 1.2.7), *p16* was thought an excellent candidate gene for involvement in this tumour, principally because the study by Kamb *et al.* (1994a)

highlighted mutations in this gene in many other tumour types and implicated it as having a widespread role in tumorigenesis

1.7 Aims of this study

The aims of this study were to use mutation analysis techniques in the following ways: firstly to characterise further the involvement of *WT1* in many forms of WT: sporadic unilateral, bilateral, familial, WAGR- and DDS-WT. This analysis is important as, in concert with studies on gene evolution, it can help elucidate the critical aspects of WT1 structure and function. This, in turn, can provide insight to the mechanisms underlying both normal and aberrant nephrogenesis and, furthermore, development of other tissues known to express this gene. Secondly, the possible involvement of *WT1* in tumorigenesis of one of these latter mentioned tissues, the mesothelium, was investigated. The mesothelial tumour type studied was MM, for which a role for *WT1* is implicated but not proven. Thirdly, the involvement of a second gene, *p16*, in this latter tumour type was further characterised. This analysis is important as high resolution studies of this candidate gene in MM are lacking. Fourthly, and similarly, the possible involvement of *p16* in WT was investigated as, again, this gene is a candidate for involvement in this tumour type but experimental evidence is lacking.

The accumulation and interpretation of data from two tumour suppressor genes, one well characterised and one a recent discovery, and two tumour types is valuable as both common and differing patterns of tumour suppressor gene action can be revealed, such as mutational mechanisms and pleiotropic effects. This in turn contributes to a greater understanding of normal developmental processes.

Chapter 2
Materials and methods

2.1 Clinical materials

2.1.1 Primary WT

The laboratory code name of each WT studied and additional relevant details are as follows:

ALSTA	unilateral tumour, male WAGR patient with partial spectrum.
AmWh	also known as NP11, unilateral tumour, female patient.
ANDMA	unilateral tumour, male patient.
ANRIA	unilateral tumour, male BWS patient.
BeMa	also known as NP9, unilateral tumour.
BeOc	also known as NP8, unilateral tumour.
CAROL	unilateral tumour, sporadic, female patient.
CRASI	unilateral tumour, male BWS patient.
DAMBA	unilateral tumour, sporadic, male patient.
DANLE	unilateral tumour, sporadic, male patient.
DARCA	unilateral tumour.
DDS2	also known as 66.1 and STEBAK, bilateral tumour, female DDS patient.
DDS3	also known as 30.1, unilateral tumour containing heterotopic ganglia, 'female' DDS patient with accompanying cerebral atrophy, pyschomotor retardation, recurrent bronchitis, dermatitis and low IgG.
DDS4	also known as JGRD, bilateral tumour containing heterotopic muscle, male DDS patient with accompanying horseshoe kidney, gross motor delay, craniosynostosis, and prominent metopic suture.
DDS7	also known as SATAY, unilateral tumour of multricentric origin and containing heterotopic muscle, male DDS patient with accompanying developmental delay, epilepsy and posterior scalp defect, contralateral kidney contains ILNR and heterotopic muscle.
GAWA	unilateral tumour.
JAHUM	unilateral tumour, sporadic, male patient.
JOMEE	unilateral tumour, sporadic, male patient.
KABEL	unilateral tumour, sporadic, female patient.
KEARK	unilateral tumour, sporadic, male patient.
KEDEV	unilateral tumour, sporadic, female patient.
MIWAT	unilateral tumour, sporadic, male patient.
MORGAN	unilateral tumour, sporadic.
NAHAS	unilateral tumour, male WAGR patient with full spectrum.

NP58	bilateral tumour, from left kidney, contralateral tumour is NP57.
PACUR	unilateral tumour, sporadic, male patient with accompanying epilepsy.
RUTPA	unilateral tumour, sporadic, female patient.
ZAGRE	unilateral tumour, sporadic, female patient.
68.1	unilateral tumour, accompanying congenital heart defect.
8RS	unilateral tumour.

2.1.2 Matched normal kidney

Matched normal kidney samples obtained from some of the above WT patients and used for study are as follows:

DDS2, DDS4, 68.1.

2.1.3 Cell cultures and cell lines from WT

Primary cell cultures (cc) and transformed cell lines (cl) derived from some of the above WT (work of A. Seawright and V. van Heyningen) and used for study are as follows:

CRASI (cc), DAMBA (cc), JOMEE (cc), NAHAS (cc) and NAHAS (cl).

2.1.4 Lymphoblastoid cell lines (LCL) from WT patients and their parents

The laboratory code name of each WT patient or parent derived LCL (work of A. Seawright and V. van Heyningen) studied and additional relevant details are as follows:

ALSTA	see 2.1.1 for details.
ANRIA	see 2.1.1 for details.
CAROL	see 2.1.1 for details.
DAMBA	see 2.1.1 for details.
DANLE	see 2.1.1 for details.
DAVAT	unilateral tumour, male patient, contralateral renal agenesis.
DYROG	unilateral tumour in duplex kidney, male patient.
FAHAS	father of NAHAS, see 2.1.1 for details.
FASTA	father of ALSTA, see 2.1.1 for details.

JAHUM	see 2.1.1 for details.
JEDOY	familial tumour, female patient with breast carcinoma in later life, daughter had WT.
JELON	unilateral tumour, female BWS patient.
JOMEE	see 2.1.1 for details.
KEARK	see 2.1.1 for details.
KECUL	unilateral tumour, male patient, contralateral kidney had duplex collecting system.
KEDEV	see 2.1.1 for details.
LISWI	familial tumour, sib died from WT.
MAHAS	mother of NAHAS, see 2.1.1 for details.
MASOU	bilateral tumour, female patient.
MASTA	mother of ALSTA, see 2.1.1 for details.
MIWAT	see 2.1.1 for details.
MORGAN	unilateral tumour, sporadic.
NAHAS	see 2.1.1 for details.
RUSSP	bilateral tumour, male patient with accompanying hypertension.
RUTPA	see 2.1.1 for details.

2.1.5 Peripheral blood and non-tumour DNA from DDS patients and their parents

The laboratory code name of each DDS patient or parent peripheral blood or non-tumour DNA studied and additional relevant details are as follows:

DDS1	also known as HADO, 'female' DDS patient.
DDS1F	father of DDS1.
DDS1Fe	fetal elements from missed abortion of DDS1M.
DDS1M	mother of DDS1.
DDS2M	also known as ABAK, mother of DDS2, see 2.1.1 for details.
DDS3	see 2.1.1 for details.
DDS3F	also known as 30.2, father of DDS3, see 2.1.1 for details.
DDS3M	also known as 30.3, mother of DDS3, see 2.1.1 for details.
DDS4	see 2.1.1 for details.
DDS5	also known as D1.1, male DDS patient with accompanying hypertension, extreme oedema, respiratory infection and pneumonia.
DDS5F	also known as D1.2, father of DDS5.
DDS5M	also known as D1.3, mother of DDS5.
DDS6	also known as ZACEV, male DDS patient with accompanying hypertensive encephalopathy and oedema.

DDS7 see 2.1.1 for details.

2.1.6 LCL from renal abnormality patients

The laboratory code name of each renal abnormality patient derived LCL (work of A. Seawright and V. van Heyningen) studied and additional relevant details are as follows:

ANBLA male patient with retroperitoneal teratoma (possible WT), child has hemihypertrophy, cousin died of childhood kidney tumour.

CORMU stillborn fetus of undetermined sex which had renal agenesis, mother and maternal grandmother both had branchio-oto-renal (BOR) syndrome, latter also had a child with bilateral renal agenesis (and one unaffected child).

CRAST female patient with unilateral renal agenesis, two children had bilateral renal agenesis, mother has unilateral renal agenesis.

DABEL male patient with large multicystic kidneys and accompanying hypotonia, enlarged cerebral ventricles, trigonocephaly and choroid cysts, half sister had similar abnormalities and died of paediatric kidney tumour.

FEWAR fetus of undetermined sex with renal multicystic dysplasia.

JOHON male patient with unilateral renal agenesis, two children with bilateral renal cystic dysplasia, cousin has child with renal multicystic dysplasia.

KIRHO female patient with renal mass (possible WT) and developmental delay, possible Perlman syndrome.

REAKI female patient with two children (different fathers) with cystic kidneys.

RUFUL female patient with bifid ureter, child had renal agenesis.

2.1.7 Peripheral blood DNA from testicular tumour families

The laboratory code name of the peripheral blood DNA from each testicular tumour family member studied and additional relevant details are as follows:

Family 1

MARBE son with right-sided intermediate teratoma and left-sided undescended testis, proband.

MICBE son with left-sided intermediate teratoma and seminoma.

ALBBE father.

MILBE mother.

Family 2

ALABO	son with right-sided seminoma and undescended testis, proband.
ANTBO	son with left-sided undifferentiated teratoma.
JOHBO	father.
LOUBO	mother.

Family 3

JOGRI	son with left-sided intermediate teratoma, seminoma and undescended testis, proband.
ANGRI	son with left-sided intermediate teratoma.

Family 4

NIGUT	son with right-sided undifferentiated teratoma and left-sided undescended testis, proband.
PEGUT	son with left-sided seminoma.
JAGUT	father with bilateral sarcoma and hernia (unspecified).
MHGUT	mother with breast carcinoma.

Family 5

JOPER	son with bilateral intermediate teratoma and undescended testes, proband.
HEPER	son with left-sided seminoma.
NANMO	mother.

Family 6

DARSH	son with right-sided teratoma and undescended testes, proband.

2.1.8 Human primary MM

The MM studied were asbestos induced unless otherwise stated. The laboratory code name of each of these tumour samples and additional relevant details are as follows:

DC	peritoneal tumour, male teenage patient, asbestos independent, peripheral blood was also available.
2125	wax embedded pleural tissue section, high tumour volume.
2125md	microdissected tumour from 2125.
3775-I	wax embedded pleural tissue section, moderate to high tumour volume.
3775-II	wax embedded pleural tissue section, moderate to high tumour volume.
3775-IImd	microdissected tumour from 37750II.

6517	wax embedded pleural tissue section, unspecified tumour volume.
6517-II	wax embedded pleural tissue section, moderate tumour volume.
10563	wax embedded pleural tissue section, low tumour volume.
12894	wax embedded pleural tissue section, unspecified tumour volume.
20348	wax embedded pleural tissue section, high tumour volume.
22574	wax embedded pleural tissue section, low tumour volume.
22574md	microdissected tumour from 22574.
23046	wax embedded pleural tissue section, moderate tumour volume.
23046md	microdissected tumour from 23046.

2.1.9 Cell lines from human MM and mesothelium

The cell lines studied were kindly made available on a collaborative basis by T. Langerak (Mero lines, TM2 and NM7) or as a gift from M. J. Garlepp (JU77 and Met5a). With the exception of Mero-123, TM2, NM7 and Met5a, the cell lines have been described previously by Versnel *et al.* (1989a), Versnel *et al.* (1989b), Manning *et al.* (1991) and Center *et al.* (1993), and the tumours of origin are known to have been asbestos induced (Hagemeijer *et al.* 1990, Manning *et al.* 1991). A summary of the basic features of each cell line is as follows:

Mero-14	MM, male patient, derived from pleural effusion, epithelial histology.
Mero-25	MM, male patient, derived from autopsy pleura, biphasic histology.
Mero-41	MM, male patient, derived from pleural effusion, epithelial histology.
Mero-48a	MM, male patient, derived from autopsy pleura, biphasic histology.
Mero-48b	MM, male patient (as above), derived from autopsy omentum, epithelial histology.
Mero-48c	MM, male patient (as above), derived from autopsy liver, epithelial histology.
Mero-72	MM, male patient, derived from biopsy pleura, biphasic histology.
Mero-82	MM, female patient, derived from pleural effusion, mesenchymal histology.
Mero-83	MM, male patient, derived from pleural effusion, biphasic histology.
Mero-84	MM, male patient, derived from pleural effusion, epithelial histology.
Mero-95	MM, male patient, derived from pleural effusion, biphasic histology.
Mero-96	MM, male patient, derived from biopsy pleura, mesenchymal histology.
Mero-123	MM, male patient, derived from biopsy, mesenchymal histology.
JU77	MM, male patient, derived from pleural effusion, epithelial histology.
TM2	*in vitro* transformed, derived from NM7.
NM7	mesothelium, female patient.
Met5a	SV40 transformed mesothelium.

2.2 Nucleic acid extraction from human tissue

2.2.1 DNA extraction

Different DNA extraction protocols were used depending on whether the samples were primary tumours or normal kidney, cell pellets from cell cultures or cell lines, peripheral blood, or wax embedded tissue sections.

For primary tumours and normal kidney: tissues were homogenised in 2ml TNES (50mM Tris pH7.5, 400mM sodium chloride, NaCl, 100mM ethylenediaminetetra-acetic acid, EDTA, and 0.5% sodium dodecyl sulphate, SDS) in a 2ml microcentrifuge tube and with a fitted hand held pestle. To this solution 250µg Proteinase K was added and samples were incubated at 55 degrees centrigrade (°C) overnight. Samples were then divided in to two microcentrifuge tubes, an equal volume of 2.6M NaCl added to each, they were shaken vigorously for 15 minutes then centrifuged at 13 000g for 5 minutes. Supernatant was removed to a clean tube and an equal volume of absolute ethanol added. Samples were inverted to mix solutions and precipitate DNA, which was then spooled and resuspended in 500µl distilled water (dH_2O). To calculate the DNA concentration a dilution was made (such as 1 in 500) and the optical density measured using an ultra violet (UV) spectrophotometer set at wavelength 260nm. The optical density reading x dilution factor (such as 500) x 50 = DNA concentration in µg/ml. DNA was stored at 4°C.

For cell pellets: frozen cells were thawed at 37°C then immediately had 1ml lysis buffer (100mM Tris pH8, 150mM NaCl, 100mM EDTA and 0.5% SDS) added. To this 10µl ribonuclease A (RNase A, 10mg/ml) was added and samples were incubated at 37°C for 1 hour, then 50µl Proteinase K (5mg/ml) was added and samples were incubated at 37°C overnight. An equal volume of phenol was added, mixed for 5 minutes then centrifuged at 3 000rpm (Sorvall RC-5B centrifuge, Du Pont) for 10 minutes. The upper (aqueous) phase was removed to a clean tube and an equal volume of 1:1 phenol-chloroform mixture was added. This was mixed and centrifuged as before. The upper phase was removed to a clean tube and an equal volume of 24:1 chloroform-isoamyl alcohol mixture added. This was mixed and centrifuged as before, then the upper phase removed to a clean tube and 0.5 volume 7.5M ammonium acetate and 2 volumes absolute ethanol added. This was inverted to mix solutions and precipitate DNA. DNA was spooled if it precipitated. If no precipitation occurred, the sample was incubated at −20°C for 30 minutes then centrifuged at 3 000rpm for 15 minutes to pellet DNA. DNA was washed twice with approximately 5ml 70% ethanol then air dried and resuspended in between 200µl and 500µl 1X TE (10mM Tris and 1mM EDTA) at 4°C overnight. DNA concentration was calculated and samples stored as described above.

For peripheral blood a Nucleon I Kit (Scotlab) and the associated protocol was used as follows. To between 0.5ml and 1.5ml blood, 3 volumes Reagent A was added. Samples were rotary mixed or shaken for 5 minutes then centrifuged at 13 000g for 5 minutes. Supernatant was discarded and 340µl Reagent B added and vortexed briefly. This solution was transferred to a 1.5ml microcentrifuge tube, 2.5µl RNase A (50µg/ml) was added and the sample incubated at 37°C for 30 minutes. To this 100µl Sodium Perchlorate Solution was added, rotary mixed or shaken at 37°C for 20 minutes then at 65°C for 20 minutes. To this 580µl –20°C chloroform was added and rotary mixed or shaken at room temperature for 20 minutes. The solution was transferred to a Nucleon Tube, ensuring that the contents flowed through the insert and to the bottom of the tube. This was centrifuged at 13 000g for 1 minute, then 45µl Nucleon Silica suspension added and centrifuged at 13 000g for 4 minutes. Without disturbing the brown layer, the upper phase was removed to a clean microcentrifuge tube and 880µl absolute ethanol was added to precipitate DNA. This was centrifuged at 13 000g for 5 minutes, the DNA pellet washed twice with approximately 500µl 70% ethanol then air dried. DNA was resuspended in 100µl dH_2O. The DNA concentration was calculated and samples stored as described above.

For wax embedded tissue sections the protocol described by Wright and Manos (1990) was used as follows. Tissue sections were placed in a microcentrifuge tube, 1ml mixed xylene was added and samples incubated at room temperature for 30 minutes, occassionally inverting to mix. Samples were then centrifuged at 13 000g for 5 minutes and the supernatant discarded. This extraction was repeated, then 500µl absolute ethanol was added to pelleted tissue, inverted to mix then centrifuged as before. Supernatant was discarded, the absolute ethanol wash repeated and tissue dried under vacuum. To this 100µl digestion buffer (50mM Tris pH8.5, 1mM EDTA and 0.5% Tween 20) and 4µl Proteinase K (5mg/ml) was added and samples incubated at 55°C for 3 hours or 37°C overnight. Samples were then centrifuged at 13 000g for 5 seconds then incubated at 95°C for 8 minutes. Samples were stored at –20°C.

2.2.2 RNA extraction

Different total RNA extraction protocols were used depending on whether the samples were primary tumours, cell pellets or peripheral blood. The Total RNA Isolation Reagent (Advanced Biotechnologies Ltd.) described below is applicable to all samples but was only available towards the latter part of this study. All solutions, glassware and plasticware were either pre-treated with 0.1% diethyl pyrocarbonate (DEPC) and/or were steriled to minimise contamination from ribonucleases.

For primary tumours a modified version of the protocol described by Promega (1991) was used as follows. Tumour was homogenised in 10ml 4M GMT (4M guanidine thiocyanate, 42mM sodiun citrate, 0.2mM 2-mercaptoethanol and 0.83% N-lauroyl sarcosine) then moved to a 30ml tube and 1ml 3M sodium acetate (NaOAc) was added and samples inverted to mix. To this 10ml phenol was added and inverted, then 2ml 24:1 chloroform-isoamyl alcohol mixture added and mixed vigorously for 10 minutes. Samples were placed on ice for 15 minutes then centrifuged at 10 000rpm (Sorvall RC-5B centrifuge), at 4°C for 20 minutes. The upper (aqueous) phase was removed to a clean tube and either 11ml isopropanol or 22ml absolute ethanol was added. Samples were incubated at −20°C for at least 1 hour then centrifuged as before. Supernatant was discarded, the pellet resuspended in 500µl 4M GMT and then moved to a microcentrifuge tube. Two volumes absolute ethanol were added and samples were incubated at −20°C for 1 hour then centrifuged at 13 000g for 10 minutes. Supernatant was discarded, the RNA pellet washed twice with 70% ethanol then dried under vacuum. RNA was resuspended in between 200µl and 500µl DEPC treated dH_2O and the concentration calculated as described for DNA in 2.2.1. RNA was aliquoted in between 20µl and 50µl volumes and stored at −70°C.

For cell pellets a modified version of the protocol described by Chomczynski and Sacchi (1987) was used as follows. Frozen cell pellets were thawed on ice and in 500µl (for $5x10^6$ cells) or 1ml (for $5x10^7$ cells) solution D (4M guanidinium thiocyanate, 25mM sodium citrate, 0.5% sarcosyl and 100mM 2-mercaptoethanol). Cells were disrupted by repeated and gentle passage through a 21g needle, then 0.1 volume 2M NaOAc pH4, 1 volume phenol and 0.2 volume 24:1 chloroform-isoamyl alcohol mixture (all volumes relative to original volume) were added, with the sample being inverted after each addition to mix solutions. This was placed on ice for 15 minutes then centrifuged at 10 000rpm (Sorvall RC-5B centrifuge), at 4°C for 20 minutes. The upper (aqueous) phase was removed to a clean tube, an equal volume of isopropanol added and samples incubated at −20°C for between 1 hour and overnight. Samples were centrifuged at 10 000rpm, at 4°C for 15 minutes, the supernatant discarded and RNA pellet resuspended by vortexing in 5ml solution D. An equal volume of isopropanol was added and incubated at −20°C for 30 minutes. This was centrifuged as before and the supernatant discarded. The RNA pellet was washed with approximately 3ml 70% ethanol, dried for 10 minutes under vacuum then resuspended in 200µl DEPC treated dH_2O. The RNA concentration was calculated and samples aliquoted and stored as described above.

For all samples Total RNA Isolation Reagent (Advanced Biotechnologies Ltd.) and the associated protocol was used as follows. Between 10mg and 100mg tissue was homogenised in 1ml Total RNA Isolation Reagent in a 2ml microcentrifuge tube and with a fitted hand held pestle. This was incubated at 4°C for 5 minutes then 200µl chloroform added and mixed vigorously for 15 seconds. Samples were placed on ice for 5 minutes then centrifuged at 13 000g, at 4°C for 15 minutes. The

upper (aqueous) phase was removed to a clean microcentrifuge tube, an equal volume of isopropanol added and sample incubated at 4°C for 10 minutes then centrifuged at 13 000g, at 4°C for 10 minutes. The supernatant was discarded and the RNA pellet washed twice with approximately 1ml 70% ethanol. Pellets were vortexed then centrifuged at 7 500g, at 4°C for 5 minutes after each wash. RNA pellets were dried for 5 minutes under vacuum then resuspended in between 50µl and 100µl DEPC treated dH_2O by vortexing for 1 minute. RNA concentration was calculated and samples aliquoted and stored as described above.

2.3 cDNA synthesis

Total RNA was reverse transcribed in a first strand synthesis reaction as follows. RNA was rapidly thawed and 20µg added to a microcentrifuge tube containing 1µl RNase inhibitor (Boehringer Mannheim) and 1µl random hexamers (pd(N)6, 100pmol/µl) (Pharmacia). Sample volume was increased to 13µl with DEPC treated dH_2O and then incubated at 70°C for 5 minutes. Samples were then placed on ice and 2µl 10X reverse transcriptase buffer (200mM Tris pH8.3, 30mM magnesium chloride, $MgCl_2$, 500mM potassium chloride, KCl and 1mg/ml bovine serum albumin), 1µl 10mM deoxyribonucleoside triphosphates (dNTP's, equimolar dATP, dCTP, dGTP and dTTP), 1µl 100mM dithiothreitol (DTT), 2µl DEPC treated dH_2O and 1µl avian myeloblastosis virus (AMV) reverse transcriptase (25U/µl) (Boehringer Mannheim) added. Samples were incubated at 42°C for 1 hour then 75°C for 8 minutes. cDNA was stored at 4°C.

2.4 Polymerase chain reaction (PCR)

2.4.1 Design of oligonucleotides

Oligonucleotides were designed to comply with a number of criteria. Within a matched pair (one sense and one antisense) oligonucleotides were of a similar length and were not shorter than 18bp (18mer oligonucleotide) or longer than 26bp (26mer oligonucleotide). Sequences that were complementary between a matched pair or that had the potential to form stem and loop structures were avoided. Similarly, sequences that were known to be internally repeated in the proposed PCR product were not incorporated. The theoretical annealing temperatures of oligonucleotides in a matched pair differed by a maximum of 2°C and were in the range of between 58°C and 76°C. The formula T_A = [4(G+C) + 2(A+T)] –5°C, where T_A is the theoretical annealing temperature, was used. The function

within the brackets calculates the melting temperature (T_M) of the oligonucleotide. Additional oligonucleotides used in this study had been designed previously by M. Little or were extracted directly from published research papers or via the Johns Hopkins University Genome Database (GDB), which was accessed through the Human Genome Mapping Project Resource Centre (HGMP-RC) computing facilities (Rysavy *et al.* 1992, not consulted).

2.4.2 Synthesis and purification of oligonucleotides

Oligonucleotides were synthesised on an Applied Biosystems 391 Synthesiser and prepared as a concentrated stock in ammonium hydroxide solution (work of A. Gallacher). To purify the oligonucleotides a precipitation protocol was used as follows. An aliquot of 300µl of stock oligonucleotide solution was transferred to a microcentrifuge tube and 30µl 3M NaOAc pH5.2 and 1ml absolute ethanol added. Samples were then vortexed and incubated at –70°C for 15 minutes, then centrifuged at 13 000g for 15 minutes. Supernatant was discarded and the oligonucleotide pellet washed twice with approximately 500µl 70% ethanol, then dried for 2 minutes under vacuum and resuspended in 200µl dH_2O. Oligonucleotide concentrations were calculated as described in 2.2.1, and samples were stored at –20°C.

2.4.3 PCR conditions

Standard, touchdown, multiplex, nested and biotinylated PCR's were used (additionally, radiolabelled PCR's were used, they are described in 2.9.2). All reactions were overlaid with mineral oil and placed in a Hybaid OmniGene thermal cycler for amplification. Different *Taq* DNA polymerases, and hence buffers, were used during this study because scientific suitability and manufacturers' obligations differed for each variant of the enzyme. The *Taq* DNA polymerases used were Ampli*Taq* (Perkin Elmer), *Pfu* DNA Polymerase (Stratagene), *Taq* DNA Polymerase (Promega), Thermostable DNA Polymerase (Advanced Biotechnologies Ltd.) and Vent DNA Polymerase (New England Biolabs).

The standard PCR used comprised 1µl genomic DNA (200ng/µl) or cDNA (1µg/µl), 2.5µl each of sense and antisense oligonucleotides (each 250ng/µl), 5µl 10X *Taq* DNA polymerase buffer, 3µl 25mM $MgCl_2$, 1µl 10mM dNTPs, 0.2µl (5U/µl) *Taq* DNA polymerase and 34.8µl dH_2O. The standard amplification programme used was 1x(94°C for 1 minute, T_A for 1 minute, 72°C for 1 minute), 30x(94°C for 30 seconds, T_A for 1 minute, 72°C for 1 minute) then 1x(94°C for 30 seconds, T_A for 1 minute, 72°C for 10 minutes), where T_A is the appropriate common annealing temperature for

the particular oligonucleotides used. If satisfactory specific amplification failed, PCR's were optimised by altering the profile of the amplification programme and/or by adding dimethyl sulphoxide (DMSO) to 10% or formamide to 1%.

Touchdown PCR's were used when no single annealing temperature produced satisfactory specific amplification. These PCR's were comprised as above except that the profile of the amplification programme incorporated multiple annealing temperatures. The initial annealing temperature was decreased by 1°C consecutively throughout the amplification programme until, at between approximately cycles 20 and 24, the lowest annealing temperature was reached and maintained for the remaining cycles.

Multiplex PCR's were used to co-amplify a *WT1* sequence and a *p16* sequence and therefore differed from standard PCR's by containing two matched pairs of oligonucleotides. Different concentrations of *WT1* and *p16* oligonucleotides were used, 1250ng of the former and 500ng of the latter, to alleviate observed differences in amplification kinetics.

For nested PCR's two matched pairs of oligonucleotides were used independently and a total of 60 cycles of amplification were performed. The first pair of oligonucleotides were used for the first 30 cycles and the second pair for the remaining 30 cycles. The second pair (or inner pair) were nested, that is their sequence was internal for products specifically amplified by the first pair (the outer pair).

The first round of PCR comprised 20µl cDNA (a complete reverse transcription reaction, see 2.3), 2µl each of sense and antisense outer oligonucleotides (each 250ng/µl), 2µl 10X *Taq* DNA polymerase buffer, 0.5µl 10mM dNTP's, 0.16µl (5U/µl) *Taq* DNA polymerase and 13.34µl dH_2O. After 30 cycles of amplification, 1µl was removed and used as template for the second round of PCR, which was a standard reaction incorporating the inner oligonucleotides.

Biotinylated PCR's were performed when specific amplification products were required for automated sequencing (see 2.10.2). These PCR's differed from the above in one aspect only, the sense or the antisense oligonucleotide was modified at the 5'-OH position by the addition of a biotin molecule. Biotinylated oligonucleotides were supplied by Oswel DNA Service (University of Edinburgh).

2.4.4 Sequences and annealing temperatures of oligonucleotides

The sequence of each olignucleotide used in this study, together with its genomic location, laboratory code name, length, T_M, orientation and origin is listed in Appendix B, Part I.

The oligonucleotide annealing temperatures used in this study, together with the non-standard amplification programme profiles used for some of the PCR's, and the lengths of the specific PCR products are listed in Appendix B, Part II.

2.5 Purification of PCR products

2.5.1 Agarose gel electrophoresis

PCR's were electrophoresed through 2% agarose gels to resolve reaction products in the size range of between approximately 150bp and 1kb. The type of agarose used differed depending on whether the products were to be recovered after electrophoresis. For PCR products that did not require further experimental analysis, gels were prepared by diluting 5ml 20X TBE (1.8M Tris, 1.8M boric acid and 40mM EDTA) with 95ml dH_2O, adding 2g standard agarose and dissolving with heat. For PCR products that did require further experimental analysis, gels were prepared by diluting 20X TAE (800mM Tris, 400mM NaOAc and 20mM EDTA, adjusted to pH8.2 with glacial acetic acid) with 95ml dH_2O, adding 2g of low melting point (LMP) agarose (Gibco BRL) and dissoloving with heat. Gels were cast with appropriately sized casting trays and well combs, solidified and submerged horizontally in 0.5% TBE or 0.5% TAE (to match type used in gel preparation). PCR's had the surface mineral oil removed and 5µl of loading buffer (200mM EDTA, 15% Ficoll and a few granules of orange G pigment, adjust to pH8.1 with sodium hydroxide, NaOH) added before being loading and electrophoresed, together with 500ng of either a 1kb DNA Ladder or a φX174 RF DNA/*Hae* III size marker (Gibco BRL, for both), at 100 volts in standard agarose gels or 50 volts in LMP agarose gels. Gels were submerged first in 0.5µg/ml ethidium bromide stain for approximately 20 minutes then dH_2O for 10 minutes. DNA was visualised by placing gels on a UV transilluminator and photographed using either a Mitsubishi video copy processor or a Polaroid MP-4 Land camera with PLUS-X pan film (Kodak). PCR products required for further experimental analysis were recovered by excision of a LMP agarose gel slice.

2.5.2 Commercial kits

Different commercial kits were used depending on the size of PCR product to be purified.

A Mermaid Kit (BIO 101) and a modified version of the associated protocol was used as follows to purify PCR products of up to 200bp in length. LMP agarose gel slices were weighed and 3

volumes Binding Solution and 10µl resuspended Glassfog resin added. Samples were incubated at 50°C for 5 minutes then at room temperature for 30 minutes, vortexing often to keep Glassfog in suspension, then centrifuged at 13 000g for 5 seconds. Supernatant was discarded and 200µl Binding Solution added. Samples were then vortexed for 1 minute and centrifuged as before. The supernatant was discarded and the pellet washed with 300µl Wash Solution, then centrifuged as before, discarding the supernatant. The wash and centrifugation steps were repeated and the supernatant discarded. Pellets were dried for 30 seconds under vacuum, resuspended in 15µl dH_2O, incubated at room temperature for 5 minutes and centrifuged as before. The supernatant was removed to a clean tube. The pellet was resuspended in 10µl dH_2O then incubated and centrifuged as before. The supernatant was removed and pooled with the previous supernatant. This pooled solution contains the purified PCR product. To estimate DNA concentration 2µl of purified PCR product solution was electrophoresed through a 2% standard agarose gel together with 500ng 1kb DNA Ladder. Purified PCR product was stored at 4°C.

A Geneclean *II* Kit (BIO 101) and a modified version of the associated protocol was used as follows to purify PCR products of 200bp in length or longer. LMP agarose gel slices were melted in a microcentrifuge tube and 3 volumes Sodium Iodide Solution added. Samples were incubated at between 45°C and 55°C for 5 minutes then placed on ice. To this 5µl resuspended Glassmilk resin was added, vortexed then placed on ice for 5 minutes, vortexing often to keep Glassmilk in suspension. Samples were centrifuged at 13 000g for 5 seconds and the supernatant removed and reserved. The pellet was washed with 500µl NEW Wash, resuspended by pipetting then centrifuged as before. This supernatant was discarded. The wash, resuspension and centrifugation steps were repeated twice, then the pellet was resuspended in an equal volume of dH_2O and incubated at between 45°C and 55°C for 5 minutes. This sample was centrifuged at 13 000g for 30 seconds and the supernatant removed to a clean tube. The reserved supernatant was added to the pellet, vortexed and placed on ice for 5 minutes, vortexing often. This was centrifuged at 13 000g for 5 seconds and the supernatant discarded. The pellet was washed, resuspended and centrifuged three times, then resuspended in dH_2O, incubated and centrifuged as before. Supernatant was removed and pooled with supernatant from the first round of purification. This pooled solution contains the purified PCR product. DNA concentration was estimated and samples stored as described above.

A Magic PCR Preps Kit (Promega) and a modified version of the associated protocol was used as follows to purify PCR products of 200bp in length or longer. LMP agarose gel slices were melted, 1ml DNA Purification Resin added, and samples vortexed for 1 minute. Samples were then pipetted into a 2ml syringe barrel with a Magic PCR Preps Mini-Column attached, the syringe plunger inserted and the contents gently expelled through the Mini-Column. The Mini-Column was removed, 2ml Column Wash were taken up into the syringe barrel, then the Mini-Column was reattached. The

Column Wash was expelled and the Mini-Column removed and transferred to a microcentrifuge tube. This was centrifuged at 13 000g for 20 seconds, then the Mini-Column was removed and left to air dry for 15 minutes. The Mini-Column was then transferred to a clean microcentrifuge tube and 25µl dH_2O added to the former. This was incubated at room temperature for 5 minutes then centrifuged as before and the Mini-Column discarded. The elutant contains the purified PCR product. DNA concentration was estimated and samples stored as described above.

2.5.3 Ultrafiltration

When required for automated sequencing PCR products were purified by ultrafiltration using Centricon-100 centrifugal concentrators (Amicon) and the associated protocol as follows. PCR products were added to a Sample Reservoir attached to a Filtrate Cup, and the sample volume increased to 2ml with dH_2O. Samples were then centrifuged at 4 000rpm (Sorvall RC-5B centrifuge) for 30 minutes. The Filtrate Cup and contents were discarded and a Retentate Cup placed on the Sample Reservoir. This was inverted and centrifuged at 4 000rpm for 10 minutes. The volume of DNA sample in the Retentate Cup was increased to 80µl with dH_2O. The DNA concentration was estimated and samples stored as described in 2.5.2.

2.5.4 Enzymatic purification

The enzyme β-Agarase I (New England Biolabs), which purifies DNA by digesting agarose, and the associated protocol was used as follows. LMP agarose gel slices were weighed and then equilibrated by adding approximately 500µl 1X β-Agarase I buffer and incubating at room temperature for 30 minutes or at 4°C overnight. The buffer was discarded and the equilibration step repeated if a 30 minute incubation was used. Agarose gel slices were then melted in a microcentrifuge tube at 65°C for 10 minutes then cooled to 40°C by incubating at this temperature for 10 minutes. To this 1.5µl β-Agarase I was added for every 50µl 2% agarose to be digested, and samples were incubated at 40°C for 1 hour. A repeat amount of β-Agarase I was added and incubated as before. Samples then had 0.1 volume 3M NaOAc pH5.2 added and were placed on ice for 15 minutes and centrifuged at 13 000g for 15 minutes. Supernatant was removed to a clean microcentrifuge tube and 3 volumes absolute ethanol were added. Samples were incubated at −70°C for 30 minutes then centrifuged at 13 000g for 10 minutes. The supernatant was discarded and the PCR product pellet washed twice with approximately 500µl 70% ethanol, then dried for 2 minutes under vacuum and resuspended in 25µl dH_2O. The DNA concentration was estimated and samples stored as described 2.5.2.

2.6 Southern blotting and probing of PCR products

2.6.1 Blotting and prehybridisation of filter

PCR's were electrophoresed through 2% standard agarose gels as described in 2.5.1 then Southern blotted as follows. Gels were washed in 0.4M NaOH for 2 minutes, Hybond N+ blotting membrane (Amersham) was applied and blotting, unwicked and with sufficient absorbent paper on top of Hybond N+ filter, was performed for between 6 hours and overnight. The filter was then removed, rinsed in 2X SSC (300mM NaCl and 30mM tri-sodium citrate) and viewed on a UV transilluminator to estimate the efficiency of DNA transfer. Filters were prehybridised in approximately 20mls Church and Gilbert solution (7% SDS and 500mM sodium phosphate buffer pH7.2) for at least 1 hour and at a temperature of 5°C below the T_M of the oligonucleotide to be used as probe. All prehybridisations were performed using a Hybaid mini hybridisation oven and glass bottles.

2.6.2 Radiolabelling probe

To prepare oligonucleotide as probe it was kinase end labelled at the 5'-OH position using [γ-^{32}P]ATP (Amersham) as follows. To 10ng of oligonucleotide, 5µl 5X polynucleotide kinase (PNK) buffer (350mM Tris pH7.6, 500mM $MgCl_2$ and 25mM DTT) and 5µl [γ-^{32}P]ATP were added, the volume was increased to 23µl with dH_2O then 2µl PNK (10U/µl) (Boehringer Mannheim) was added. Samples were incubated at 37°C for 30 minutes.

2.6.3 Hybridisation and washing of filter

Filters were hybridised and washed as follows. Radiolabelled oligonucleotide probe was added directly to prehybridising filter in existing Church and Gilbert solution and hybridised overnight at the prehybridisation temperature. After hybridisation the solution was discarded and the filter washed in approximately 20mls 6X SSC containing 0.1% SDS, at the hybridisation temperature for 20 minutes. Solution was discarded and wash repeated. Solution was discarded again and wash repeated but at the T_M of the oligonucleotide probe and for 10 minutes. Filters were stored wrapped in cellophane.

2.6.4 Signal detection

Hybridised probe was detected using autoradiography. Filters were exposed to X-OMAT AR film (Kodak) for between 2 and 48 hours and the film was then developed using a Fiji RG II X-ray film processer. Alternatively, filters were exposed to a Phosphor Screen (Molecular Dynamics) for between 20 minutes and 6 hours, and the image processed and visualised using a Molecular Dynamics Phosphorimager SP and the associated ImageQuant computer software.

2.7 The HOT technique of chemical modification and cleavage of mismatched DNA

2.7.1 Formation of radiolabelled DNA heteroduplexes

Purified PCR product amplified, with specific oligonucleotides, from a known wild type genomic DNA or cDNA template was the sole radiolabelled component in the chemical modification and cleavage reactions performed in this study. For the radiolabelling reaction, 100ng of wild type PCR product was kinase end labelled with [γ-^{32}P]ATP as described in 2.6.2. This was then precipitated and used in a modified version of the protocols described by Cotton *et al.* (1988) and Condie *et al.* (1993) as follows. For precipitation, 1.5µl mussel glycogen (1mg/ml), 25µl dH_2O, 5µl 3M NaOAc, 1µl 5mM EDTA and 150µl absolute ethanol were added and the sample incubated at −70°C for 15 minutes, then centrifuged at 13 000g for 15 minutes and the supernatant discarded. Pellets were washed twice with approximately 500µl 70% ethanol, then dried for 2 minutes under vacuum and resuspended in 10µl 1X TE. The wild type PCR product is now labelled and at a concentration of 10ng/µl. One µl of this was added to a microcentrifuge tube containing 100ng purified PCR product amplified, with the same oligonucleotides as above, from a potential mutant template. To this 2µl 5X Salts (0.5M Tris pH8 and 1.5M NaCl) were added and the sample volume increased to 10µl with dH_2O. Samples were covered with mineral oil and incubated at 100°C for 5 minutes then 65°C overnight.

Mineral oil was removed, 50µl SE (0.3M NaOAc and 0.1mM EDTA) and 50µl chloroform added then sample vortexed and centrifuged at 13 000g for 2 minutes. The upper phase was removed to a clean microcentrifuge tube. To the lower phase a further 50µl SE and 50µl chloroform was added, and the sample vortexed and centrifuged as before. The upper phase was removed and pooled with the previous. This extraction was repeated until the pooled upper phases reached a volume of 200µl. To the pooled sample 1.5µl mussel glycogen (10mg/ml) and 750µl absolute ethanol were added. Samples were incubated at −70°C for 15 minutes then centrifuged at 13 000g for 15 minutes and the

supernatant discarded. Pellets were washed twice with approximately 500µl 70% ethanol, dried for 2 minutes under vacuum then resuspended in 13µl 1X TE. This is the labelled DNA heteroduplex solution.

2.7.2 Modification of mismatched DNA

Hydroxylamine (HA) reaction: HA solution was prepared fresh by adding 3.36ml dH_2O to 0.94g hydroxylamine hydrochloride (Aldrich), vortexing, then titrating to pH6 by adding 1.02ml diethylamine (Aldrich) and inverting to mix. Seven µl labelled DNA heteroduplex solution was added to 20µl of HA solution, vortexed to mix, incubated at 37°C for 1 hour (reactions performed during the early stages of this study were incubated at 37°C for 2 hours) and then placed on ice.

Osmium tetroxide (OsO_4) reaction: Six µl labelled DNA heteroduplex solution was added to 2.5µl 10X OsO_4 buffer (100mM Tris pH7.7, 10mM EDTA and 15% pyridine). Then 15µl 0.8% OsO_4 (freshly dilute 4% OsO_4 [Aldrich] with dH_2O) was added, inverted to mix, incubated at 37°C for 5 minutes (reactions performed during the early stages of this study were incubated at 37°C for 10 minutes) and then placed on ice.

To completed HA and OsO_4 reactions 200µl SE, 5µl dextran, molecular weight $2x10^6$ (10mg/ml) and 750µl absolute ethanol were added. Samples were incubated in a methanol-dry ice bath for 15 minutes, then centrifuged at 13 000g for 15 minutes and the supernatant discarded. Pellets were washed twice with approximately 500µl 70% ethanol then dried for 2 minutes under vacuum.

2.7.3 Cleavage of modified mismatched DNA

To the dried pellets from the HA and OsO_4 reactions 50µl 1M piperidine (freshly dilute 10M piperidine [Aldrich] with dH_2O) was added, vortexed for 1 minute then incubated at 90°C for 30 minutes. To this 200µl SE and 750µl absolute ethanol were added and the samples incubated in a methanol-dry ice bath for 15 minutes. Samples were then centrifuged at 13 000g for 15 minutes and the supernatant discarded. Pellets were washed twice with approximately 500µl 70% ethanol, dried for 2 minutes under vacuum then resuspended in 5µl 1X TE and 2µl stop mix (200mM EDTA, 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol FF).

2.7.4 Resolution of reaction products

Reaction products were resolved by denaturing polyacrylamide gel electrophoresis (PAGE), using 38.5cm x 20cm 6% acrylamide gels. To prepare casting plates the inner surface of a front glass plate was treated with dimethyldichlorosilane then wiped with absolute ethanol, and the inner surface of a back plate treated with a solution made by adding 30µl γ-methacryloxypropyltrimethoxysilane to 10ml absolute ethanol, mixing and then adding 30µl glacial acetic acid diluted with 300µl dH_2O. This solution was alllowed to evaporate then the surface of the plate was wiped with absolute ethanol. Treated plates were assembled and then gel prepared by adding 180µl 1% ammonium persulphate and 50µl N,N,N',N'-tetramethylethylenediamine (TEMED) to 30ml 6% acrylamide-7M urea sequencing solution (Severn Biotech Ltd.). This solution was inverted to mix then poured immediately and a sharks tooth comb inserted. When solidified, gels were assembled vertically with a heat dissipater fitted and 1X TBE as running buffer.

Reaction samples were incubated at 100°C for 2 minutes then loaded in gel, together with a sequencing reaction as a size marker, and electrophoresed at a constant power of 30 Watts for either 1hour and 20 minutes (to resolve products of between approximately 1bp and 250bp) or 4 hours (to resolve products of between approximately 200bp and 450bp). The glass plates were then separated and the acrylamide gel, adhered to the back plate, placed in fix solution (10% methanol and 10% glacial acetic acid) for 30 minutes, then rinsed in dH_2O and dried at 80°C for between 1hour and 5 hours.

Radiolabelled reaction products were detected and visualised by autoradiography or Phosphorimager analysis, as described in 2.6.4. Gels were exposed to film for between 12 hours and 1 week or to a Phosphor Screen for between 2 hours and 3 days.

2.8 Restriction enzyme digests

Different restriction enzymes and their accompanying buffers were used to digest purified or precipitated PCR products in this study. PCR products to be digested were precipitated as follows. Surface mineral oil was discarded and 50µl chloroform added. Samples were vortexed then centrifuged at 13 000g for 5 minutes. The lower phase was removed to a clean tube and to this 5µl 3M NaOAc pH5.2 and 2 volumes absolute ethanol were added. Samples were incubated at –70°C for 15 minutes then centrifuged at 13 000g for 15 minutes and the supernatant discarded. PCR product pellets were washed twice with approximately 500µl 70% ethanol, dried for 2 minutes under vacuum and resuspended in 10µl dH_2O. Eight µl were used for the digest reaction and the remaining 2µl for the control reaction, lacking restriction enzyme.

Restriction enzyme digest reactions were performed as follows. Between 200ng and 500ng of purified, or 8µl of precipitated, PCR product was added to 2µl 10X restriction enzyme reaction buffer and 2µl restriction enzyme (between 10U/µl and 20U/µl). Sample volume was increased to 20µl with dH_2O then incubated at the appropriate temperature for between 2 hours and overnight.

Reaction products were resolved by agarose gel electrophoresis, as described in 2.5.1. Different types and concentrations of agarose were used to resolve digest reaction products of different sizes. Two percent and 4% standard agarose gels were used to resolve products of between approximately 150bp and 1kb or approximately 100bp and 600bp, respectively. Four percent and 6% NuSieve agarose (FMC BioProducts) gels were used to resolve products of between approximately 50bp and 350bp. All gels were prepared and submerged in TBE as described in 2.5.1.

2.9 Allele loss studies

2.9.1 Restriction fragment length polymorphism (RFLP) analysis

RFLP analysis was performed using different restriction enzymes and purified or precipitated PCR products. Restriction enzyme digest reactions were performed and the products resolved as described in 2.8.

2.9.2 Microsatellite analysis

Microsatellite analysis was performed using PCR's with a proportion of either the sense or the antisense oligonucleotide radiolabelled. For the radiolabelling reaction, 200ng of oligonucleotide was kinase end labelled with [γ-^{32}P]ATP as described in 2.6.2. This reaction was then precipitated by adding 2.5µl 3M NaOAc pH5.2 and 75µl absolute ethanol, and incubating at −70°C for 15 minutes. Samples were then centrifuged at 13 000g for 15 minutes and the supernatant discarded. Pellets were washed twice with approximately 500µl 70% ethanol then air dried and resuspended in 50µl dH_2O. PCRs were set up as described in 2.4.3, except 1µl dH_2O was omitted and 1µl radiolabelled oligonucleotide (4ng/µl) added. PCRs were overlaid with mineral oil and amplified as described in 2.4.3. PCR products were resolved by denaturing PAGE using 38.5cm x 20cm 8% acylamide gels, as described for 6% acylamide gels in 2.7.4.

Radiolabelled PCR products were detected and visualised by autoradiography or Phosphorimager analysis, as described in 2.6.4. Gels were exposed to film for between 12 hours and 1 week or to a Phosphor Screen for between 2 hours and 3 days.

2.10 Sequencing of PCR products

2.10.1 Direct sequencing

The Sequenase Version 2.0 Kit (USB Corporation) and a modified version of the protocol described by Winship (1989) was used as follows for double stranded PCR product DNA sequencing. Termination mixes were prepared by diluting 9 volumes of each of the four dGTP Termination Nucleotide Mixtures (A, C, G and T) with 1 volume DMSO. Two µl of each termination mix was added to a separate 0.5ml microcentrifuge tube and stored at 4°C. Template mix was prepared in a 0.5ml microcentrifuge tube by adding 300ng of purified PCR product to 1µl oligonucleotide sequencing primer (0.5pmol/µl) (oligonucleotide length in bp x 330 = g/mole), 2µl 5X Sequenase Buffer and 1µl DMSO. The volume was increased to 10µl with dH_2O and template mix placed on ice. Labelling mix was prepared by adding 2µl diluted Labelling Nucleotide Mixture (diluted 1 in 15 with dH_2O) to 1µl 0.1M DTT and 0.5µl [^{35}S]dATPαS (Amersham), then placed on ice. Enzyme was prepared by adding 1µl Sequenase T7 DNA Polymerase (13U/µl) to 5µl Enzyme Dilution Buffer, then placed on ice. The template mix was removed from ice and placed at room temperature for 1 minute, then incubated at 99.9°C for 3 minutes. When this incubation had proceeded for 2 minutes the four termination mixes were incubated at 37°C. At the end of the 3 minute incubation template mix was placed into liquid nitrogen for a few seconds then 3.5µl labelling mix and 1.5µl enzyme added. To thaw the frozen template mix it was agitated with the enzyme pipette tip. When thawed sufficiently to allow pipetting the sample was incubated at room temperature for 50 seconds then 3µl were added immediately to each of the four termination mixes, now at room temperature. Reactions were incubated at 37°C for 5 minutes then 4µl stop mix was added. Reaction products were resolved by denaturing PAGE using 38.5cm x 20cm 6% acrylamide gels, as described in 2.7.4. Samples were electrophoresed for between 1hour, 20 minutes and 4 hours.

Radiolabelled reaction products were detected and visualised by autoradiography, as described in 2.6.4. Gels were exposed to film for between 12 hours and 1 week.

2.10.2 Automated sequencing

The PRISM Sequenase Terminator Single-Stranded DNA Sequencing Kit (Applied Biosystems) and a modified version of the associated protocol (A. Brown, personal communication) was used as follows. To 20µl Dynabeads (Dynal Ltd.) 100µl wash solution (250mM Tris pH8 and 0.1% Tween 20) was added. Samples were vortexed then the Dynabeads pelleted with a magnet. The supernatant was discarded, the wash repeated and the supernatant discarded again. Dynabeads were resuspended in 40µl dH_2O, then 40µl ultrafiltration purified biotinylated PCR product (between

12.5ng/µl and 25ng/µl) and 40µl 6M lithium chloride (LiCl) were added. Samples were placed on a rotary shaker at 14 000rpm (Ika Vibrax-VXR shaker, Scotlab) for 30 minutes. The Dynabead-DNA complexes were then pelleted and washed as before. The supernatant was discarded, 100µl dH_2O added and the samples vortexed then pelleted as before., The supernatant was discarded and Dynabead-DNA complexes resuspended in 20µl 0.1M NaOH (Analar grade) and incubated at room temperature for 4 minutes. Samples were pelleted and washed as before, the supernatant discarded and 100µl TE7.5 added. Samples were vortexed and pelleted as before, the supernatant discarded and 14µl dH_2O added. To this 1µl oligonucleotide sequencing primer (0.8µM, of the opposite orientation to that of the biotinylated oligonucleotide) and 5µl 5X SS buffer (equal volumes of MOPS Buffer and Manganese-Isocitrate Solution) were added and the sample incubated at 65°C for 2 minutes then cooled to room temperature for approximately 30 minutes. To this 4µl Sequenase Terminator Mix was added and sample incubated at 37°C for 2 minutes, then 1µl Sequenase T7 DNA Polymerase (Version 2.0, 1.5U/µl), was added, mixed and incubated at 37°C for 10 minutes. Dynabead-DNA complexes were pelleted as before, the supernatant discarded and pellets washed as before. The supernatant was discarded and pellets washed five times. Then 100µl 1X TE was added and the sample vortexed and pelleted as before. The supernatant was discarded and Dynabead-DNA complexes resuspended in 4µl automated sequencing stop mix (5 volumes deionised formamide and 1 volume 50mM EDTA containing 30mg/ml dextran blue).

Samples were incubated at 90°C for 2 minutes then loaded and electrophoresed in a 6% acylamide gel in an Applied Biosystems 373A DNA Sequencer (work of A. Brown and A. Gallacher). Sequence data was processed and visualised using the associated 373 DNA Sequencing System computer software (Applied Biosystems).

2.11 Computer analysis of DNA sequences

The computer programmes and the database used were accessed through the HGMP-RC computing facilities (Rysavy *et al.* 1992, not consulted). Restriction enzyme DNA recognition sequences were identified using the Seqed, Map and Mapsort functions within the University of Wisconsin's Genetics Computer Group (GCG) programme. Protein motifs were investigated by using the BLAST programme (Altschul *et al.* 1990) to search the National Biomedical Research Foundation protein database (NBRF-Protein).

Chapter 3

Searching for constitutional mutations in *WT1* in individuals with Wilms' tumour, renal abnormalities or testicular tumours.

3.1 Mutation analysis techniques

3.1.1 Detection of gross mutations

Cytogenetic analysis techniques are capable of detecting many mutations, such as deletions and insertions, that span approximately 3Mb or longer. With the increasing sophistication of these techniques, incorporating methods such as chromatin extension and fluorescence *in situ* hybridisation (FISH), the limits of resolution are continually improving. Mutations that are not of a suitable length or arrangement to be detected by these techniques may be resolved with Southern blotting and hybridisation analysis techniques, which can detect chromosomal abnormalities if they generate a pattern of restriction fragments that alters from that normally observed. Early studies which applied these techniques to WT rarely detected mutations at the *WT1* locus. For example, Cowell *et al.* (1991), Huff *et al.* (1991), Brown *et al.* (1992) and Tadokoro *et al.* (1992a), using Southern blotting and a *WT1* probe, detected mutations in a total of only 3 out of 170 (1.8%) WT. In accord with these findings, many of the WT in this study were initially analysed in this way and no mutations were detected (work of A. Seawright and K. Pritchard-Jones 1992, Ph.D. thesis). These results indicate that the majority of *WT1* mutations predicted to be present in this tumour type escape detection with these techniques.

To gain resolution beyond the Mb and kb ranges, mutation detection methods involving PCR analysis techniques are required. PCRs are designed to amplify sequences of between approximately 150bp and 1kb. Mutations that span approximately 40bp or longer and similarly alter the length of a specific PCR product can be distinguished from specific PCR products of wild type length after appropriate gel electrophoresis. PCRs can also detect more discrete mutations, such as point mutations, if they are present at one of two critical positions. Firstly, mutations that disrupt the sequence where either one or both of the oligonucleotides anneal can be detected if they cause amplification to be abolished or to be primed from inappropriate sequence. This feature is utilised by multiplex PCR when used to detect deletions affecting one of the target sequences (Ballabio *et al.* 1990). Secondly, mutations that disrupt sequence at the exon-intron boundary can be detected if they cause aberrant splicing, as this will generate specific PCR product of altered length when cDNA rather than genomic DNA template is amplified. PCR techniques were used extensively for much of the mutation analysis presented in this study.

3.1.2 Detection of point mutations

If the above techniques fail to detect mutations, point mutation analysis techniques are required. These techniques are capable of detecting discrete mutations, including those that alter only a single

base pair, and therefore provide maximum resolution for mutation analysis (reviewed by Prosser 1993). Sequencing techniques are increasingly being adapted for rapid point mutation analysis however, even with the continuing advances in automated methods, this application is currently only appropriate if a limited number of samples or relatively short lengths of sequence are to be scanned for unknown point mutations, or if the presence of a specific, previously identified mutation is suspected. Similarly, specific restriction enzyme digest assays can also be used to detect recurrent, previously identified mutations which create or destroy restriction enzyme recognition sites. These two techniques were used extensively for much of the *WT1* point mutation analysis presented in this study.

When large numbers of samples or relatively long lengths of sequence are to be scanned for unknown point mutations different techniques should be considered. Point mutations form sites of mismatch when incorporated in to heteroduplexes (one wild type strand and one mutant strand) and these sites can be enzymatically cleaved by RNase A (RNA-RNA or RNA-DNA heteroduplexes)(Myers *et al.* 1985b) or *E. coli* mismatch repair enzymes such as MutY (DNA-DNA heteroduplexes)(Lu and Hsu 1992). Heteroduplexes can also be distinguished from homoduplexes by their altered mobility after non-denaturing PAGE (Keen *et al.* 1991, Perry and Carrell 1992). This technique is called heteroduplex analysis and is very similar to the single strand conformation polymorphism (SSCP) technique which, again after non-denaturing PAGE, detects the altered mobility of wild type and mutant single strands (Orita *et al.* 1989). Furthermore, the altered mobility of duplexes which contain at least one mutant strand may be detected with techniques, based on variant forms of PAGE, termed denaturing gradient gel electrophoresis (DGGE) (Myers *et al.* 1985a), temperature gradient gel electrophoresis (TGGE) (Wartell *et al.* 1990) and constant denaturant gel electrophoresis (CDGE) (Hovig *et al.* 1991).

None of the above techniques were used in this study for a variety of reasons. For many, the rate of mutation detection is less than 100% (RNase A cleavage, heteroduplex analysis and SSCP), the optimal length of the PCR product to be scanned, between approximately 150bp and 400bp, is relatively short (heteroduplex analysis, SSCP, DGGE, TGGE and CDGE) or the necessary materials were unavailable during this study (*E. coli* mismatch repair enzymes, DGGE, TGGE and CDGE). Additionally, although all of the above techniques based on PAGE can detect point mutations, they do not indicate the location of these within the scanned sequence.

Two techniques have been described that provide a mutation detection rate of virtually 100% and can scan for unknown point mutations in PCR products that, at up to 2kb, are relatively long. Furthermore, as both techniques are based on chemical modification of bases at mismatched sites, the location of any mutations detected is indicated. The first of these techniques is the carbodiimide (CDI) technique, where mismatched bases in heteroduplexes are modified by this chemical, which then blocks the procession of radiolabelled primer extension reactions (Ganguly and Prockop 1990). The length of the truncated products of primer extension indicates the location of the blockage and therefore that of the modified base within the heteroduplex. This technique was initially considered

appropriate for this study however, after repeated attempts to establish it for detection of all possible forms of single base pair mutation, it was found to be unworkable (in common with other groups) and was therefore dismissed for point mutation analysis. The second technique, the HOT technique of chemical modification and cleavage of mismatched DNA, had been established successfully (work of J. Prosser) and was used extensively for much of the *WT1* point mutation analysis presented in this study.

3.1.3 The HOT technique of chemical modification and cleavage of mismatched DNA

The HOT technique was developed by Cotton *et al.* (1988) to permit detection of all forms of naturally occurring single base pair mutations. Other point mutations, such as deletions and insertions, can also be detected with this technique. The term HOT is an acronym derived from the two modifying chemicals used, hydroxylamine (HA) and osmium tetroxide (OsO_4). Both chemicals specifically modify bases located at sites of mismatch within heteroduplexes (RNA-DNA and DNA-DNA) and both act by attacking the pyrimidine carbon double bond at C-5,C-6. HA modifies mismatched C bases and OsO_4 modifies mismatched T bases and, to a far lesser extent, mismatched C bases also. Modification of purine bases is not involved or required by this technique as one or both strands in a heteroduplex will always contain a mismatched pyrimidine. A GC base pair can be mutated by transition to AT or by transversion to TA or CG. For each event the C on the wild type strand, mismatched as AC, TC or CC, will be available for modification by HA. The remaining possible single base pair mutation events comprise an AT base pair mutated by transition to GC or by transversion to TA or CG. For each of these events the T on the wild type strand, mismatched as GT, TT or CT, and will be available for modification by OsO_4. Four of the mismatches, TC, CC, TT and CT, will be available for modification by both HA and OsO_4. When a mismatched pyrimidine base is modified it causes the adjacent sugar-phosphate backbone to become sensitive to chemical cleavage by piperidine. Base modification and strand cleavage therefore both occur at the location of the mutation. PAGE can then be used to resolve cleaved and uncleaved strands, and these in turn can be visualised by silver staining (Saleeba *et al.* 1992) or autoradiography (Figure 3.1.1). For all of the possible modifications to be detectable by autoradiography, both strands of the heteroduplex have to have been radiolabelled. In practice, it is most common for the radiolabel to be present on only one strand of the heteroduplex, usually the wild type strand, as this does not significantly alter the mutation detection rate of virtually 100%. Additionally, by radiolabelling strands of one type only they can be combined with an excess number of unlabelled strands of the opposite type to promote formation of substrate radiolabelled heteroduplexes and diminish formation of redundant radiolabelled homoduplexes.

The only mutations which are refractive to detection when using the HOT technique are those that infrequently create a GT mismatch in a sequence context that assists stability at the mismatched

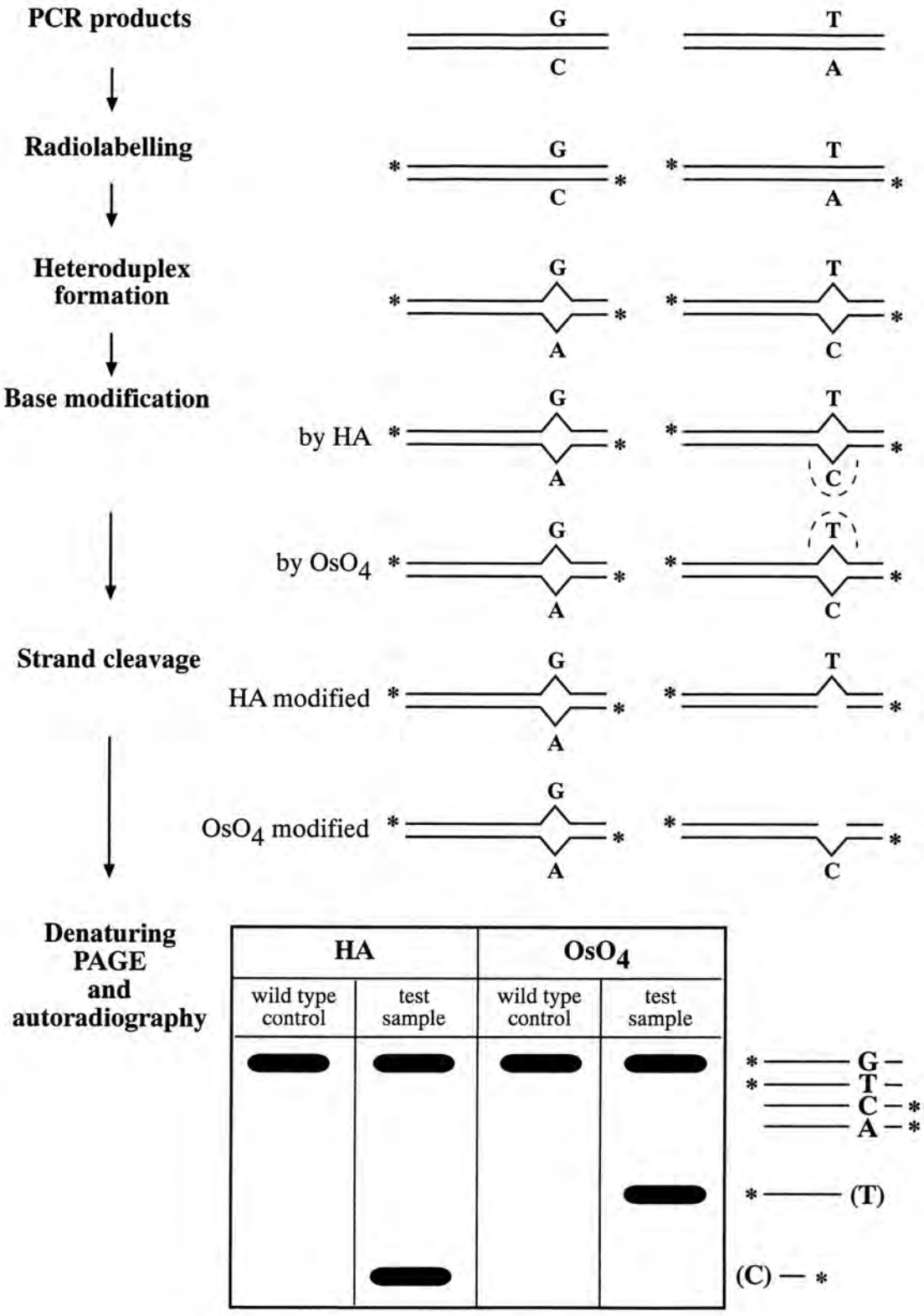


Figure 3.1.1 The HOT technique. The method that uses DNA-DNA heteroduplexes with both types of strand radiolabelled is illustrated, and demonstrates the detection of a GC→TA transversion mutation.

site (Forrest *et al.* 1991). However, with silver staining or appropriate strand labelling, this mutation can still be detected as the reciprocal AC mismatch will be available for modification by HA. When a mutation is detected its exact nature is determined by sequencing the identified region using specific PCR product that has been independently amplified from the same template source. The location of the mutated base and the nature of the mutation should be concordant when HOT technique and sequencing results are compared. All of the HOT technique point mutation analysis presented in this study was performed on DNA-DNA heteroduplexes that were radiolabelled on the wild type strand only.

3.2 Individuals with WT and/or renal abnormalities

3.2.1 Possible involvement of constitutional *WT1* mutations

At the outset of this study mutations specifically affecting *WT1* had been detected in only three cases of WT (Haber *et al.* 1990, Cowell *et al.* 1991, Huff *et al.* 1991) (see Table 1.3.1). These early reports suggested that such mutations were infrequent in unilateral and bilateral WT and that, when present, they destroyed the potential to encode four ZFs and therefore, presumably, the capacity for sequence-specific DNA binding by WT1. Similar analysis of many more WT samples was required to extend these initial findings and establish the frequency at which *WT1* mutations contribute to WT, the mutational mechanisms used and the functional significance predicted for any resulting protein product. I chose to perform a *WT1* mutation analysis study on unilateral, bilateral or familial WT individuals considered candidates for containing constitutional mutations of this gene.

LCL derived from three individuals with unilateral WT, DAVAT, DYROG and KECUL, were selected, as was normal kidney from a fourth individual with unilateral WT, 68.1. In accord with Knudson's two hit model these individuals would be predicted to most likely contain only somatic *WT1* mutations and therefore present as homozygous wild type for this gene at the constitutional level. The study of these individuals was however appropriate as, in addition to WT, they had accompanying abnormalities which also may have developed as a consequence of *WT1* mutations. DAVAT had contralateral renal agenesis, DYROG had a duplex kidney and KECUL had a duplex collecting system. As these are all defects of the renal system it is possible that constitutional *WT1* mutations have had a pleiotropic effect at this site, resulting in the development of WT and renal abnormalities. Individual 68.1 had a congenital heart defect and this may again have resulted from a pleiotropic effect of constitutional *WT1* mutations, in this instance on the development of the kidney and the pericardium. It is possible that, if such pleiotropic effects do occur, the exact nature of the *WT1* mutation and/or the genetic background dictates which tissues are affected.

Bilateral and familial WT are similar in that if they contain *WT1* mutations these will, in general, be present constitutionally at the heterozygous level, representing the first hit event. LCL derived from two individuals with bilateral WT, MASOU and RUSSP, and two individuals with familial WT, JEDOY and LISWI, were available and selected for study.

Two further LCL, derived from individuals ANBLA and KIRHO, were selected as both had tumours that are possible but undiagnosed WT and accompanying features suggestive of constitutional *WT1* mutation. ANBLA had a retroperitoneal tumour, had fathered a child with hemihypertrophy and also had a first cousin who had a paediatric kidney tumour (fatal). KIRHO, a possible Perlman syndrome individual (see Table 1.2.1), had a renal mass and developmental delay.

Involvement of *WT1* mutations in development of renal abnormalities distinct from WT has not been reported. As this gene is of critical importance in kidney development it is possible that in this tissue a different class of *WT1* mutations contributes to such abnormalities as compared to the ZF altering mutations associated with tumorigenesis. This putative different class of *WT1* mutations may involve preferential mutation of exons 1 to 6, encoding the TD. I chose to investigate this possibility by, again, analysing *WT1* at the constitutional level in suitable individuals.

In addition to the three individuals with WT and renal abnormalities, DAVAT, DYROG and KECUL, a number of LCL derived from individuals with renal abnormalities in the absence of WT were studied. These individuals are CORMU, CRAST, DABEL, JOHON and RUFUL. The presence of a contributing constitutional mutation in each of these individuals is suggested by the fact the they all have family members with identical or similar renal abnormalities. CORMU (sex undetermined) and the mother and maternal grandmother had/has branchio-oto-renal (BOR) syndrome, which comprises abnormal branchial, auditory and renal development; the latter individual also had a child with bilateral renal agenesis, CRAST and her mother have unilateral renal agenesis, the former also had two children with bilateral renal agenesis, DABEL and his half sister had large, multicystic kidneys, the latter also had a paediatric kidney tumour (fatal), JOHON has unilateral renal agenesis and has two children with bilateral multicystic dysplasia and a first cousin once removed with multicystic dysplasia, and RUFUL has bifid ureter and had a child with renal agenesis. Two further LCL, derived from individuals FEWAR and REAKI, were selected. FEWAR had multicystic dysplasia without a known familial predisposition and REAKI is unaffected but has two children, with different fathers, with cystic kidneys.

PCR amplification of individual *WT1* exons using intronic oligonucleotides C155 and 945/E194 (exon 7), C323 and 796/G667 (exon 8), 798/G26 and 801/G25 (exon 9) and C911 and C912 (exon 10), and subsequent point mutation analysis using the HOT technique had been performed previously for all the above samples except 68.1 and no mutations were detected (work of M. Little). The same exons of *WT1* from 68.1 were similarly analysed and, again, no mutations were detected (work with M. Little). These exons were preferentially analysed as they encode the ZFs. The possible

presence therefore of constitutional mutations affecting exons 1 to 6 in these samples was further investigated.

3.2.2 PCR analysis of *WT1*

LCL DNA from the above individuals, or normal kidney DNA from 68.1, was analysed for *WT1* mutations by PCR amplification of individual exons using oligonucleotides C145/F518 and C146/F519 (exon 1), C147 and C148 (exon 2), C149 and C150 (exon 3), C486 and C152 (exon 4), C153 and C154 (exon 5) and C178 and C177 (exon 6). With the exception of C145/F518, all of the oligonucleotides are intronic. Only specific PCR products of wild type length were generated (Table 3.2.1).

3.2.3 Point mutation analysis of *WT1*

As PCR analysis did not detect any mutations in the above samples, they were subjected to *WT1* point mutation analysis using the HOT technique. Exons 4, 5 and 6 were scanned against a known wild type DNA, FATO, with HA and OsO_4. No constitutional *WT1* mutations were identified in individuals with WT alone or WT and renal/other abnormalities (Table 3.2.2) or with renal abnormalities alone (Table 3.2.3).

3.3 Individuals with testicular tumours

3.3.1 Possible involvement of constitutional *WT1* mutations

Although *WT1* mutations are known to contribute to development of gonadal abnormalities such as cryptorchidism involvement of such mutations in gonadal tumours is less well defined. As expression of *WT1* in the mature gonad is confined to male-specific Sertoli cells and female-specific granulosa cells, it is possible that mutations in this gene can contribute to the derived tumour types, classified as sex cord-stromal tumours. The other major classification of gonadal tumours comprises the germ cell tumours (Adkins and Jaffe 1988), which can present in males as seminomas, in females as dysgerminomas and in both sexes as teratomas. Germ cells do not express *WT1* and may therefore be insensitive to mutations in this gene. It is however possible that, because of the numerous reciprocal interactions that occur between Sertoli cells and spermatocytes/spermatids (reviewed by

Patient	WT	abnormality renal/other	WT1 exons amplified	results
DAVAT	+ u	+/–	1,2,3,4,5,6	all wt
DYROG	+ u	+/–	1,2,3,4,5,6	all wt
KECUL	+ u	+/–	2,3,4,5,6	all wt
68.1	+ u	–/+	2,3,4,5,6	all wt
MASOU	+ b		2,3,4,5,6	all wt
RUSSP	+ b		1,2,3,4,5,6	all wt
JEDOY	+ f		2,3,4,5,6	all wt
LISWI	+ f		1,2,3,4,5,6	all wt
ANBLA	(rp)		1,2,3,4,5,6	all wt
KIRHO	(rm)	–/+	2,3,4,5,6	all wt
CORMU		+/–	2,3,4,5,6	all wt
CRAST		+/–	2,3,4,5,6	all wt
DABEL		+/–	1,2,3,4,5,6	all wt
FEWAR		+/–	1,2,3,4,5,6	all wt
JOHON		+/–	2,3,4,5,6	all wt
REAKI		(+)/–	2,3,4,5,6	all wt
RUFUL		+/–	2,3,4,5,6	all wt

Table 3.2.1 Constitutional mutation analysis of *WT1* in individuals with WT and/or renal abnormalities. Mutation analysis was performed using PCR amplification. The data detailed in the 'results' column relates only to the length of the specific PCR products generated. + - present; – - not present; (+) - present in offspring only; u - unilateral; b - bilateral; f - familial; (rp) - retroperitoneal tumour, possible WT; (rm) - renal mass, possible WT; wt - wild type.

Patient	WT	other tumour	abnormality renal	abnormality other	WT1 exon scanned	HA result	OsO_4 result
DAVAT	+ u		+		4	wt	wt
					5	wt	wt
					6	wt	wt
DYROG	+ u		+		4	wt	wt
					5	wt	wt
					6	wt	wt
KECUL	+ u		+		4	wt	wt
					5	wt	wt
					6	wt	wt
68.1	+ u			+ he	4	wt	wt
					6	wt	wt
MASOU	+ b				4	wt	wt
					5	wt	wt
					6	wt	wt
RUSSP	+ b				4	wt	nd
					5	wt	wt
					6	wt	wt
JEDOY	+ f	+ br			4	wt	wt
					5	wt	wt
					6	wt	wt
LISWI	+ f				5	wt	wt
					6	wt	wt
ANBLA		+ rp			4	wt	wt
					5	wt	wt
					6	wt	wt
KIRHO		+ rm		+ dd	4	wt	wt
					5	wt	wt
					6	wt	wt

Table 3.2.2 Constitutional point mutation analysis of *WT1* in individuals with diagnosed or possible WT. Mutation analysis was performed using the HOT technique. + - present; u - unilateral; b - bilateral; f - familial; br - breast; rp - retroperitoneal, possible WT; rm - renal mass, possible WT; he - heart; dd - developmental delay; wt - wild type and nd - not determined.

Patient	renal abnormality in patient	renal abnormality in family member(s)	WT1 exon scanned	HA result	OsO_4 result
CORMU	+	+	4	wt	wt
			5	wt	wt
			6	wt	wt
CRAST	+	+	4	wt	wt
			5	wt	wt
			6	wt	wt
DABEL	+	+	4	wt	wt
			5	wt	wt
			6	wt	wt
FEWAR	+		4	wt	wt
			5	wt	wt
			6	wt	wt
JOHON	+	+	4	wt	wt
			5	wt	wt
			6	wt	wt
REAKI		+	4	wt	wt
			5	wt	wt
			6	wt	wt
RUFUL	+	+	4	wt	wt
			5	wt	wt
			6	wt	wt

Table 3.2.3 Constitutional point mutation analysis of *WT1* in individuals from renal abnormality families. Mutation analysis was performed using the HOT technique. + - present and wt - wild type.

McGuinness and Griswold 1994) and between granulosa cells and oocytes (reviewed by Eppig 1991), *WT1* mutations expressed by the somatic cells of the gonad influence germ cell tumorigenesis. Samples that permit investigation of the latter proposal were available and were analysed in a preliminary study for constitutional mutations of *WT1*. These samples derived from 18 individuals, male and female, from 6 testicular tumour families. Eleven of the males had germ cell tumours, and six of these individuals additionally had cryptorchidism, a further indication that constitutional *WT1* mutations may be present. The remaining individuals, all parents of the above, did not have gonadal tumours however, because of possible inherited predisposition for this tumour type, these individuals were also considered candidates for carrying constitutional *WT1* mutations. Family 1 comprised a son with teratoma and cryptorchidism, a son with teratoma and seminoma, father and mother; individuals MARBE, MICBE, ALBBE and MILBE, respectively. Family 2 comprised a son with seminoma and cryptorchidism, a son with teratoma, father and mother; individuals ALABO, ANTBO, JOHBO and LOUBE, respectively. Family 3 comprised a son with teratoma, seminoma and cryptorchidism, and a son with teratoma; individuals JOGRI and ANGRI, respectively. Family 4 comprised a son with teratoma and cryptorchidism, a son with seminoma, father with bilateral sarcoma and hernia (unspecified) and mother with breast carcinoma; individuals NIGUT, PEGUT, JAGUT and MHGUT, respectively. Family 5 comprised a son with teratoma and cryptorchidism, a son with seminoma, and mother; individuals JOPER, HEPER and NANMO, respectively. And lastly, Family 6 comprised a son with teratoma and cryptorchidism; individual DARSH.

3.3.2 PCR and point mutation analysis of *WT1*

Peripheral blood DNA from the above individuals was analysed for *WT1* mutations by PCR amplification of individual exons using intronic oligonucleotides C486 and C152 (exon 4), C153 and C 154 (exon 5) and C178 and C177 (exon 6). All of the samples amplified for each of these exons and generated only specific PCR products of wild type length. Samples from all of the members of families 1, 2, 3 and 5 were subsequently subjected to point mutation analysis using the HOT technique. Exons 4 and 6 were scanned against a known wild type DNA, FATO, with HA and OsO_4. No constitutional *WT1* mutations were detected. Upon completion of this analysis, further PCR and point mutation analysis of *WT1* in all of the testicular tumour family members was halted in anticipation of receiving RNA/cDNA samples, which provide templates more convenient for such analysis.

3.4 Discussion

The possible presence of constitutional *WT1* mutations in individuals with WT, renal abnormalities or testicular tumours has been investigated. Firstly, DNA from 17 individuals with WT and/or renal abnormalities; including unilateral, bilateral and familial WT, was analysed. Individual *WT1* exons were PCR amplified and subjected to point mutation analysis using the HOT technique; no mutations were detected in any of the samples. Mutations may however have escaped detection as some exons were not amplified in all samples and some were not subjected to analysis using the HOT technique. Furthermore, with the exception of exon-intron junction sequences, analysis of the non-coding regions of *WT1* was omitted.

The 5' region of *WT1* is very GC rich, making it refractive to PCR amplification. It is most probably for this reason that exon 1 did not amplify in WT samples KECUL, 68.1, MASOU, JEDOY and KIRHO, and renal abnormality samples CORMU, CRAST, JOHON, REAKI and RUFUL. The first 362bp (44%) of exon 1 were excluded from analysis in all samples, however this is exclusively untranslated sequence. Recently a new mutational mechanism has been identified in exon 1, comprising discrete deletions and insertions at sites in close proximity to dinucleotide and trinucleotide repeats (non-expanding) and the tetranucleotide 5'-CCTG-3' (Huff *et al.* 1995). These types of sequence have been identified in other genes as hotspots for recombination (reviewed by Steinmetz *et al.* 1987) and deletion (Krawczak and Cooper 1991). In the *WT1* study, 4 out of 80 (5%) WT individuals, a relatively high proportion, contained this type of mutation either constitutionally or somatically. These mutations all created a frameshift, predicted to cause termination of translation within the TD. Gessler *et al.* (1994) have also reported an exon 1 frameshift deletion mutation in a unilateral WT. Similarly, mutations in exons 2 and 3 have been detected by other researchers subsequent to this study (see Table 1.3.1 and Figure 1.3.3). Three of these mutations are missense mutations which may switch the TD from a repressor to an activator of transcription (Park *et al.* 1993a, Park *et al.* 1993c, Gessler *et al.* 1994), suggesting that even in the presence of correct sequence-specific DNA binding, altered WT1 can contribute to tumorigenesis. As point mutation analysis was not performed on exons 1, 2 and 3 in this study, the possible presence of similar mutations in the samples detailed here must not be overlooked.

Exons 4, 5 and 6 were preferentially selected for point mutation analysis as, second to those encoding the ZFs, these three exons contain the most highly conserved sequence of *WT1*. During this study four mutations in this region of the gene were reported in WT; two frameshift deletion mutations in exons 4 and 6 (Pelletier *et al.* 1991b) and two genomic deletions encompassing exon 6 (Huff *et al.* 1991, Tadokoro *et al.* 1992a). Mutations in exon 5 remain to be reported and therefore this exon is the only coding region of *WT1* to always present as wild type, suggesting that preservation of this sequence is an absolute requirement for cell viability. Similarly, mutations which affect exon 5

alternative splicing are unknown. In this study, inclusion of the exon-intron junction sequences for analysis indicates that exons 4, 5 and 6 should be correctly spliced, as should exons 7, 8, 9 and 10. In support of this, PCR amplification of the TD encoding sequence from cDNA from RUSSP and LISWI detected both the −17aa and +17aa isoforms. There is no evidence therefore that the ratio of the four mRNA isoforms is perturbed in any of the present samples.

In addition to the 17aa and KTS alternative splices involving exons 5 and 9, respectively, splicing of exons 2, 4 and 8 will maintain the correct open reading frame of *WT1*. As exon 2 was not subjected to HOT technique analysis it is possible that mutations causing its aberrant splicing are present in many of the samples detailed here. Detection of this type of TD alteration in WT has been reported (Haber *et al.* 1993). The significance of these findings is, however, unclear; firstly, in these tumours the ratio of aberrant to wild type transcripts is low, and the presence of mutations could not be demonstrated at the genomic level, and secondly, independent studies indicate that low levels of this aberrant transcript are present in normal individuals (A. Seawright, personal communication).

As the majority of non-coding sequence was omitted from analysis, mutations that affect the untranslated sequences of exons 1 and 10, or the promoter region will have escaped detection. This aspect of mutation analysis was excluded from these studies as the consequences that such mutations have on gene expression can frequently only be recognised with mRNA studies and for many of the present samples only constitutional DNA was available. Interestingly, a recent report has indicated a paucity of mutations within the promoter region of *WT1* (Grubb *et al.* 1995). When present, however, such mutations may have the potential to alter expression levels in some or all of the correct tissues or to create an ectopic expression pattern. Additionally, a complete lack of mutations may still not exclude such alterations of *WT1* expression as epigenetic features, such as hypermethylation of 5' sequences, may also elicit these effects (reviewed by Counts and Goodman 1995). Reduced expression as a result of hypermethylation has been reported for the von Hippel-Lindau (*VHL*) gene in clear cell renal carcinoma (CCRC) (Herman *et al.* 1994), *RB1* in retinoblastoma (Greger *et al.* 1994) and, as described in 1.5.8, *p16* in various tumour types (Merlo *et al.* 1995). Mutations abolishing expression of one copy of *WT1* are not predicted in these samples as these would cause WT1 haplo-insufficiency and therefore a WAGR syndrome phenotype.

As only approximately 10% of unilateral and bilateral WT are predicted to contain *WT1* mutations it is possible that the six individuals in this study with these forms of tumour contain mutations in other critical genes, such as the putative *WT2* gene at 11p15.5. None of the WT from these individuals are known to be anaplastic and therefore the presence of constitutional heterozygous *p53* mutations, constituting Li-Fraumeni syndrome, is not indicated. Individuals with this syndrome, who are predisposed to development of many tumour types, only rarely develop WT (Hartley *et al.* 1993). Although unremarkable, the apparent lack of *WT1* mutations in the unilateral WT individuals in this study is perhaps surprising as each had an associated abnormality of the renal system or heart. The similar lack of detectable mutations in the two familial WT individuals is, again, unremarkable,

even though during this study Pelletier *et al.* (1991b) reported an inherited *WT1* mutation (see Table 1.3.1). Furthermore, it is conceivable that the two possible WT samples, ANBLA and KIRHO, are other types of renal tumour not associated with *WT1* mutations.

In addition to the above results that provide no evidence to support the proposal that *WT1* mutations may contribute to renal abnormalities, the results from the eight individuals with such abnormalities in the absence of WT reinforces this. Interestingly, Kumer *et al.* (1992) have defined a locus for BOR syndrome on chromosome 8q and it is possible that it is this and not *WT1* that is mutated in CORMU. Additionally, it is feasible that REAKI, who is unaffected, may be a germline mosaic, containing only wild type *WT1* in her haematopoietic system, therefore making analysis of LCL unsuitable.

Similarly to the study of individuals with WT and/or renal abnormalities, DNA from 18 individuals from 6 testicular tumour families was analysed to a more limited extent; no constitutional WT1 mutations were detected. A number of more recent reports also detail *WT1* mutation analysis studies in a total of 119 gonadal tumour samples and, in agreement with this study, no mutations were detected. Bruening *et al.* (1993) used SSCP point mutation analysis techniques to scan exons 1 to 10 of *WT1* in ovarian tumours, a tumour type previously identified as frequently containing deletions of chromosome 11p13 and 11p15 (Viel *et al.* 1992). Similarly, Coppes *et al.* (1993b) scanned exons 6 to 10 in granulosa, Sertoli and Leydig cell sex cord-stromal tumours, and Looijenga *et al.* (1994) scanned exons 2 and 6 to 10 in seminoma and non-seminoma male germ cell tumours, which, again, are tumour types previously identified as frequently displaying LOH at chromosome 11p13 and 11p15 (Lothe *et al.* 1993). It was unfortunate that no sex cord-stromal tumours were available for analysis in this study, however limited direct sequence analysis of cDNA from four ovarian carcinoma cell lines, COV362, COV413, COV434 and COV446 (gift from T. Langerak, described by van den Berg-Bakker *et al.* 1993) identified a possible mutation; the presence of only +KTS sequence in COV434, which, interestingly, is the only cell line derived from a granulosa cell tumour. A further form of gonadal tumour is gonadoblastoma and two individuals with such tumours (one of which was associated with a granulosa cell tumour) have been found to contain *WT1* mutations. These tumours are however remarkable as they developed in a DDS context (see Table 1.3.4) (Pelletier *et al.* 1991a). Predisposition for gonadoblastoma rather than WT, in association with nephropathy and PGD, comprises a syndrome distinct from DDS, the Frasier syndrome (Moorthy *et al.* 1987). Indeed, one of the individuals described by Pelletier *et al.* (1991a) as having DDS may in fact have been misdiagnosed and may actually have Frasier syndrome. In contrast however, an independent study that analysed *WT1* exons 8 and 9 in three diagnosed Frasier syndrome individuals detected no mutations (Poulat *et al.* 1993), suggesting that, in common with the other forms of gonadal tumour, involvement of *WT1* mutations in tumorigenesis of this tissue is rare. Similarly, a tumour resected from testis and believed to be germ cell in origin has been shown histologically to be a WT (Gillis *et al.* 1994). However, analysis of *WT1* in this tumour, by Southern blotting and SSCP analysis of exons

2 and 6 to 10, did not detect any mutations. Furthermore, loci at 11p15.5 rather than 11p13 are implicated in this tumour as biallelic expression of *H19* and *IGF2* was detected.

Overall, this study has shown that, although no mutations in *WT1* were detected, our findings are in agreement with those subsequently reported for unilateral, bilateral and familial WT and for gonadal tumours. Furthermore, the possible involvement of such mutations in renal abnormalities distinct from WT remains unsupported. Finally, it is interesting to note that the current spectrum of *WT1* mutations forms a reciprocal pattern, that is that in general these mutations appear to contribute to tumours but not abnormalities of the renal system, and conversely, to abnormalities but not tumours of the gonadal system.

Chapter 4

Analysis of first and second hit *WT1* mutations in WAGR syndrome

4.1 Contribution of *WT1* mutations to WAGR syndrome

The constitutional heterozygous 11p13 deletions invariant in WAGR syndrome render the *WT1* and *PAX6* genes hemizygous, thereby causing WT predisposition and aniridia, respectively. These deletions may also disrupt flanking genes, a feature which was originally thought to elicit the remaining two components of WAGR syndrome; GU abnormalities and mental retardation. The former is now known to result from the pleiotropic effect of constitutional hemizygous *WT1* on the developing genital system, whereas the genetic basis of the latter remains undetermined.

As described in 1.2.4.2, development of WT, GU abnormalities and mental retardation is not fully penetrant in this syndrome. With respect to *WT1*, the reason(s) why the hemizygous state elicits GU abnormalities in only a proportion of WAGR syndrome individuals and with variable expressivity is not fully understood. Certainly, genetic background is involved, as clearly demonstrated by the fact that the developing genital system in males is more sensitive than that in females to *WT1* mutations. The GU abnormalities characteristic of this syndrome, most commonly hypospadias and cryptorchidism, are relatively mild and, as described in 1.3.6, are thought to develop because of WT1 haploinsufficiency.

As mentioned, only a proportion of WAGR syndrome individuals develop WT. Tumorigenesis is not fully penetrant as a requirement for a somatic second hit, in addition to the constitutional first hit *WT1* deletion, is predicted. Again as described in 1.3.6, the most obvious form of second hit in this syndrome is functional loss of the remaining copy of *WT1*. Homozygosity for the constitutional deletion is rarely observed in WAGR syndrome as this is expected to be cell lethal (as they are such large deletions). A second hit affecting the remaining copy of *WT1* will, therefore, be more subtle, for example intragenic deletion or insertion. At the outset of this study second hit *WT1* mutations had been identified in only three WAGR-WT (Brown *et al.* 1992, Baird *et al.* 1992a) (Table 1.3.2). Each of these mutations is intragenic and they create premature stop codons, which at the protein level are predicted to produce WT1 with either no ZFs, one ZF or only a residual N-terminal peptide structure, and therefore incapable of sequence-specific DNA binding.

4.2 Predicted involvement of second hit *WT1* mutations in WAGR-WT from two individuals

4.2.1 Phenotype of each WAGR syndrome individual

To investigate further the involvement of second hit *WT1* mutations in this form of WT I chose to perform a mutation analysis study of this gene in tumours from WAGR syndrome individuals.

Samples from two such individuals, ALSTA and NAHAS were available for analysis. ALSTA has partial spectrum WAGR syndrome, consisting of unilateral WT, aniridia and micropenis (not apparent until late childhood). In contrast, NAHAS has full spectrum WAGR syndrome, consisting of unilateral WT, aniridia, cryptorchidism (right testis), speech retardation and microcephaly. Both individuals are XY males and contain 11p13 deletions that are sub-microscopic. The mother and father of each individual are phenotypically normal.

4.2.2 PCR analysis of the remaining copy of *WT1*

Although previous analysis of ALSTA-WT and NAHAS-WT had detected expression of *WT1*, these tumours had been temporarily thawed prior to the present study. Because of this the quality of these tissues is poor and hence only low yields of RNA could be recovered from them. Standard PCR techniques were inadequate for analysis of this material and therefore reverse transcription followed by nested PCR (RT-nested PCR) was used. This PCR strategy offers increased sensitivity as it can incorporate 60 cycles of amplification, in comparison to the standard 30 cycles. The TD encoding sequence and the ZF encoding sequence were amplified from cDNA in two overlapping fragments, 5'TD and 3'ZF, respectively. The 5'TD fragment was amplified using outer oligonucleotides D609 (exon 1) and D610 (exon 6) and inner oligonucleotides B920 (exon 1) and B918 (exon 6), and the 3'ZF fragment amplified using outer oligonucleotides C582 (exon 5) and C583 (exon 10) and inner oligonucleotides B919 (exon 6) and B917 (exon 10). Collectively, these two fragments cover all but the first 256bp of coding sequence (Figure 4.2.1).

The 5'TD and 3'ZF regions amplified from ALSTA-WT and NAHAS-WT samples and only specific PCR products of wild type length were generated. Similarly, amplification of the 5'TD region detected both the −17aa and +17aa isoforms in each tumour sample.

4.2.3 Point mutation analysis of the remaining copy of *WT1*

As PCR analysis did not detect any mutations in the above samples, they were subjected to point mutation analysis using direct sequencing. Additionally, similar analysis of individual exons from NAHAS-WT DNA was performed when ambiguities were detected in the cDNA sequence. As a result of this approach much of the coding sequence of *WT1* in this tumour has been analysed repeatedly. Specifically, sequencing of the 5'TD region was primed with oligonucleotides B920, B918, 434/F25 (exons 1-2) and B700/E976 (exons 5-6), and similarly the 3'ZF region was primed with oligonucleotides B919 and B917. Individual exons from NAHAS-WT DNA were PCR amplified using oligonucleotides F516 and F517 (exon 1), C147 and C148 (exon 2), C153 and C154 (exon 5), C178 and C177 (exon 6), C155 and 945/E194 (exon 7), 798/G26 and 801/G25 (exon 9) and C911 and

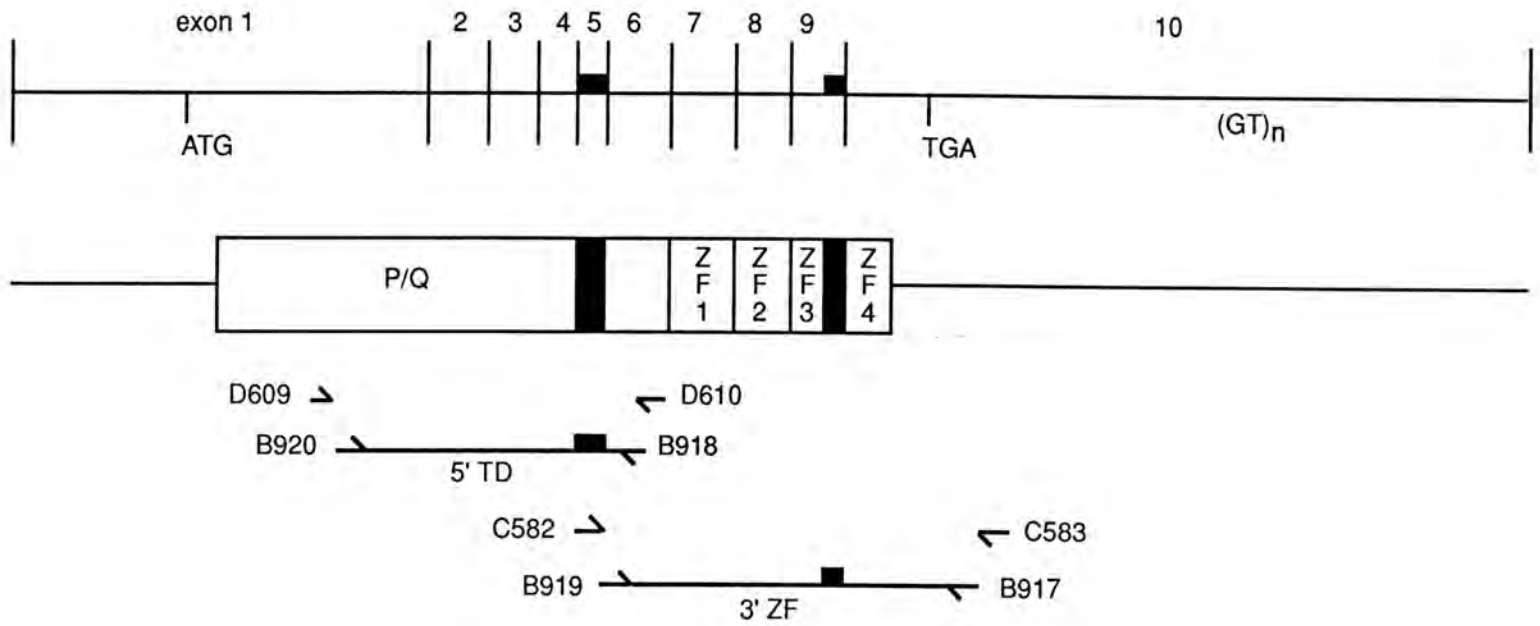


Figure 4.2.1 Strategy for PCR amplification of *WT1* coding sequence from cDNA. The coding sequence of *WT1* is PCR amplified as two overlapping fragments, 5'TD and 3'ZF, using the oligonucleotides detailed. The 5'TD fragment incorporates alternative splice I, 17aa, and therefore generates specific PCR products of 568bp and 619bp. Similarly, the 3'ZF fragment incorporates alternative splice II, KTS, generating specific PCR products of 590bp and 599bp. The alternative splices are illustrated as solid boxes. The first 256bp of coding sequence are excluded from amplification.

C912 (exon 10). With the exception of F516, each of the latter oligonucleotides is intronic. Individual exons were sequenced using one or both of the appropriate PCR oligonucleotides as primer.

Sequencing of the 3'ZF region with oligonucleotide B917 as primer identified two species of specific PCR product, the –KTS and +KTS isoforms, in both ALSTA-WT and NAHAS-WT samples. Additionally, in ALSTA-WT this analysis detected a 2bp deletion in exon 10. This frameshift mutation, which alters the sequence starting at nucleotide 1696 from AGAG to AG, destroys the normal stop codon and creates a new one at 201bp downstream of the former (Figure 4.2.2a). It is proposed that this deletion is a second hit as it was confirmed in ALSTA-WT DNA and, by analysis of LCL cDNA, was found to be not present in the remaining copy of *WT1* present constitutionally or in the mother, MASTA or the father, FASTA. This latter analysis of *WT1,* in non-expressing cells, was possible as RT-nested PCR permits, and was actually originally designed for, amplification of illegitimate transcripts. These transcripts are those generated by breakthrough transcription, a process which produces minute quantities of tissue-specific mRNA in non-expressing cells (it is estimated that for each tissue-specific gene there may be as few as one mRNA per 100 to 1 000 such cells) (reviewed by Kaplan *et al.* 1992). As illegitimate transcripts have the same sequence as the corresponding ones present in expressing cells, their use in mutation analysis is appropriate, and, indeed, often vital.

Despite repeated analysis, no second hit *WT1* mutations were detected in NAHAS-WT. Inclusion of the genomic amplification of exon 1 increased the 5' boundary of sequence analysis by only 5bp, as oligonucleotides F516 and B920 are overlapping. Similarly, the genomic amplification of exon 10 increased the 3' boundary by only 21bp of untranslated sequence, as C912 and B917 are overlapping. Definite wild type sequence was obtained for all but the first 15bp (excluding B920) of the 5'TD region and for all but the last 1bp (excluding B917) of the 3'ZF region.

4.3 Predicted consequence of the second hit *WT1* mutation in ALSTA-WT

As the second hit *WT1* mutation in ALSTA-WT causes a frameshift which increases the potential coding sequence by 201bp, it is predicted that translation of this sequence will produce protein with an extended C-terminus (Figure 4.2.2b). This aberrant form of WT1 would lack the 10 residues that normally follow ZF4, RNMTKLQLAL, and these would be substituted by 77 inappropriate residues, KHDQTPAGALRGLPRGPFSVPGSTVCELLSSLTLHSSSLKRKLQLIFFI QLPRQDTGASGNYQVCLEELVSALPTFS. No known protein motifs are present in the latter residues. Nonetheless, it is possible that, given the terminal position of these residues, they cause aberrancy by imparting an additional function to the protein, the TD and ZFs of which may retain the correct conformation and permit sequence-specific DNA binding. Alternatively, aberrant WT1 that is incapable of such DNA binding may be generated if these inappropriate residues interfere with protein folding and/or block access of the ZFs to the target nucleotides in the major groove.

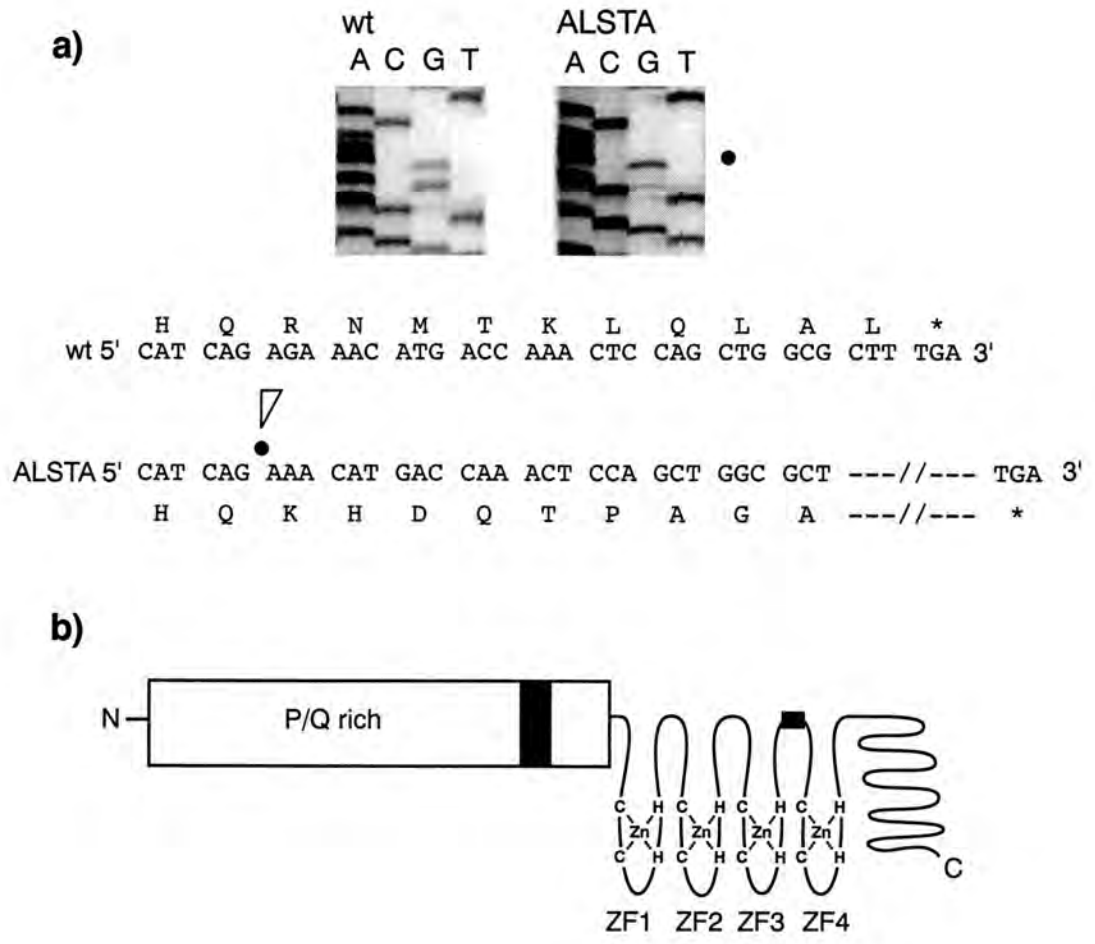


Figure 4.2.2 Second hit *WT1* mutation detected in ALSTA-WT. a) sequence analysis detected a 2bp (GA) deletion in exon 10. The resulting frameshift destroys the normal stop codon and creates the potential to encode 77 inappropriate residues. The first H residue illustrated, preceding the frameshift, is the most C-terminal zinc co-ordinating residue. wt - wild type. **b)** schematic illustration of the predicted structure of WT1 in ALSTA-WT, the protein is extended at the C terminus. Only the +17aa+KTS isoform is illustrated, but the remaining three isoforms are also predicted to be present.

Experiments to analyse the DNA binding properties of this predicted aberrant form of WT1 were initiated, however these could not be completed within the duration of this study. The mutant coding sequence, in the +17aa-KTS and +17aa+KTS isoforms, has been unidirectionally cloned in to the pET protein expression vector, but confirmation that PCR-generated mutations are not present and subsequent protein expression and mobility shift assays remain to be performed.

4.4 Possible involvement of 11p15.5 loci in WAGR-WT

As described previously and demonstrated by ALSTA, the most obvious mutational mechanism for development of WAGR-WT is a second hit causing functional loss of the remaining copy of *WT1*. It is however possible that, although a second hit is present, a locus other than *WT1* is affected. Evidence for such a mechanism is presented by Henry *et al.* (1989) and Jeanpierre *et al.* (1990), who describe a total of three WAGR-WT displaying somatic LOH at 11p15.5. As described in 1.2.6, a putative gene for WT predisposition, *WT2*, maps to this region, and loci here frequently display LOH (of the maternal homologue) in other forms of WT and are particularly implicated in development of BWS-WT. It is possible therefore that constitutional deletion of *WT1* followed by somatic LOH at the more distal region 11p15.5 (affecting *WT2*?) has caused development of these three WAGR-WT. Unfortunately the status of the remaining copy of *WT1* in these tumours has not been reported, however for one it was demonstrated that the deletion and the LOH affected the maternally derived homologue of 11p (Jeanpierre *et al.* 1990).

The apparent lack of a mutation in the remaining copy of *WT1* in NAHAS-WT suggests that a second hit affecting a different locus may be present in this tumour. To investigate the possible involvement of a second hit affecting 11p15.5, I chose to analyse this region in NAHAS for LOH. Three polymorphic marker sequences that map to 11p15.5 were selected, comprising the variable number tandem repeat (VNTR) sequence 3' of the Ha-*ras* gene (Decorte *et al.* 1990) and the *IGF2* RFLP sequences exon 4 *Apa I* (Tadokoro *et al.* 1991) and exon 9 *Ava II* (Gloudemans *et al.* 1993). LCL and WT DNA from NAHAS was PCR amplified for these marker sequences using oligonucleotides G517 and G518 (VNTR), G16 and G17 (*Apa I*) and G20 and G21 (*Ava II*). Specific PCR products were generated for each of the marker sequences, and those of the RFLP markers were subsequently digested with the appropriate restriction enzyme. Comparison of the final reaction products from each marker sequence between LCL and WT identified heterozygosity but no differences. This analysis therefore provides no evidence for the presence of 11p15.5 LOH in NAHAS-WT and, as with analysis of *WT1*, has not detected a second hit in this tumour.

4.5 Does the mental retardation component of WAGR syndrome result from imprinting at the *WT1* locus?

4.5.1 Evidence for such a mechanism

Expression of *WT1* in foetal brain, an ectoderm derived tissue, was first reported by Pritchard-Jones *et al.* (1990), who describe the level of expression as very weak. More recently Jinno *et al.* (1994) have also reported expression of this gene in foetal brain and, as mentioned in 1.3.2.3, provide evidence for monoallelic expression, suggesting that *WT1* may be imprinted in a subset of expressing tissues. The specific temporal and spatial pattern of *WT1* expression within the brain is not known, although the latter mentioned study does report that, by using immunohistochemical techniques, expression has been identified in the region in which the earliest stages of differentiation occur. In the developing mouse, expression in the brain is detected for a period of only 24 hours and is localised to the fourth ventricle (hindbrain) (Armstrong *et al.* 1992), whereas in rat brain expression persists postnatally and is localised to the area postrema (Sharma *et al.* 1992). As the functions of these regions of the brain are not known, in humans or rodents, suggested roles for *WT1* in development of this tissue are purely speculative.

Nonetheless, with the apparent identification of monoallelic expression of *WT1* in this tissue, albeit in only two samples, Jinno *et al.* (1994) have put forward an imprinting model implicating this feature in the mental retardation component of WAGR syndrome. In their model the important factor is not the size of the constitutional 11p13 deletion, but its parental origin. It is proposed that only the maternally derived copy of *WT1* is active in foetal brain and therefore constitutional deletions of paternal origin should not disturb expression (as the remaining copy of *WT1* is maternally derived and therefore active) whereas those of maternal origin should abolish expression (as the remaining copy of *WT1* is paternally derived and therefore inactive). Accordingly, it is suggested that the former mutations will not elicit mental retardation and that the latter will. To support this model the authors describe two WAGR syndrome individuals; both have paternally derived 11p13 constitutional deletions and no evidence of mental retardation. This report by Jinno *et al.* (1994) remains the only one to suggest that *WT1* is imprinted and, consequently, the only one implicating this model as the cause of mental retardation in WAGR syndrome.

4.5.2 Possible explanation for the phenotypic difference between ALSTA and NAHAS

Of the two WAGR individuals available for analysis in this study, ALSTA has not developed mental retardation whereas NAHAS has. One reason for this phenotypic difference, evident from the original model for mental retardation in this syndrome, is that a putative gene for mental retardation,

flanking either *WT1* or *PAX6*, is disrupted by the constitutional 11p13 deletion in NAHAS but left intact by the similar deletion in ALSTA. Previous analysis to map the extent of these deletions has been performed by FISH, using probes that included the marker sequences *FSHB*, ZF17, D11S1312, D11S112, D11S914 (listed centromere distal to proximal, all distal of *WT1*), and YAC y23IC9, D11S672, D11S1776 and D11S102 (also listed centromere distal to proximal, all proximal of *WT1*) (work of J. Fantes). This analysis defined the proximal breakpoints of the deletions in both ALSTA and NAHAS; the former lies within D11S672 and the latter within the more distal marker, y23IC9 (Fantes *et al.* 1995, see Appendix C). Similarly, the distal breakpoints were defined as lying between ZF17 and the more proximal marker D11S112 for ALSTA, and proximal to D11S914 for NAHAS. Marker sequences spanning the distal breakpoints were not identified. The extent of the 11p13 deletion in ALSTA was therefore found to exceed that of NAHAS both proximally and distally. Additionally, similar analysis was performed on LCL samples from both sets of parents; two copies of each marker sequence were identified in all of these individuals, indicating that the deletions in both ALSTA and NAHAS arose *de novo*.

As the size of the constitutional deletion in ALSTA has been found to exceed and wholly encompass that of NAHAS, it is obvious that the mental retardation affecting the latter individual does not result from deletion of an additional gene, as, if this were so, it is expected that ALSTA would be similarly affected. The phenotypic difference between these two individuals must therefore be elicited by a distinct mechanism and the imprinting model proposed by Jinno *et al.* (1994) was considered a suitable candidate. Applying this model to the present study, it is possible that the larger deletion, that of ALSTA, is of paternal origin, and the smaller deletion, that of NAHAS, is of maternal origin. Consequently, the remaining copy of *WT1* in ALSTA would be maternally derived, therefore active and not eliciting mental retardation, whereas in NAHAS the opposite pattern would exist, that is the remaining copy of *WT1* would be paternally derived and therefore inactive, eliciting mental retardation. It is important to note here that in this model the direction of *WT1* imprinting has not actually been deduced for foetal brain but is presumed from similar analysis on placentae. Therefore, for the present study the critical aspect is whether the deletions in ALSTA and NAHAS differ in parental origin, which, respectively, could be paternal versus maternal (as described) or maternal versus paternal.

4.5.3 Determining the parental origin of the constitutional 11p13 deletions in ALSTA and NAHAS

To investigate the parental origin of the constitutional 11p13 deletions in ALSTA and NAHAS I chose to analyse the status of polymorphic markers that map within the known deletion interval, that is between the marker sequences ZF17 and y23IC9. Eleven polymorphic markers were selected,

comprising microsatellite sequences D11S1312 (Gyapay *et al.* 1994), D11S995 (Browne *et al.* 1993), *PAX6* $(GT)_n$ (Martha *et al.* 1993) and *WT1* $(GT)_n$, and *WT1* RFLP sequences exon 10 *Hinf I* (A) (Hoban and Kelsey 1991), intron 9 *Hha I*, intron 7 *Taq I* (B) (Tadokoro *et al.* 1993), exon 7 *Afl III* (Groves *et al.* 1992, Tadokoro *et al.* 1993), intron 5 *Apa LI*, intron 5 *Taq I* (A) and intron 3 *Hinf I* (B) (Tadokoro *et al.* 1993).

LCL DNA from ALSTA, MASTA and FASTA, and from NAHAS, his mother, MAHAS, and father, FAHAS, was PCR amplified for each of these polymorphic markers using oligonucleotides E460 and E461 (D11S1312), E221 and E222 (D11S995), G276 and F825 (*PAX6* $(GT)_n$), 716 and 718 (*WT1* $(GT)_n$), D157 and D158 (*Hinf I* -A), G192 and G193 (*Hha I*), G190 and G191 (*Taq I* -B), C155 and 945/E194 (*Afl III*), G188 and G189 (*Apa LI* and *Taq I* -A), and G186 and G187 (*Hinf I* -B). Repeated attempts to amplify *PAX6* $(GT)_n$ were unsuccessful and hence analysis of this microsatellite sequence was not possible. Specific PCR products were generated for the remaining marker sequences, and those of the RFLP markers were subsequently digested with the appropriate restriction enzyme.

Heterozygosity for at least one of the polymorphic markers from D11S1312, D11S995, *WT1* $(GT)_n$, *Hinf I* (A) and *Hinf I* (B) was identified in MASTA, FASTA and MAHAS, however only D11S995 was informative for determining the parental origin of the 11p13 deletions (Figure 4.5.1). In ALSTA and NAHAS the maternally derived copy of the latter microsatellite sequence is present whereas the paternally derived copy is absent. This demonstrates that in both of these individuals the constitutional 11p13 deletion is present on the paternally derived homologue (Figure 4.5.2). This in turn demonstrates that the imprinting model proposed by Jinno *et al.* (1994) to explain the cause of mental retardation in WAGR syndrome can not be accurate, as paternally (or maternally) derived deletions are not specific for either the absence or presence of this trait.

This analysis also confirms that both deletions arose *de novo* and has further defined the location of the distal breakpoints, and hence size, of each deletion. For ALSTA, the distal breakpoint was known to lie between the marker sequences ZF17 and D11S112 (M2 and M4 in Figure 4.5.2). With the identification of heterozygosity for D11S1312 (M3 in Figure 4.5.2), the region between this marker and ZF17 must lie out with the deletion, locating the position of the distal breakpoint as between D11S1312 and D11S112. This has been confirmed by FISH analysis with probe YAC yFO9162, which contains D11S1312 at the 3' end and crosses the breakpoint (work of J. Fantes, Fantes *et al.* 1995, see Appendix C). The size of the 11p13 deletion in ALSTA is therefore now estimated to be approximately 2.5Mb. For NAHAS, the distal breakpoint was known only to lie proximal of D11S914 (M5 in Figure 4.5.2), however with D11S995 (M6 in Figure 4.5.2) now identified as lying within the deletion, the position of the distal breakpoint must be located between these two marker sequences. This could be analysed further using yFO9162, which extends from D11S1312 to this more proximal region. The size of the 11p13 deletion in NAHAS is therefore now estimated to be approximately 1.8Mb.

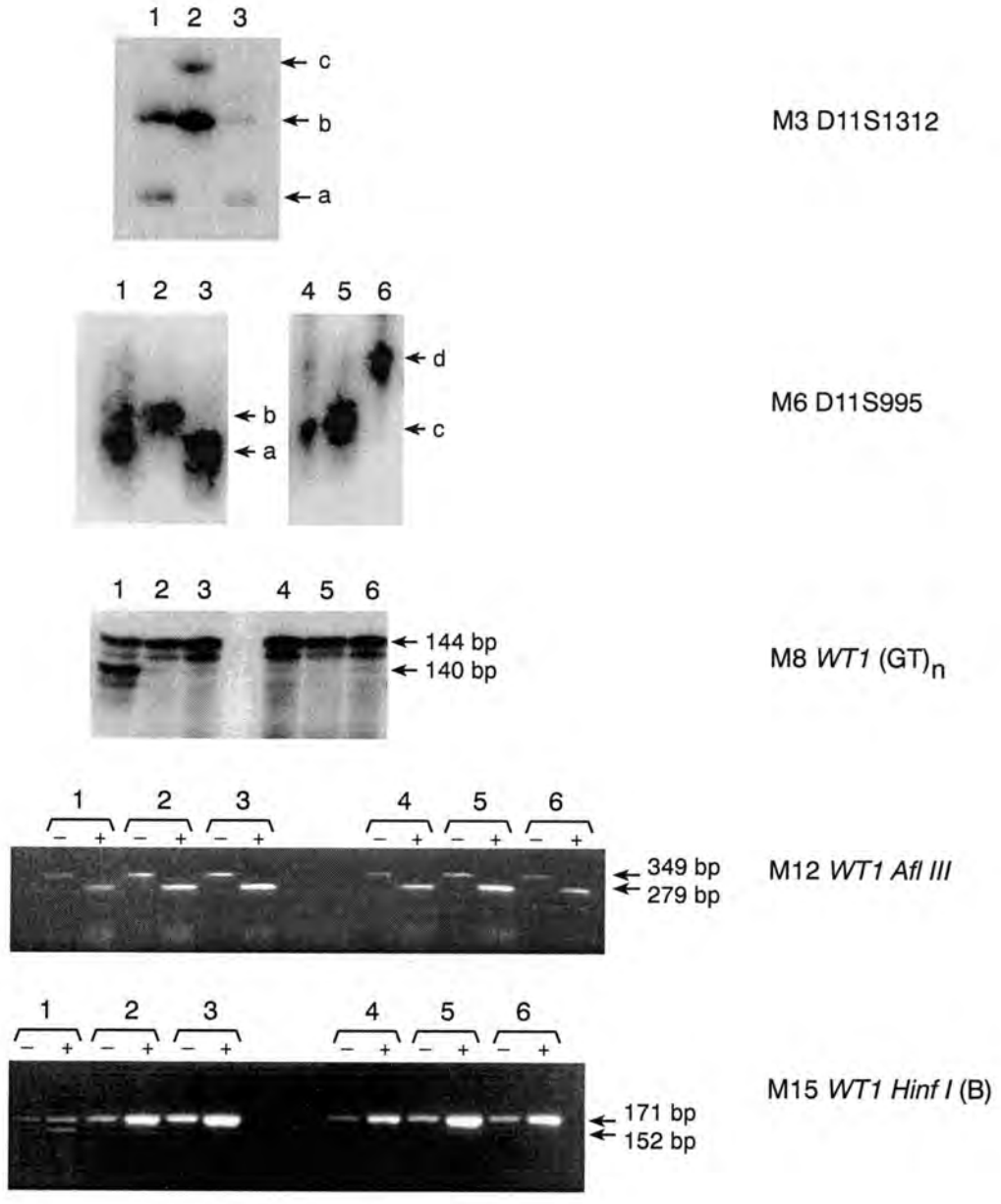


Figure 4.5.1 Status of 11p13 polymorphic marker sequences in WAGR individuals ALSTA and NAHAS, and their parents. Results from five of the eleven selected polymorphic marker sequences are shown. Heterozygosity in at least one individual is demonstrated for D11S1312, D11S995 (lane 4 is distorted), *WT1* $(GT)_n$ and *WT1 Hinf I* (B). For all marker sequences lane 1 - MASTA; lane 2 - FASTA; lane 3 - ALSTA; lane 4 - MAHAS; lane 5 - FAHAS; lane 6 - NAHAS. a, b, c, d, 144 and 140 - allele designation for appropriate marker sequence; 279bp - allele b of *WT1 Afl III* marker; 171bp and 152bp - alleles a and b, respectively, of *WT1 Hinf I* (B) marker; – - no restriction enzyme; + - restriction enzyme; M3, M6, M8, M12 and M15 - marker sequence numbers for reference to Figure 5.4.2.

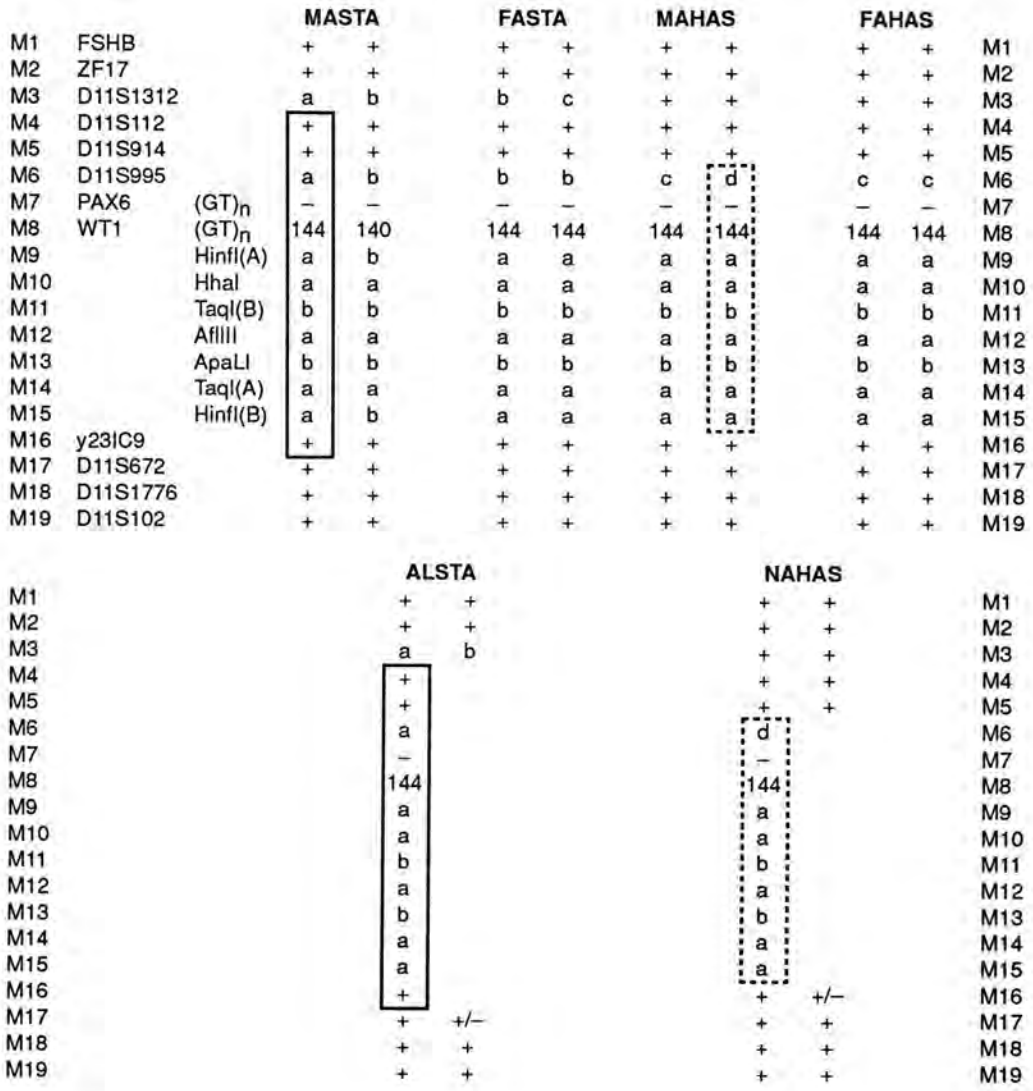


Figure 4.5.2 Parental origin and extent of the constitutional 11p13 deletions in WAGR individuals ALSTA and NAHAS. Analysis of marker sequences in ALSTA and NAHAS, and in their parents, demonstrates that the constitutional 11p13 deletions in both WAGR syndrome individuals are of paternal origin. ALSTA is deleted for M4 D11S112 to M16 y23IC9, and NAHAS for M6 D11S995 to M15 *WT1 Hinf I* (B) (all inclusive). M1 to M19 - marker sequence numbers, these are listed centromere distal to proximal; M1 to M5 map to the p13p14 boundary, M6 to M19 map to the distal end of p13. + - marker present, by FISH analysis (work of J. Fantes); +/– - marker crosses breakpoint, by FISH analysis (work of J. Fantes); – - analysis not possible; a, b, c, d, 144, 140 - allele designation for polymorphic marker sequences analysed in this study.

4.6 Discussion

The involvement of second hit *WT1* mutations in WAGR-WT has been investigated at the mRNA level by PCR amplification and sequencing of cDNA. Two unilateral tumours of this type were analysed and in one, ALSTA-WT, a second hit was detected; a 2bp deletion in exon 10. This mutation is predicted to produce protein with an extended C-terminus. The additional, inappropriate residues in this aberrant form of WT1 may destroy the capacity for sequence-specific DNA binding, however it is also possible that such binding is retained as the deletion does not interrupt the coding sequence for the TD and ZFs. This type of *WT1* mutation has not been reported previously in any form of WT. If the TD and ZFs do adopt the correct conformation it would suggest that an alternative pathway to loss of sequence-specific DNA binding, for example gain of function, exists for somatic *WT1* mutations in WAGR-WT. Second hit *WT1* mutations have now been reported for eight WAGR-WT (Table 1.3.2 and Figure 1.3.2) and all but one of these mutations are predicted to cause loss of sequence-specific DNA binding. The exceptional mutation is a missense mutation in exon 3, which encodes the change G201D (Park *et al.* 1993c). In *in vitro* transcription assays this TD mutation, cloned in to the murine +17aa-KTS isoform, has been demonstrated to switch Wt1 from a repressor to an activator, however functional analysis of the DNA binding properties of the human equivalent have not been reported. This latter form of analysis has been initiated for WT1 in ALSTA-WT, where, unfortunately, *in situ* protein analysis is not feasible (as this tumour has been temporarily thawed).

No second hit *WT1* mutations were detected in the one other WAGR-WT analysed, NAHAS-WT. It is possible that such a mutation is present but has escaped detection as the first 290bp of coding sequence and the majority of the untranslated sequence have not been analysed. The start of the coding sequence was omitted from analysis because, as previously described, this region of *WT1* is very GC rich and therefore refractory to PCR amplification and sequencing. However, the recent identification of mutations in this region, including somatic mutations, in a relatively high proportion of WT individuals (Huff *et al.* 1995) suggests that attempts to analyse this remaining portion of coding sequence in NAHAS-WT is appropriate. Mutations are not expected within the promoter region of *WT1*, as expression has been detected previously, and the identification of both alternative splices suggests that all four mRNA isoforms are present. With reference to the latter, it is important to note that the 17aa and KTS alternative splices were identified independently. However, as this tumour contains only one copy of *WT1* it is extremely unlikely that there is a cryptic mutation present which can cause misrepresentation of the isoforms by affecting both alternative splices in a partial manner, for example abolishing only the −17aa−KTS or +17aa+KTS isoform.

All of the eight WAGR-WT reported to contain second hit *WT1* mutations are unilateral tumours, the most common form to develop in this syndrome. The apparent lack of a similar mutation in NAHAS-WT is therefore remarkable, although there is one case of bilateral WAGR-WT reported where second hit *WT1* mutations, predicted to be different in each tumour, were found to be not

present (Baird *et al.* 1992a). Analysis of *WT1* in these two tumours was performed exclusively at the DNA level and involved SSCP analysis and sequencing of exons 1 to 10, including sequencing of the last 60bp of the 5' untranslated sequence and of the exon-intron junction sequences. The latter analysis indicates that alternative splicing is normal in these tumours and therefore all four mRNA isoforms should be present. Mutations affecting the promoter region are not evident as expression of *WT1* was demonstrated, however this required 40 cycles of PCR as the quality of both WT is reported to be poor.

The apparent lack of a somatic *WT1* mutation in NAHAS-WT suggested the possible involvement of a second hit affecting 11p15.5 loci, including the putative gene *WT2*. LOH for 11p15.5 loci, analysed using three appropriate polymorphic markers, was however found to be not present in this tumour. Similar analysis has been detailed for eight out of ten of the WAGR-WT reported (Brown *et al.* 1989, Baird *et al.* 1992a, Gessler *et al.* 1993, Gessler *et al.* 1994), including the bilateral tumours, and, concordant with NAHAS-WT, alteration was found to be confined to 11p13. Therefore, the only examples of known alteration at both 11p13 and 11p15.5 in WAGR-WT are the three unilateral tumours reported by Henry *et al.* (1989) and Jeanpierre *et al.* (1990), where subsequent point mutation analysis of the remaining copy of *WT1* in each tumour has not been reported. Involvement of LOH at 11p15.5 in this form of WT is therefore predicted to be infrequent, although it has to be emphasised that the number of such tumours analysed is low. Detailed analysis of this distal region should however be considered if all attempts to demonstrate functional loss of the remaining copy of *WT1* in WAGR-WT identifies only wild type sequence.

Previous analysis has demonstrated that the constitutional 11p13 deletion in NAHAS is smaller, and completely internal, to that in ALSTA. This cytogenetic difference is particularly interesting given that the smaller deletion elicits the more severe phenotype; NAHAS has full spectrum WAGR syndrome whereas ALSTA has only a partial spectrum as he is not mentally retarded. Two models to explain the genetic basis of this latter component of WAGR syndrome, the flanking gene model and the imprinting model, have been tested in ALSTA and NAHAS. The flanking gene model proposes disruption to a putative gene for mental retardation, mapping either proximal of *WT1* or distal of *PAX6*. The imprinting model, proposed recently by Jinno *et al.* (1994) and based on the apparent monoallelic expression of *WT1* in foetal brain, suggests development of mental retardation only when the constitutional 11p13 deletion is of maternal origin. With reference to the latter model, Huff (1994) reviewed all reported cases of WAGR syndrome where the parental origin of the constitutional 11p13 deletion and the phenotype was known. A total of nine such cases are detailed, comprising three maternally derived deletions with mental retardation, three paternally derived deletions with mental retardation and three paternally derived deletions with no mental retardation (two of which were cited by Jinno *et al.* (1994) to support the imprinting model). Additionally, for seven of these cases the extent of the constitutional deletion was known and it is noted that the individuals carrying the smaller deletions are those with no signs of mental retardation. On the basis of this data the author discounts

the imprinting model and favours the flanking gene model. Analysis of the constitutional 11p13 deletions present in the two WAGR syndrome individuals in the present study clearly demonstrates that neither of these models is correct. If the imprinting model were correct ALSTA and NAHAS should differ in parental origin of the constitutional 11p13 deletion and, if the flanking gene model were correct, ALSTA and not NAHAS should display mental retardation. The pattern observed in these two WAGR individuals is in fact the opposite to the above. Firstly, the parental origin of the constitutional 11p13 deletions in ALSTA and NAHAS does not differ, in both individuals the deletion is present on the paternally derived homologue, hence discounting the imprinting model. The predominance of paternally derived deletions in WAGR syndrome is actually not surprising as, in general, the mutation rate in male gametes is much higher than that in female gametes (Redfield 1994). Secondly, it is NAHAS and not ALSTA that displays mental retardation, hence discounting the flanking gene model.

Although the flanking gene model cannot be accurate, it is however possible that the size of 11p13 deletion is still the critical feature in development of mental retardation. For the latter, rather than the actual size being the key factor there may be various but specific sizes that alter the gross structure of the chromatin and hence affect gene expression, that is a periodicity may exist and it is the disruption of this that is all important. Alternatively, and perhaps more likely given the complexity of the trait, it may be the genetic background of the constitutional 11p13 deletion which dictates development of mental retardation in WAGR syndrome.

Moreover, as a consequence of discounting the imprinting model as the cause of mental retardation, this study has found no evidence to support the proposal of Jinno *et al.* (1994) that *WT1* is imprinted in a subset of expressing tissues. This proposal is not considered absolute for two main reasons. Firstly, biallelic expression of *WT1* was demonstrated in kidney (as expected) and placentae matched for the foetal brain samples displaying monoallelic expression, however all of this analysis was based on PCR amplifications and only the placentae were analysed with cDNA specific oligonucleotides and also at the DNA level. Secondly, the identification of monoallelic and biallelic expression of *WT1* in independent and apparently normal placentae, termed polymorphic imprinting by the authors, is ambiguous; imprinting is generally understood to be a precisely controlled regulatory mechanism and, in normal development, all tissues displaying monoallelic expression manifest this pattern consistently.

In summary, this study has analysed the *WT1* locus in two WAGR syndrome individuals; first and second hits have been characterised in ALSTA whereas only the first hit has been characterised in NAHAS. This analysis has identified a number of important features; a novel form of *WT1* mutation on ALSTA-WT, an apparent lack of a somatic mutation or involvement of previously implicated 11p15.5 loci in NAHAS-WT, and that the mental retardation component of WAGR syndrome is elicited by a mechanism distinct from both the flanking gene and imprinting models.

Chapter 5
Involvement of *WT1* mutations in Denys-Drash syndrome

5.1 Contribution of *WT1* mutations to DDS

Abnormal development of the renal and gonadal systems underlies DDS, manifesting, as described in 1.2.4.3, as WT, DMS nephropathy and PGD. With the identification of *WT1* expression in these developing tissues, the possible involvement of this gene in DDS was recognised (Pritchard-Jones *et al.* 1990). More specifically, expression of constitutional mutations of *WT1* in the podocyte cells of the glomerulus may influence the associated but non-expressing mesangial cells, which, in turn, may function abnormally and effect DMS. Constitutional mutations of *WT1* are also considered sufficient to elicit PGD, which, similar to WAGR-GU abnormalities, display variable penetrance and are more prominent in XY compared to XX individuals, again implicating the involvement of genetic background in this phenotype. However, dissimilar to the GU abnormalities that develop in WAGR syndrome, the penetrance of such abnormalities in DDS is very high and the severity of these dictates that ambiguities between the genotypic and phenotypic sex of an individual are common. Furthermore, the presence of rudimentary structures such as streak gonad in DDS indicates that *WT1* plays a critical role during the earliest stages of gonadal development, perhaps preceding sex determination (reviewed by Bogan and Page 1994). In DDS tumorigenesis may also be highly penetrant and, as implicated in many cases of WT, a somatic second hit in addition to a constitutional first hit *WT1* mutation is predicted.

At the outset of this study Pelletier *et al.* (1991a) reported mutation analysis of *WT1* in ten DDS individuals; a constitutional missense mutation was detected in each. Nine of these mutations are within exon 9 and, of these, seven are identical, encoding the change R394W. The two other exon 9 mutations both alter residue D396, and the remaining mutation, within exon 8, alters R366, the ZF2 equivalent of R394 (see Table 1.3.4). WT from three of these individuals was analysed and in each tumour a second hit removing the wild type copy of *WT1*, that is LOH, was identified. The constitutional *WT1* mutations associated with DDS therefore appeared to be of a specific type, altering residues within the fingertip region of ZF3 and ZF2. This region of a ZF is known to confer sequence-specificity for DNA binding and, in EGR1, it is known that the residues equivalent to WT1 R366 and R394 are critical as they form hydrogen bonds with the G bases in the target sequence, 5' GCGGGGGCG 3'. Similarly, the residue equivalent to D396 is also critical as it stabilises the interactions between the R residue and G base (Pavletich and Pabo 1991). Pelletier *et al.* (1991a) demonstrated by using mobility shift assays that, when cloned in to the murine –17aa–KTS isoform, the DDS *WT1* exon 9 mutations prevent the ZFs binding to the EGR1 DNA target sequence. It was therefore proposed that, rather than simply causing loss of sequence-specific DNA binding, these mutations, when present constitutionally, may elicit the severe abnormalities associated with DDS by acting in a dominant manner. For example, as the residues conferring DNA sequence-specificity are altered, binding to inappropriate sequences may be present. An alternative mechanism, that DDS may be elicited by the mutations acting in a dominant negative manner, was also stated.

To investigate further the involvement of *WT1* mutations in DDS, I chose to perform a mutation analysis study of this gene in individuals with this syndrome. Analysis was focused at the constitutional level and, when possible, was extended to DDS-WT.

5.2 Analysis of *WT1* in seven individuals with diagnosed or suspected DDS

5.2.1 Phenotype of each individual

Although a rare syndrome, samples from seven individuals with diagnosed or suspected DDS were available for analysis; DDS1, DDS2, DDS3, DDS4, DDS5, DDS6 and DDS7. By ascertainment, all of these individuals has (or had) nephropathy, and further, DDS3 and DDS7 had unilateral WT, DDS2 and DDS4 bilateral WT, and DDS1, DDS5, and DDS6 are/was tumour-free (each underwent bilateral nephrectomy). PGD has been described for the five individuals with a karyotype known to be XY, one of whom, DDS1, is female (externally). For the remaining two individuals, DDS2 and DDS3, an apparently normal female phenotype is observed, the karyotype of the former is known to be XX. Interestingly, abnormalities affecting the brain and skull directly (cerebral atrophy, hypertensive encephalopathy, craniosynostosis, prominent metopic suture and posterior scalp defect) and perhaps in a less direct manner (pyschomotor retardation, gross motor delay, developmental delay and epilepsy) are prominent in these individuals, as are respiratory illness and oedema. Furthermore, it is also interesting to note that the bilateral WT in DDS7 developed within a horseshoe kidney. All of the features described above for individuals DDS1 to DDS7 are detailed in Table 5.2.1. Two of these individuals are known to have died; DDS3, at age 15 months, from end stage renal failure (ESRF), and DDS5, at age 10 months, from respiratory infection.

5.2.2 PCR and point mutation analysis of *WT1*

5.2.2.1 Exon 9 studies

The indication that *WT1* exon 9 is a hotspot for missense mutations in DDS suggests that this exon should always be the first to be analysed in detail. Accordingly, DNA from individuals DDS1 to DDS6 was PCR amplified for exon 9 using intronic oligonucleotides 798/G26 and 801/G25, and cDNA from DDS7-WT was PCR amplified for the 3'ZF fragment using oligonucleotides B919 (Exon 6) and B917 (exon 10) (see Figure 4.2.1). For each sample only specific PCR products of wild type length were generated. Direct sequencing of these products, using as primer one or both of the intronic oligonucleotides used for PCR or, for cDNA, oligonucleotides 792 (exon 7) or B451 (exon 8),

detected mutations in three of the samples, DDS1, DDS2 and DDS6. Both DDS1 and DDS6 contain a C to T transition mutation at nucleotide 1560, encoding the change R394W, whereas DDS2 contains a G to A transition mutation at nucleotide 1566, encoding the change D396N. These missense mutations present heterozygously at the constitutional level, and in DDS2-WT as mutant sequence only (Figure 5.2.1a).

The presence of either of these missense mutations destroys the single recognition site for the restriction enzyme *Rsr II*, 5' CGGA/TCCG 3', which is present in *WT1* exon 9. Consequently, exon 9 specific PCR product, normally digested by this enzyme in to two fragments of 182bp and 167bp, will remain full length (349bp) if either missense mutation is present. This reaction can therefore be used to screen for these mutations and was used to confirm their presence in DDS1 and DDS6. As expected, digested and full length reaction products were generated from the heterozygous, constitutional samples and only full length reaction products were generated from the WT sample (Figure 5.2.1b, lanes 2, 6, 7 and 9). It is important to note that, as lack of digestion is the critical feature, this type of screen should not be used exclusively for point mutation detection.

5.2.2.2 Exon 8 studies

For the DDS individuals found not to contain exon 9 mutations, DDS3, DDS4, DDS5 and DDS7, exon 8 was selected for detailed analysis. DNA from the first three DDS individuals was PCR amplified for this exon using intronic oligonucleotides C323 and 796/G667, and, as before, for each sample only specific PCR products of wild type length were generated. DDS7-WT was analysed at the cDNA level, as described in 5.2.2.1. Direct sequencing of these products, again using as primer one or both of the intronic oligonucleotides used for PCR or oligonucleotide 792 (exon 7), detected mutations in three of the samples, DDS3, DDS4 and DDS5. DDS3 contains a T to G transversion mutation at nucleotide 1458, encoding the change C360G, DDS4 contains a C to T transition mutation at nucleotide 1464, creating a stop codon at residue R362, and DDS5 contains a C to G transversion mutation at nucleotide 1499, encoding the change H373Q. Therefore, two missense mutations and one nonsense mutation have been detected in exon 8. These mutations present heterozygously at the constitutional level in DDS3 and DDS5 (later also demonstrated for DDS4, work of K. Miyagawa), and in tumour samples only mutant sequence is present in DDS4-WT, however, remarkably, heterozygosity is retained in DDS3-WT (Figure 5.2.2a). This latter analysis suggests that an independent mutation within *WT1* may be present in DDS3-WT, causing compound heterozygosity. To investigate this possibility DNA from this WT was PCR amplified using intronic oligonucleotides C486 and C152 (exon 4), C178 and C177 (exon 6), C155 and 945/E194 (exon 7), and C911 and C912 (exon 10) and the specific PCR products, all of wild type length, were subsequently analysed using direct sequencing and/or the HOT technique; no mutations were detected. Collectively therefore, analysis of DDS3-WT has found no evidence for a second hit *WT1* mutation in exons 4, 6, 7, 8, 9 and 10.

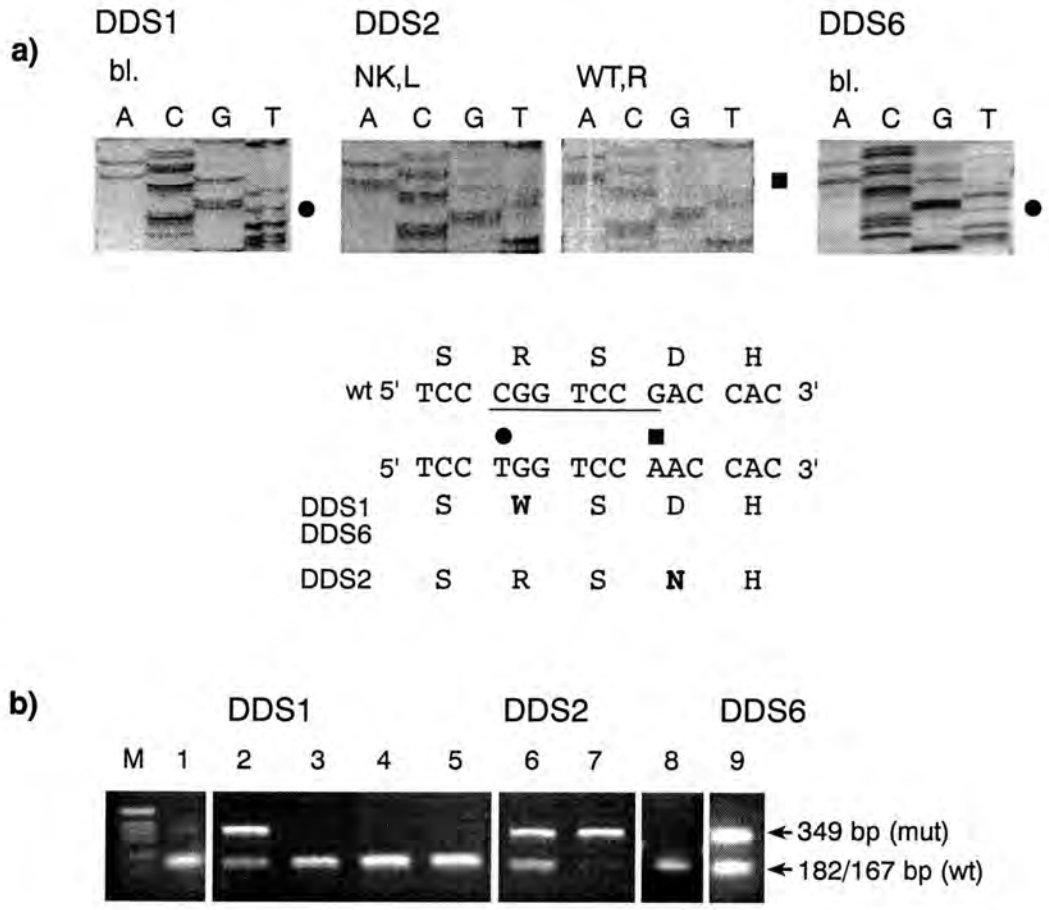


Figure 5.2.1 ***WT1* exon 9 mutations detected in DDS.** **a**) sequence analysis detected missense mutations in DDS1, DDS2 and DDS6. The position of the nucleotide changes are indicated by a circle for DDS1 and DDS6, and by a square for DDS2. The residue changes are R394W and D396N, the inappropriate residues are drawn in bold. bl - blood; NK,L - normal kidney, left; WT,R - Wilms' tumour, right; wt - wild type; the recognition site for *Rsr II* is underlined. **b**) *Rsr II* digest patterns of exon 9 PCR product from DDS1, DDS2, DDS6 and associated samples. Full length product (349bp) indicates the presence of either of the above mutations, digested product (182bp and 167bp) indicates wild type sequence. Lane 1 - known wild type, positive control; lane 2 - DDS1; lane 3 - DDS1M; lane 4 - DDS1F; lane 5 - DDS1Fe, lane 6 - DDS2, normal kidney; lane 7 - DDS2-WT; lane 8 - DDS2M; lane 9 - DDS6. All samples are constitutional unless stated. M - 1kb size marker.

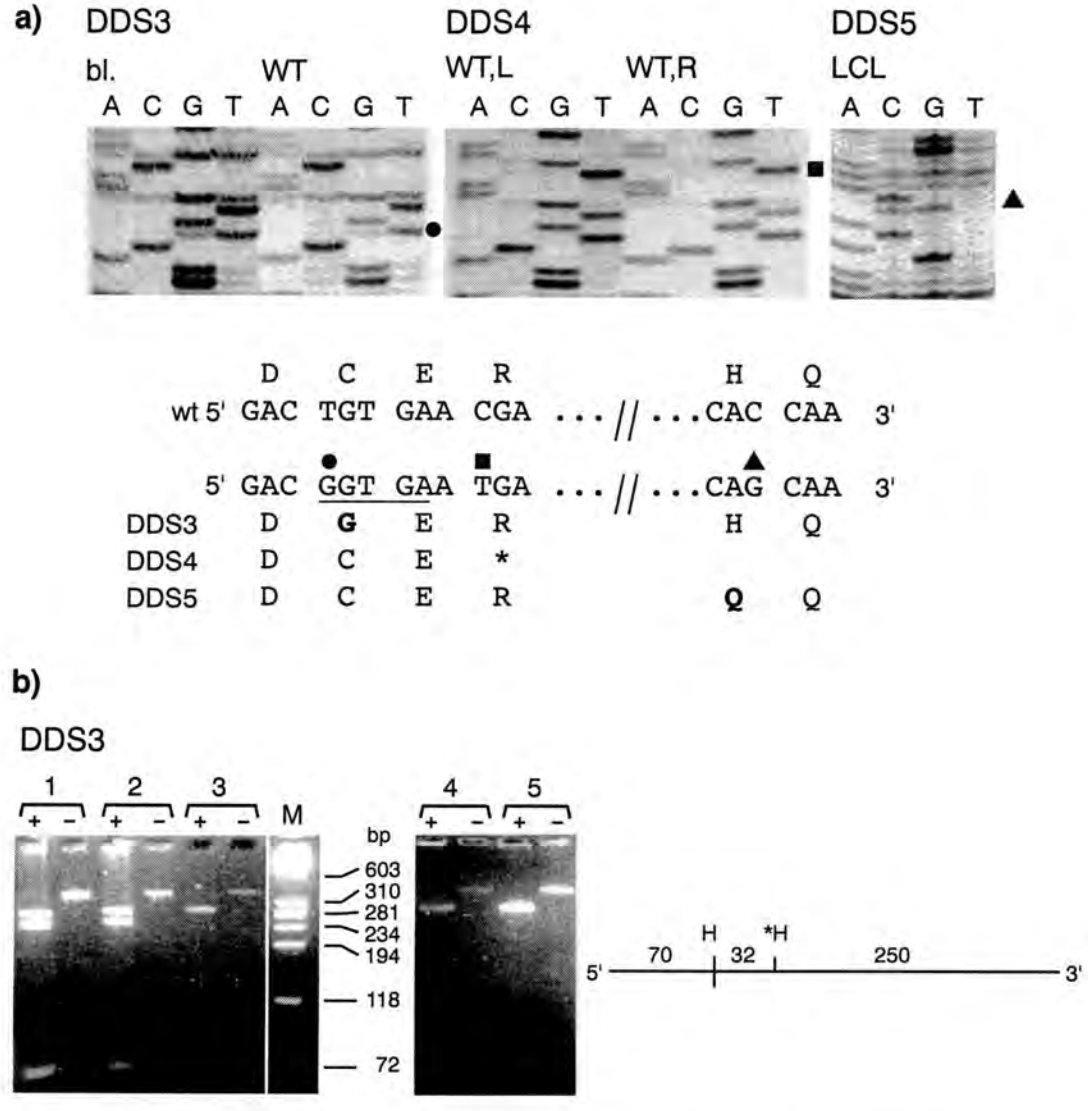


Figure 5.2.2 ***WT1* exon 8 mutations detected in DDS.** **a**) sequence analysis detected missense mutations in DDS3 and DDS5, and a nonsense mutation in DDS4. The nucleotide changes are indicated by a circle for DDS3, a square for DDS4 and a triangle for DDS5. The residue changes are C360G, R362* and H373Q, the inappropriate residues or change are drawn in bold. bl - blood; WT,L - Wilms' tumour, left; WT,R - Wilms' tumour, right; wt - wild type; the recognition site for *Hph I* is underlined. **b**) *Hph I* digest patterns of exon 8 PCR product from DDS3 and associated samples. The mutation identified in DDS3 creates a second recognition site for *Hph I*, *H. Products of 282bp indicate wild type sequence, those of 250bp and 32bp indicate mutant sequence. A 70bp band is invariant. Lanes 1 - DDS3; lanes 2 - DDS3-WT; lanes 3 - known wild type, positive control; lanes 4 - DDS3F; lanes 5 - DDS3M. All samples are constitutional unless stated. The invariant 70bp band is too faint to be visible in lanes 3, 4 and 5. − - no *Hph I;* + - *Hph I*; M - φ*X*174 *Hae III* size marker.

The missense mutation in DDS3 creates a second recognition site for the restriction enzyme *Hph I*, 5' GGTGA(8/7) 3', in exon 8. Consequently, exon 8 specific PCR product, normally digested by this enzyme in to two fragments of 282bp and 70bp, will be digested in to three fragments of 250bp, 70bp and 32bp if this mutation is present. This digest reaction was used to confirm the presence of the missense mutation in DDS3 and, as wild type and mutant reaction products were generated from the constitutional and tumour samples, further demonstrates that heterozygosity for this mutation is retained in DDS3-WT (Figure 5.2.2b, lanes 1 and 2).

5.2.2.3 LOH studies

Comparison of the sequence and digest results obtained from constitutional and tumour samples from DDS2 and DDS3 illustrates that, as described in 5.2.2.1 and 5.2.2.2, LOH for *WT1* in WT is restricted to the former individual. To analyse the possible presence of LOH in DDS4-WT, constitutional DNA from three tissues and tumour DNA from the right and left WT was PCR amplified for the *Hinf I* RFLP marker in *WT1* exon 10 (Hoban and Kelsey 1991) using exonic oligonucleotides D157 and D158. Only specific PCR products of wild type length were generated and subsequently digested. Heterozygosity is observed for this marker in each of the constitutional samples, however only one copy is observed in the tumour (Figure 5.2.3), demonstrating that, similar to DDS2-WT, LOH for *WT1* is present in DDS4-WT. This result was confirmed by subsequent sequence analysis of DDS4, as mentioned in 5.2.2.2 (work of K. Miyagawa).

5.2.2.4 Summary

A summary of all of the results relating to individuals DDS1 to DDS7 is presented in Table 5.2.1; the apparent lack of mutations in DDS7-WT is discussed in 5.4. Furthermore, initial point mutation analysis of DDS1 and DDS2, using the HOT technique, was performed by M. Little, and much of the subsequent analysis of these individuals and of DDS3, DDS4 and DDS5, as described above, was performed with M. Little.

5.2.3 Analysis of *WT1* in parental samples

Eight parental samples, associated with DDS1, DDS2, DDS3 and DDS5, were available for analysis. The DDS1 parental samples were from both the mother and father, and, additionally, from evacuated products of conception from the mother, samples DDS1M, DDS1F and DDS1Fe, respectively. DDS2 was conceived by *in vitro* fertilisation (IVF) involving an anonymous sperm donor, and therefore only a sample from the mother, sample DDS2M, was appropriate and available for analysis. For DDS3 and DDS5 samples from both sets of parents were available, samples DDS3M and DDS3F, and DDS5M and DDS5F, respectively. All of the parental samples comprised normal, non-gonadal material. To determine if any of the parental samples contained a mutation identical to

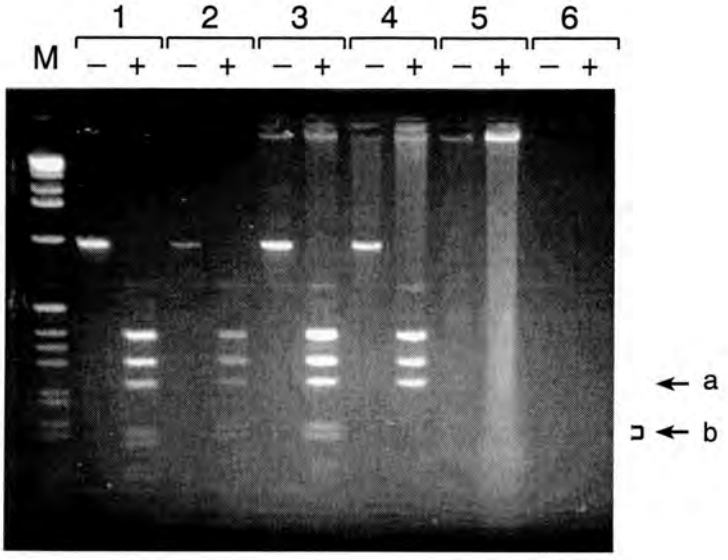


Figure 5.2.3 Detection of LOH in DDS4-WT. The *Hinf I* polymorphism in *WT1* exon 10 was used to analyse heterozygosity in DDS4-WT. Two alleles, a and b, are present constitutionally but only allele a is present in the tumour, demonstrating LOH at 11p13 in this tissue. Lanes 1 - fibroblast DNA; lanes 2 - blood DNA; lanes 3 - normal kidney DNA; lanes 4 - left WT DNA; lanes 5 - right WT DNA, degraded; lanes 6 - no template for PCR and digest reaction, negative control. – - no *Hinf I;* + - *Hinf I.*

	DDS1	DDS2	DDS3	DDS4	DDS5	DDS6	DDS7
Karyotype	46, XY	46, XX	nd	46, XY	46, XY	46,XY	46, XY/46,Xp+,Y [a]
PGD	Externally-normal female, Mullerian and Wolffian structures, no gonads,abnormal uterus	Externally-normal female	Externally-normal female	Micropenis, cryptorchidism	Hypospadias	Hypospadias	Severe hypospadias, bifid scrotum
WT	– [b]	+ b	+u	+ b	– [b]	– [b]	+u [c]
DMS	+	+	+	+	+	+	+
Other features			Cerebral atrophy, pyschomotor retardation, recurrent bronchitis, dermatitis, low IgG	Gross motor delay, craniosynostosis, prominent metopic suture, horseshoe kidney	Hypertension, extreme oedema, respiratory infection, pneumonia	Hypertensive, encephalopathy, oedema	Developmental delay, epilepsy, posterior scalp defect
Heterotopic elements in WT			Ganglia	Muscle			Muscle
Mutation	C to T	G to A	T to G	C to T	C to G	C to T	Not identified
Amino acid change	R394W [d]	D396N	C360G	R362* [e]	H373Q	R394W	
Location	ZF3	ZF3	ZF2	ZF2	ZF2	ZF3	
Con. mutation	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous	
Tumour LOH	–	Yes	No	Yes	–	–	

Table 5.2.1 Summary of the features of the seven DDS individuals studied. nd - not determined; – - absent; + - present; b - bilateral; u - unilateral; a - Xp11.2>p11.22, WT was exclusively 46,Xp+,Y/46,Xp+,Y,del(9)(q22.3q32); *b* - bilateral nephrectomy; *c* - contralateral kidney contains ILNR and heterotopic muscle elements; *d* - mutation previously reported by Baird *et al.* (1992b); *e* - mutation confirmed by Clarkson *et al.* (1993).

the one previously identified in the associated DDS individual, DNA from each sample was analysed as described in 5.2.2.1 and 5.2.2.2, that is it was PCR amplified for exon 8 or exon 9 and then either digested with the appropriate restriction enzyme (DDS1M, DDS1F, DDS1Fe, DDS2M, DDS3M and DDS3F) or sequenced (DDS5M and DDS5F). All of the parental samples generated only wild type results, some of which are shown in Figure 5.2.1b (lanes 3, 4, 5 and 8) and Figure 5.2.2b (lanes 4 and 5). This apparent lack of constitutional *WT1* mutations in the parental samples suggests that the mutations detected in this gene in DDS1, DDS2, DDS3 and DDS5 arose *de novo*. The presence of germline mosaicism for the appropriate *WT1* mutation in the parents does, of course, remain a possibility. Finally, although samples were not available from the parents of DDS4 and DDS6, it is presumed that the *WT1* mutations detected in these two individuals also arose *de novo*.

5.3 Apparent lack of a *WT1* mutation in DDS7

As a *WT1* mutation was not detected in exons 8 and 9 in cDNA from DDS7-WT, analysis of the remaining regions of coding sequence was appropriate. To accompany the previously amplified 3'ZF fragment, the 5'TD fragment was PCR amplified using oligonucleotides B920 (exon 1) and B918 (exon 6) (see Figure 4.2.1). The 5'TD and 3'ZF fragments were also PCR amplified using oligonucleotides M3897 (exon 1) and M3896 (exon 6), and M3895 (exon 6) and M3898 (exon 10), to permit automated sequencing. Additionally, when ambiguities were detected in the cDNA sequence, individual exons were PCR amplified from DNA using intronic oligonucleotides C147 and C148 (exon 2), C153 and C154 (exon 5), C178 and C177 (exon 6), C323 and 796/G667 (exon 8), 798/G26 and 801/G25 (exon 9), and C911 and C912 (exon 10). For each PCR, only specific products of wild type length were generated and analysis of the 5'TD fragment detected both the −17aa and +17aa isoforms. All specific PCR products were subsequently analysed by direct and/or automated sequencing, using as primer one or both of the oligonucleotides used for PCR or, for automated sequencing, oligonucleotides H193 (exon 1), 434/F25 (exon 1-2), B700/E976 (exon 5-6) or H195 (exon 6). Sequence analysis of the 3'ZF fragment, using oligonucleotide B917 as primer, detected both the −KTS and +KTS isoforms. Definite sequence was obtained for all but the first 167bp (excluding B920 and M3897) of the 5'TD region, that is from the start of exon 2, and for all of the 3'ZF region (excluding B917 and M3898); no mutations were detected.

Although analysis of cDNA has demonstrated that 17aa and KTS isoforms are present, it is possible that the relative level of each splice form is aberrant because of a mutation in only one copy of one of the splice sites. The sequence obtained from the DNA samples does, however, indicate that such a mutation is not present; analysis of the exon-intron junction sequences of exons 5 detected only wild type splice acceptor and donor sites, and similarly for exon 9 only wild type sequence was

detected for the splice acceptor site, KTS encoding sequence and the following splice donor site. Collectively therefore, analysis of cDNA and DNA suggests that the relative level of each alternative splice is normal. This analysis does not however eliminate the possibility that the overall level of the 17aa and KTS alternative splices may be aberrant , for example this level may be decreased because of lack of expression from one copy of *WT1*. To analyse this possibility constitutional DNA, WT DNA and WT cDNA from DDS7 was PCR amplified and digested for the *Hinf I* RFLP marker in *WT1* exon 10 (Hoban and Kelsey 1991), as described in 5.2.2.3. Heterozygosity for this marker sequence (alleles a and b, as shown in Figure 5.2.3) was detected in each sample, demonstrating that in DDS7-WT two copies of *WT1* are retained and are expressed, and suggesting that the overall level of expression is not aberrant. As cDNA specific oligonucleotides were not used for this analysis the possibility that amplification from the tumour cDNA derived from contaminating DNA was considered, however subsequent attempts to amplify from this cDNA sample using intronic oligonucleotides failed to generate any PCR products, suggesting the purity of the sample is acceptable for this form of analysis.

Therefore, despite repeated and detailed analysis, this study has found no evidence for intragenic mutations, aberrant splicing or an altered expression level of *WT1* in individual DDS7.

5.4 Predicted consequences of the *WT1* mutations identified in DDS1 to DDS6

The *WT1* exon 9 mutations detected in DDS1, DDS2 and DDS6, encoding the change R394W or D396N, are identical to eight of the mutations reported by Pelletier *et al.* (1991a). As described in 5.1, *in vitro* experiments have indicated that these missense mutations, positioned within the fingertip region, abolish binding of WT1 to the EGR1 DNA target sequence, an aberrancy mediated by the inappropriate residues altering the DNA sequence-specificity of ZF3. Again as described previously, this loss of sequence-specific DNA binding is compatible with either a dominant or dominant negative mechanism. The *WT1* exon 8 mutations detected in DDS3, DDS4 and DDS5, encoding the changes C360G, R362stop and H373Q, respectively, differ from those of exon 9 as they do not directly affect the residues at the fingertip region. Disruption to the ZFs is, however, still predicted. The C360G and H373Q missense mutations each alter a residue critical for zinc co-ordination, hence destroying the potential to form ZF2. The consequence of these mutations is therefore predicted to be either loss of sequence-specific DNA binding, if the remaining three ZFs are not compromised, or, if they are compromised, complete loss of DNA binding. The presence of the latter type of mutation in DDS is indicated by the remaining mutation detected, R362stop. This nonsense mutation creates the potential to encode only the TD and ZF1, and, if translated, is predicted to produce WT1 with no capacity to bind DNA. The position of each of the residues predicted to be altered by the mutations in DDS1 to DDS6 is shown in Figure 5.4.1, which similarly includes the mutations reported by Pelletier *et al.*

(1991a) and all subsequent reports (but one) of *WT1* mutation in DDS (also detailed in Table 1.3.4 and Figure 1.3.3).

The phenotype of DDS4, who carries the R362stop mutation, is not remarkable when compared to that of other DDS individuals. This suggests that all of the constitutional missense mutations that elicit DDS adopt the same mechanism as that indicated by the nonsense mutation, that is, they do not result in binding to inappropriate DNA target sequences but instead cause complete loss of DNA binding. In other forms of WT this consequence of constitutional *WT1* mutation is also predicted and, indeed, is clearly demonstrated by the constitutional heterozygous deletions of this gene in WAGR syndrome. However, the fact that the abnormalities associated with the latter syndrome are relatively mild suggests that complete loss of DNA binding does not elicit the severe abnormalities such as DMS and extreme PGD that feature in DDS. The *WT1* mutations that elicit DDS must therefore function by a mechanism distinct from complete loss of or inappropriate DNA binding, indicating that it is a dominant negative mechanism that is involved in this syndrome.

Dominant negative mutations involve the mutant form of a product disrupting the function of the wild type form when both forms are present in the same cell. Such mutations were first described by Muller (1932), who used the term antimorphs and, from analysis of the *brown* and *ebony* genes in *Drosophila*, describes '..antagonistic mutant genes, having an effect actually contrary to that of the gene from which they were derived by mutation..' This type of mutation has subsequently been described by Herskowitz (1987), who details models for the action of dominant negatives. Applying one such model directly to this study, it is proposed that, at the constitutional, heterozygous level, a dominant negative mutation in *WT1* will result in there being 50% mutant WT1 and 50% wild type WT1. The level of functional wild type protein will however be less than 50% as a proportion of it will be functionally inactivated because of interference from the mutant form. For WT1, interference may comprise 'homodimerisation' between the wild type and mutant proteins, perhaps mediated through the TD (as this domain contains the two putative leucine zipper like motifs and is, most likely, the only one present in the mutant form of WT1 predicted for DDS4). It appears therefore that the severity of the abnormalities associated with constitutional *WT1* mutations may be determined by a dosage effect, the critical aspect of which is the proportion of WT1 that can function normally. In individuals with WAGR syndrome (and non-syndrome associated WT) the constitutional level of functional wild type WT1 is 50%, eliciting only relatively mild abnormalities, whereas in DDS this level is predicted to be less than 50%, and hence far more severe abnormalities develop.

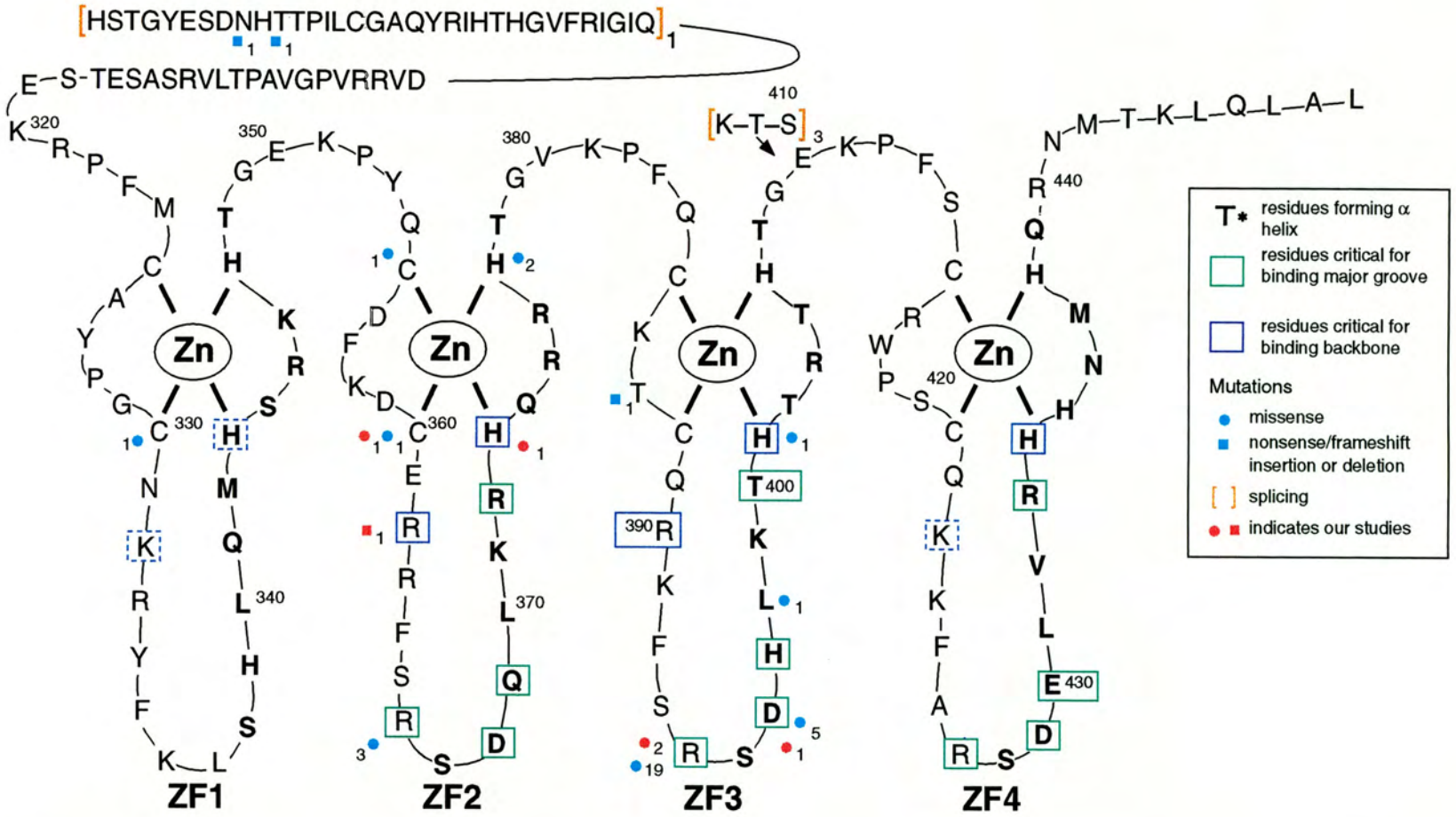


Figure 5.4.1 Position of the residues predicted to be altered by *WT1* mutations detected in DDS. The residues associated with the *WT1* mutations identified in DDS1 to DDS6 are indicated, together with all but one of the DDS *WT1* mutations reported (Y221*, Bardeesy *et al.* 1994b). *- letters in bold.

Discussion

The possible presence of constitutional *WT1* mutations in seven diagnosed or suspected DDS individuals, DDS1 to DDS7, has been investigated. Each individual was ascertained as a DDS patient as they displayed DMS and WT or PGD; two individuals developed the full spectrum of abnormalities. For some of these individuals, for example DDS1 and DDS6, a definite diagnosis of DDS was considered dependent on the status of *WT1*. By PCR amplification and sequence analysis six *WT1* mutations were detected; three exon 9 and two exon 8 missense mutations, and one exon 8 nonsense mutation. The three exon 9 missense mutations encode the changes R394W (in two cases) and D396N, and, even with the many DDS *WT1* mutations reported during this study, these mutations still represent the two most commonly identified *WT1* mutations in DDS, with 19 and 5 cases, respectively, from a total of 42 reported (Pelletier *et al.* 1991a, Bruening *et al.* 1992, Baird *et al.* 1992b, Coppes *et al.* 1992b, Poulat *et al.* 1993, Sakai *et al.* 1993, Clarkson *et al.* 1993, Tsuda *et al.* 1993, Nordenskjold *et al.* 1994) (see Table 1.3.5). One of the R394W mutations in this study, detected in DDS1, is included in the latter figures as it was also detected by Baird *et al.* (1992b). These authors refer to DDS1 as individual GOS368, and it is by this code name that DDS1 was originally reported by Jadresic *et al.* (1991). The remaining two missense mutations detected, in exon 8, encode the changes C360G and H373Q. These mutations differ from the other missense mutations as, rather than altering fingertip residues that confer sequence-specific DNA binding, they each destroy a critical zinc co-ordinating residue and hence formation of ZF2. Rather than causing inappropriate DNA binding, the latter mutations may cause such major disruption to the ZFs that the aberrant forms of WT1 produced display complete loss of DNA binding.

The concept that complete loss, as opposed to alteration, of DNA binding can elicit a DDS phenotype is clearly demonstrated by the nonsense mutation identified, which encodes the change R362stop. This mutation, which was subsequently confirmed by Clarkson *et al.* (1993), destroys the potential to encode ZF2, 3 and 4, and is therefore predicted to produce WT1 consisting of only a TD and, perhaps, ZF1. The generation of severe phenotypes from such a mutation indicated that, in addition to not binding DNA, mutant WT1 in DDS acts in a dominant negative manner. During this study an exon 6 nonsense mutation was reported by Baird *et al.* (1992b) and subsequently a third example of this type of mutation, in exon 3, was reported by Bardeesy *et al.* (1994b). The first set of authors did not however state that such mutations implicate a dominant negative mechanism in DDS.

The proposal that complete loss of DNA binding is a feature of DDS *WT1* mutations has been confirmed recently by Little *et al.* (1995). This study demonstrates that the presence of previously reported missense and nonsense mutations in ZF2 and ZF3 prevents the mutant form of WT1 binding to four different putative DNA target sequences, including that of EGR1. The involvement of dominant negative mutations in DDS has been further implicated by a recent study by Reddy *et al.*

(1995), who demonstrate that a peptide comprising only the first 182 residues of WT1 is sufficient to disrupt wild type WT1 function. This N-terminus region of WT1 is encoded by exons 1 and 2 only and, although not containing either of the putative leucine zipper like motifs, binds to wild type WT1 and inhibits the activation properties of the latter. It is thought that sequence-specific DNA binding is not compromised for the mutant-wild type WT1 dimers. Interestingly, all of the intragenic DDS *WT1* mutations reported do not affect exons 1 and 2 (see Figure 1.3.3) and therefore all have the potential to encode the minimum region required for dominant negative action. Furthermore, it is perhaps significant that the only DDS *WT1* mutation predicted to actually truncate the TD, an exon 3 nonsense mutation encoding Y221stop, elicits a relatively mild and incomplete DDS phenotype (Bardeesy *et al.* 1994b). This suggests that, although 182 residues are sufficient, the dominant negative mutants that contain a full length TD may be more effective, hence their prominence in the more complete forms of DDS.

This dominant negative model cannot however explain all cases of DDS. It is predicted that perturbation of the isoform ratio is the critical feature in three cases displaying an intron 9 splicing mutation, as the potential to encode the +KTS isoforms is abolished. Additionally, two individuals with DDS in combination with WAGR syndrome have been reported (Jadresic *et al.* 1991, Baird *et al.* 1992b, Henry *et al.* 1993). Both of these individuals carry constitutional 11p13 deletions that render *WT1* hemizygous, therefore implicating the additional involvement of other factors, such as genetic background, in these unusual cases of DDS.

In the present study a *WT1* mutation was not detected in individual DDS7. It is possible that such a mutation is present as the first 439bp of coding sequence, that of exon 1, and the untranslated sequence has not been analysed in detail. As the 5'TD fragment amplified from DDS7-WT appears wild type in length, deletions or insertions spanning approximately 40bp or longer are not predicted for exon 1. However, to investigate the possible presence of more subtle mutations, analysis of this exon, although technically difficult, should be pursued. It is important to note, with respect to dominant negative mutations, that even if an exon 1 mutation creates a frameshift, the potential to encode 182 wild type residues will be destroyed. Furthermore, the presence of a *WT1* mutation in DDS7 is indicated by the fact that in the associated WT and the contralateral kidney heterotopic muscle has developed. WT1 is thought to inhibit myogenesis and therefore development of heterotopic muscle suggests that this inhibitory function of WT1 has been lost, perhaps as a result of direct mutation. Evidence for this proposal derives from analysis of a number of WT, including DDS4-WT; heterotopic muscle is present in this bilateral tumour only in the regions that display LOH for the constitutional *WT1* mutation detected in the present study, R362stop (work of K. Miyagawa). It is also possible that a *WT1* mutation affecting the isoform ratio is present but undetected in DDS7. This possibility exists as, although the presence, and the relative and overall levels of expression of the 17aa and KTS alternative splices was analysed and identified no mutations, the presence of all four isoforms has not been demonstrated by using a single PCR. It is possible therefore that there is a lack

of any one of the isoforms or of both the −17aa−KTS and +17aa+KTS isoforms or both the −17aa +KTS and +17aa−KTS isoforms.

Collectively therefore, the analysis of DDS7 suggests that, if a *WT1* mutation is present, the DDS phenotype may have resulted from a mechanism distinct from the dominant negative and altered isoform ratio mechanisms. Perhaps similar to DDS7, an apparent lack of *WT1* mutation was identified in one of the DDS individuals, GOS374, reported by Baird *et al.* (1992b). The analysis of *WT1* in this study was performed exclusively on constitutional DNA and involved SSCP analysis and sequencing of exons 1 to 10 and the exon-intron junction sequences. GOS374 did not develop WT but did display additional features, comprising cleft palate, nystagmus and mental retardation, which, interestingly, have been previously reported in part for two individuals known to have *WT1* mutations, one had unilateral WT (possibly as part of a mild BWS phenotype) (Park *et al.* 1993a) and one WAGR syndrome (Santos *et al.* 1993) (see Table 1.3.1 and Table 1.3.2). Cytogenetically visible deletion is not present in GOS374 and, unfortunately, as constitutional heterozygosity and cDNA analysis was not performed, the presence and expression of two copies of *WT1* in this individual can not be confirmed and the presence of all four isoforms is indicated by DNA sequence only.

The possibility that DDS7 is a non-*WT1* DDS individual is, although novel, a valid consideration. This individual does contain a constitutional, cytogenetic X chromosome abnormality, Xp11.2>p11.22. Loci mapping in the proximity of this region include one for mental retardation and cerebral atrophy, at p11-q21, one for gonadotropin deficiency and cryptorchidism, at p21, and the androgen receptor gene, at Xcen-q22 (HGM 1995). It is possible that disruption to one or more of these loci contributes to the phenotype of DDS7. Furthermore, in DDS7-WT an additional chromosome abnormality, del9q22, is observed. This chromosomal region has not been previously implicated in development of WT but may, however, be significant in this tumour. It is not known if a similar abnormality is present in the ILNR within the contralateral kidney.

Analysis of parental samples has indicated that the constitutional *WT1* mutations detected in individuals DDS1 to DDS6 arose *de novo*. This finding is in agreement with similar analysis detailed in many of the other DDS cases reported. Moreover, LOH for *WT1* has been observed for all of the reported DDS-WT tested and similarly, LOH was identified in DDS4-WT and DDS7-WT. The remaining tumour in this study, DDS3-WT, is however remarkable as heterozygosity for the constitutional exon 8 mutation (encoding the change C360G) is retained. It is possible that as *WT1* exons 1, 2, 3 and 5 have not been analysed, this WT may display compound heterozygosity. This form of independent first and second hits has been identified previously in a unilateral WT (Varanasi *et al.* 1994). Alternatively, DDS3-WT may contain a second hit at a locus other than *WT1*, this has not been analysed.

Finally, our understanding of the complex genetic mechanisms that contribute to DDS may be aided by the recent establishment of a mouse model for this syndrome. Chimaeric mice, generated with embryonic stem cells containing a *WT1* exon 9 frameshift mutation, can develop segmental mesangial sclerosis and, in one case, WT, and mice heterozygous for this mutation develop DMS (Patek *et al.* submitted). With respect to the genetic basis of an additional feature, the mental retardation frequently observed in DDS has been proposed to involve *WT1* imprinting (Jinno *et al.* 1994), however results from previous analysis (see Chapter 4) has indicated that this proposal is in fact not relevant to DDS and therefore this feature was not investigated in the present study.

Chapter 6
Searching for *WT1* mutations in malignant mesothelioma

6.1 Possible involvement of *WT1* mutations in MM

As described in 1.6, expression of *WT1* in the mesothelium (Pritchard-Jones *et al.* 1990) and detection of *WT1* point mutations in mesothelial abnormalities (Park *et al.* 1993b, Devriendt *et al.* 1995) indicates that alteration of this gene may contribute to tumorigenesis of this tissue. Further molecular evidence for such involvement of *WT1* is provided by reports of overexpression in MM of *IGF2* (reviewed by Macauley 1992) and *PDGF-A* (Gerwin *et al.* 1987, Versnel *et al.* 1988), both putative target genes for WT1 transcriptional repression. However, as mentioned in 1.4.4.4, the frequent gain of chromosome 7p in this tumour type may contribute significantly to overexpression of the latter gene. Similarly, gain of chromosome 11, a less frequent event but observed by three independent studies (Tiainen *et al.* 1988, Tiainen *et al.* 1989, Hansteen *et al.* 1993), may influence expression levels of *WT1* and *IGF2*. Rearrangements confined to 11p are however only rarely observed (Gibas *et al.* 1986, Hagemeijer *et al.* 1990), suggesting that if *WT1* mutations do contribute to MM they are infrequent and/or they present as more subtle alterations. To investigate the possible involvement of *WT1* mutations in this tumour type I chose to perform a mutation analysis study of this gene on cell lines derived from pleural and peritoneal MM.

6.2 Analysis of *WT1* in MM

6.2.1 MM analysed

Eight human MM cell lines were available for analysis, Mero-14, Mero-25, Mero-48b, Mero-48c, Mero-83, Mero-84, Mero-96 and Mero-123. Five of these were derived from pleurae; Mero-14, -25, -83, -84 and -96, two from peritoneum (from the same individual); Mero-48b and -48c, and one was of unspecified origin; Mero-123.

6.2.2 *WT1* expression in normal and malignant mesothelial cell lines

RNA from a mesothelial cell line, Met5a, and four mesothelioma cell lines, JU77, LO68, NO36 and ONE58, had been available for previous analysis by northern blotting; expression of *WT1* was detected in each cell line (work of K. Miyagawa).

6.2.3 PCR analysis of *WT1*

For all of the above Mero cell lines only low yields of RNA were available for analysis and therefore RT-nested PCR was used to amplify the TD encoding sequence, 5'TD, and the ZF encoding sequence, 3'ZF. As described in 4.2.2, the 5'TD region was amplified from cDNA using outer oligonucleotides D609 (exon 1) and D610 (exon 6) and inner oligonucleotides B920 (exon 1) and B918 (exon 6), and the 3'ZF region similarly amplified using outer oligonucleotides C582 (exon 5) and C583 (exon 10) and inner oligonucleotides B919 (exon 6) and B917 (exon 10). For both the 5'TD and 3'ZF regions only specific PCR products of wild type length were generated. The 5'TD region failed to amplify from Mero-25 and -96, however in the remaining six cell lines both the −17aa and +17aa isoforms were detected.

6.2.4 Point mutation analysis of *WT1*

As PCR analysis did not detect any mutations in the above samples, they were subjected to point mutation analysis using the HOT technique. Purified +17aa 5'TD products and 3'ZF products were scanned against a known wild type cDNA, EMBK, with HA and OsO_4. Strand cleavage subsequent to HA modification was detected for the 5'TD products from Mero-48b, -48c and -123, indicating mismatch at a wild type C nucleotide (Table 6.2.1 and Figure 6.2.1a). For each of these samples, the radiolabelled cleaved fragment was 134bp in length. Direct sequence analysis of cDNA, using oligonucleotide B920 as primer, detected an identical base change in all three samples; an exon 1 C to T transition at nucleotide 768 (Figure 6.2.1b). This is a novel transition and converts codon 130 from AAC to AAT, both of which encode a N residue. This transition is therefore considered a silent base change and is not thought to be functionally significant.

6.3 Exon 1 base change studies

If a silent base change presents at a frequency ≥ 5% it constitutes a polymorphism. To determine if the C to T transition at nucleotide 768 is polymorphic 77 control DNA samples, 54 of which were from parents from 36 CEPH families (families 17, 23, 28, 35, 37, 45, 66, 102, 104, 884, 1331, 1332, 1333, 1334, 1341, 1344, 1345, 1346, 1347, 1349, 1350, 1362, 1375, 1377, 1408, 1413, 1416, 1418, 1420, 1421, 1423, 1424, 13291, 13292, 13293 and 13294) were selected for analysis. For each of these samples exon 1 was PCR amplified using oligonucleotides C145/F518 and

MM cell line	site of tumour	WT1 cDNA region scanned	HA result	OsO_4 result
Mero-14	pl	5'TD	wt	wt
		3'ZF	wt	wt
Mero-25	pl	3'ZF	wt	wt
Mero-48b	pt(o)	5'TD	mut	wt
		3'ZF	wt	wt
Mero-48c	pt(l)	5'TD	mut	wt
		3'ZF	wt	wt
Mero-83	pl	5'TD	wt	wt
		3'ZF	wt	wt
Mero-84	pl	5'TD	wt	wt
		3'ZF	wt	wt
Mero-96	pl	3'ZF	wt	wt
Mero-123	nd	5'TD	mut	wt
		3'ZF	wt	wt

Table 6.2.1 **Point mutation analysis of *WT1* in MM cell lines.** Mutation analysis was performed using the HOT technique. pl - pleurae, pt(o) - peritoneum (omentum), pt(l) - peritoneum (liver), nd - not determined, 5'TD - exons encoding transregulatory domain , 3'ZF - exons encoding ZF DNA binding domain, wt - wild type and mut - mutant.

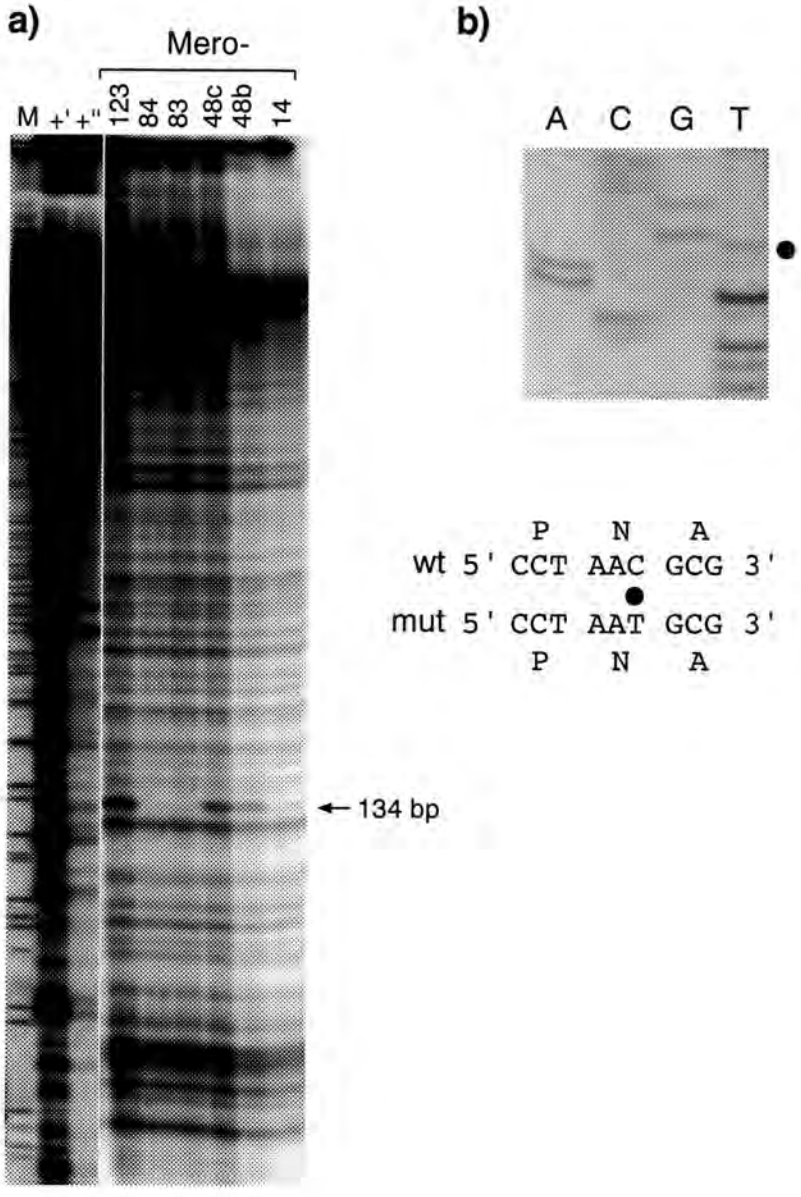


Figure 6.2.1 Detection of a base change in *WT1* exon 1. a) detection of a cleaved fragment, 134bp in length, using the HOT technique. This fragment was specific for HA modification reactions and was detected in MM cell lines Mero-48b, -48c and-123. + - positive controls, these were 3'ZF products and comprised radiolabelled WT33 scanned against EMBK (·) and radiolabelled EMBK scanned against WT33 (-); M - size marker **b)** direct sequence analysis of cDNA from these three cell lines detected a C to T transition at an identical location in each (only Mero-48b is shown); nucleotide 768, within exon 1. wt - wild type; mut - mutant.

C146/F519 or F516 and F517, which generate only specific PCR products. The DNA recognition sequence of the restriction enzyme *Tha I*, 5' CGCG 3', is present in these products and one such site is destroyed if nucleotide 768 is T (5' TGCG 3'). Digestion of these products with this enzyme was therefore used to determine the frequency of transition. Eight out of 77 (10.4%) samples were either C/T or T/T, however further analysis to establish Mendelian inheritance of this apparent polymorphism (using CEPH families 28, 35 and 1362) generated ambiguous results; it appears that the sequence context of this CG or TA base pair can make it refractive to *Tha I* digestion. For this reason the exon 1 C to T transition could not be confirmed as polymorphic; extensive sequence analysis would be required and this was not performed. In contrast, Tadokoro *et al.* (1993) have reported this transition and calculated a frequency of observed heterozygosity of 40% (in normal Japanese).

6.4 Discussion

The possible involvement of *WT1* mutations in MM has been investigated. Eight MM cell lines, derived from pleurae and peritoneum, were analysed for point mutations in this gene using PCR amplification and the HOT technique. Virtually the entire coding sequence was analysed at the cDNA level; no mutations considered functionally significant were detected. One alteration, a silent base change in exon 1 comprising a C to T transition at nucleotide 768, was detected. As the first 256bp of coding sequence were omitted from this study, as they are refractory to PCR amplification, it is possible that mutations are present in this region and have escaped detection. Similarly, mutations affecting the untranslated sequences or the promoter region may be present, perhaps causing the observed levels of mRNA to be aberrant and/or affecting translation of WT1.

The analysis of these MM cell lines was performed as part of a collaborative study with T. Langerak and colleagues (Rotterdam). By Southern and northern blotting experiments these researchers also identified a lack of alteration at the *WT1* locus and confirmed expression of this gene in mesothelial cell lines and in the Mero cell lines detailed. Furthermore, in the latter samples the four mRNA isoforms were detected and the level of expression of *WT1* was found to not correlate with cell morphology (mesenchymal or epithelial) or with the level of expression of *IGF2* or *PDGF-A*. This study therefore provides no evidence for contribution of *WT1* mutations to MM. Expression of this gene in human MM cell lines has also been reported by Walker *et al.* (1994) and Amin *et al.* (1995). Additionally, the latter study detected expression of *WT1* in primary MM and demonstrated that, in both this form of tumour sample and in the cell lines, WT1 is present. Preliminary studies involving Southern blotting and sequence analysis of *WT1* in three of the MM cell lines found mutations to be not present. These results are, therefore, consistent with the finding of the present study, as are those reported by Park *et al.* (1993b), where SSCP analysis of *WT1* exons 2 to 10 found mutations to be not

present in a total of 32 MM samples. In addition, the present study provides no evidence for a role for *WT1* in mesenchymal to epithelial cell type transitions or for *IGF2* or *PDGF-A* being true *in vivo* targets for WT1.

It is however possible that the lack of detectable *WT1* mutations stems from experimental bias, as only one type of MM are known to have been analysed: the asbestos status of the samples reported by Amin *et al.* (1995) is not detailed, however all of the present samples and those reported by Park *et al.* (1993b) are derived from asbestos induced tumours. This bias suggests that it is perhaps only the asbestos-independent form of MM that is sensitive to *WT1* mutations. As asbestos-independent MM are rare it would be difficult to perform parallel studies on a comparable number of samples, however analysis of such tumours is vital. Interestingly, in the study by Park *et al.* (1993b) one mesothelial lesion was analysed in concert with the 32 MM. The lesion was the only sample to have developed in an asbestos-independent manner and was the only one in which a *WT1* mutation was detected; an exon 6 missense mutation encoding the change S273G. This mutation, demonstrated in *in vitro* transcription assays to switch WT1 from a repressor to an activator, was not present constitutionally and was homozygous within the lesion. More specifically, the lesion was a multicystic peritoneal (omentum) mesothelioma which, as a developmental abnormality of borderline malignancy, is distinct from MM. Subsequent to this present study the research group I am part of obtained a portion of an asbestos-independent MM which had developed in the peritoneum of a teenage male. Preliminary analysis has demonstrated *WT1* expression in this tumour (work of A. Seawright). I believe that this tumour, DC, is an excellent candidate for containing a *WT1* mutation and, if this proposal were confirmed, would represent the first direct evidence for involvement of such mutations in MM.

Finally, it is conceivable that in the mesothelium the effects of *WT1* mutations are similar to those observed in gonadal tissue, that is these mutations may contribute to abnormalities but not to tumorigenesis of these tissues.

Chapter 7

Investigating *p16* as a putative Wilms' tumour predisposition gene

7.1 Possible involvement of *p16* mutations in WT

As described in 1.6.3, the lack of *WT1* mutations in the majority of WT, primarily those that are not syndrome-associated, implicates the involvement of other genes in this form of tumorigenesis. The location and hence the identity of many of these putative WT predisposition genes is indicated by the non-random chromosomal rearrangements that are observed in this tumour type, such as the repeated alterations at 11p15.5 highlighting the *IGF2* and *H19* genes. Rearrangements affecting chromosome 9p are not a common feature of WT (reviewed by Slater and Mannens 1992) and therefore for this tumour type the possible involvement of specific genes mapping to this region has not been a principal area of research. However, with the recent identification of *p16*, the central role of this gene in cell cycle regulation and its frequent mutation in many different types of tumour cell line, the potential importance of loci at 9p21 in all forms of tumorigenesis has been highlighted (Kamb *et al.* 1994a, Nobori *et al.* 1994). The involvement of *p16* in WT has not been reported. To investigate the possibility that this gene is a WT predisposition gene I chose to perform a *p16* mutation analysis study on different forms of WT.

7.2 Analysis of *p16* in WT

7.2.1 WT analysed

A total of 30 tumours were selected, comprising 21 unilateral WT; AmWh, ANDMA, BeMa, BeOc, CAROL, DAMBA, DANLE, DARCA, GaWa, JAHUM, JOMEE, KABEL, KEARK, KEDEV, MIWAT, MORGAN, PACUR, RUTPA, ZAGRE, 68.1 and 8RS, 1 bilateral WT; NP58, 2 WAGR-WT; NAHAS and ALSTA, 4 DDS-WT; DDS2, DDS3, DDS4 and DDS7, and 2 BWS-WT; CRASI and ANRIA. In addition to these primary tumours, primary cell cultures derived from DAMBA-, JOMEE-, NAHAS- and CRASI-WT, and a cell line derived from NAHAS-WT were available and selected.

7.2.2 Mutational mechanisms of *p16*

The initial studies of *p16* indicated that virtually all rearrangements affecting this locus comprise homozygous deletions and only a minority present as point mutations (see 1.5.8). For this reason I chose to analyse only the former mutational mechanism in this study. Furthermore, the initial studies also indicated that when homozygous deletions are present they most often eliminate the entire

gene. This feature suggested that, for this study, analysis limited to exon 2 would be appropriate, as this is the central exon and contains the majority of the coding sequence. This region of *p16* was therefore selected to analyse the possible presence of homozygous deletions at this locus in the tumour samples detailed.

7.2.3 PCR analysis of *p16*

Intronic oligonucleotides G386 and G387 were used to PCR amplify *p16* exon 2. One species of PCR product only was generated and this was shown to be specific by restriction enzyme digests; digestion with *Ava I*, *Bgl I*, *Hind III* or *Xma I* generated restriction fragments of the expected length (252bp/170bp/87bp, 334bp/175bp, 420bp/89bp and 257bp/252bp, respectively). As these oligonucleotides were to be used in PCRs investigating possible homozygous deletions, inclusion of a positive control for amplification was imperative. I chose to use multiplex PCR, enabling simultaneous amplification of both *p16* and a positive control, thereby ensuring that if *p16* alone failed to amplify it was a feature of the template and not a PCR artefact. *WT1* was selected as a suitable internal positive control and intronic oligonucleotides C323 and 796/G667 (exon 8) were found to be compatible for co-amplification with oligonucleotides G386 and G387. This multiplex PCR was used to analyse DNA from the WT samples detailed; only specific products of wild type length were generated and both *p16* exon 2 and the internal positive control amplified in all samples. Results from some of the samples are shown in Figure 7.2.1. This analysis demonstrates that homozygous deletions of *p16* exon 2 are not present.

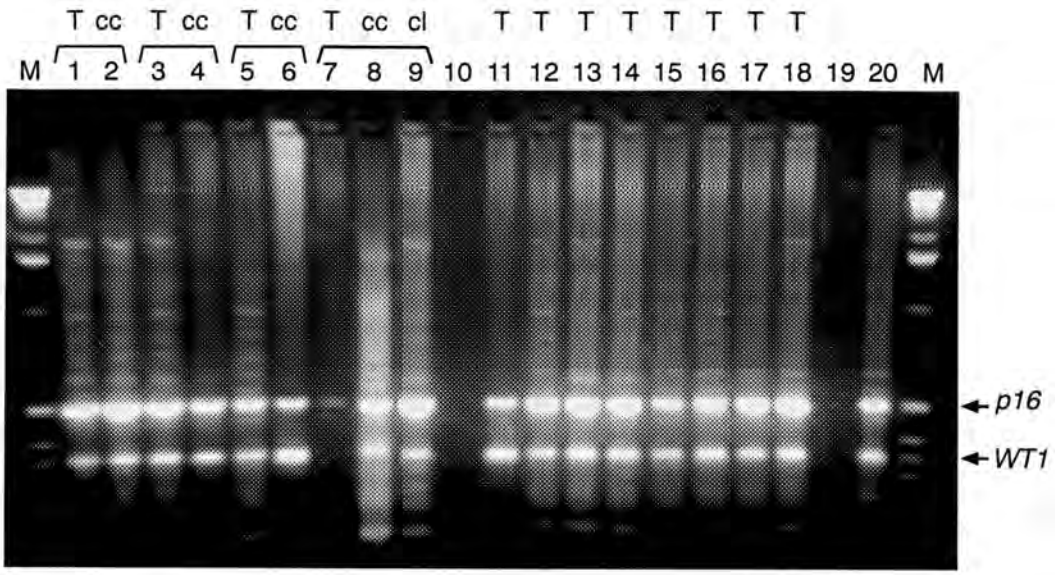


Figure 7.2.1 Multiplex PCR analysis of *p16* in WT. *p16* exon 2 was co-amplified with an internal positive control, *WT1* exon 8, from WT samples. Specific PCR products were generated from all of the samples, demonstrating a lack of homozygous deletions. Lanes 1 and 2 - CRASI; lanes 3 and 4 - DAMBA; lanes 5 and 6 - JOMEE; lanes 7 to 9 - NAHAS; lane 10 - no template, negative control; lane 11 - MORGAN; lane 12 - JAHUM; lane 13 - RUTPA; lane 14 - KEARK; lane 15 - KEDEV; lane 16 - KABEL; lane 17 - MIWAT; lane 18 - DANLE; lane 19 - no template, negative control; lane 20 - known wild type, positive control. T- primary WT; cc - WT primary cell culture; cl - WT cell line; M - 1kb size marker.

7.3 Discussion

The possible involvement of homozygous deletions of *p16* in primary WT has been investigated. DNA from 30 unilateral, bilateral and syndrome-associated WT was analysed by multiplex PCR amplification; no homozygous deletions were present. In primary tumours in general the frequency of this type of mutation is low as compared to that observed in tumour cell lines, and therefore the lack of such mutations in primary WT is unremarkable. Analysis of a comparable number of WT derived cell lines was not possible as such samples are difficult to establish and hence are limited in number. However, four primary cell cultures and one cell line, all derived from matched WT, were available for analysis and, concordant with the results from primary WT, no homozygous deletions were present. These results suggest that *p16* homozygous deletion is not a feature of WT cells either *in vivo* or *in vitro*. Five of the selected WT, ZAGRE, ALSTA, DDS2, DDS3 and DDS4, were known to contain *WT1* mutations (Little *et al.* 1992b, Chapters 4 and 5). Additionally, 11 out of 11 LCL DNA from WT individuals (ANRIA, CAROL, DAMBA, DANLE, JAHUM, JOMEE, KEARK, KEDEV, MIWAT, MORGAN, RUTPA) amplified by multiplex PCR also failed to show *p16* homozygous deletion, as did 11 out of 13 similar samples (ALSTA, DAVAT, DYROG, JELON, KECUL, KIRHO, LISWI, and NAHAS, and renal abnormality individuals CORMU, DABEL and REAKI) amplified by standard PCR. The LCL included one derived from a familial WT individual (LISWI), suggesting that, as with the above samples, rearrangements at this locus do not contribute to this form of tumour.

It is possible that in all of the WT samples and LCL analysed deletions of *p16* are present but have escaped detection. These deletions would include those that affect only the flanking exons, exons 1 and 3, or that cause hemizygosity. This last feature would be missed in this study as the PCRs used were not quantitative. Similarly point mutations, together with mutations and alterations of the promoter sequence, such as the 5' hypermethylation pattern detected for *p16* in various tumour types by Merlo *et al.* (1995), may be present.

In summary, although the full spectrum of genes involved in development of WT remains to be determined, this study has found no evidence for inclusion of *p16* as one such gene.

Chapter 8

Deletion analysis of *p16* and surrounding loci in malignant mesothelioma

8.1 Possible involvement of 9p21 loci in MM

Six studies have reported loss of 9p sequences in MM, including loss involving the region 9p21-22 (Tiainen *et al.* 1988, Tiainen *et al.* 1989, Center *et al.* 1993, Cheng *et al.* 1993, Hansteen *et al.* 1993, Taguchi *et al.* 1993), but only one further study has focused on the *p16* locus within this region (Cheng *et al.* 1994). Therefore, although *p16* has been implicated from analysis of tumour cell lines as an important tumour suppressor gene, little is known of its potential involvement in MM. The report by Cheng *et al.* (1994), as described in 1.5.7, detected 1 intronic *p16* point mutation and 39 homozygous deletions encompassing this locus, from a total of 63 MM samples. Approximately two thirds of the samples analysed were cell lines and these were found to contain 88% of the mutations detected. This study therefore suggests that, similar to analyses of other tumour types reported, homozygous deletion does appear to be the preferred mutational mechanism of *p16* in MM. Furthermore, this study resolved the extent of the smallest homozygous deletions to 1Mb, indicating that, in addition to *p16*, many genes at 9p21 may be affected in these tumour samples. To investigate the possibility that it is the *p16* locus that is of critical importance in 9p21 deletions in MM, I chose to perform a more detailed homozygous deletion analysis study of this region, focused on the *p16* locus, in MM cell lines and primary MM.

8.2 Homozygous deletion analysis of *p16* in MM cell lines

8.2.1 Cell lines analysed

Fifteen human MM cell lines were available for analysis, Mero-14, Mero-25, Mero-41, Mero-48a, Mero-48b, Mero-48c, Mero-72, Mero-82, Mero-83, Mero-84, Mero-95, Mero-96, Mero-123, TM2 and JU77. Eleven of these were derived from pleurae; Mero-14, -25, -41, -48a, -72, -82, -83, -84, -95, -96 and JU77, two were derived from peritoneum; Mero-48b and -48c (from the same individual), one was of unspecified origin; Mero-123, and one, not a true MM, derived from spontaneous transformation of a mesothelial cell line; TM2. Two human mesothelial cell lines, NM7 and Met5a, were also available for analysis. NM7 is the source of TM2, whereas Met5a is independent of all of the MM cell lines detailed. Additionally, three glioblastoma cell lines were available, U87, U373 and A172.

8.2.2 PCR analysis of *p16*

As described in 7.2.2 and 7.2.3, analysis of *p16* exon 2 was considered appropriate to investigate the possible presence of homozygous deletions at this locus and comprised PCR amplification using intronic oligonucleotides G386 and G387 multiplexed with those of an internal positive control, C323 and 796/G667 (*WT1* exon 8). This multiplex PCR was used to analyse DNA from all of the cell lines detailed; for each sample specific PCR product of wild type length was generated for the internal positive control, but specific PCR product for *p16* exon 2 was generated from only the mesothelial cell lines, NM7 and Met5a, and not from any of the MM cell lines. (Figure 8.2.1). This multiplex PCR strategy therefore indicates that *p16* exon 2 is homozygously deleted in all of the MM cell lines; Mero-14, -25, -41, -48a, -48b, -48c, -72, -82, -83, -84, -95, -96 and -123, and TM2 and JU77. Similarly, homozygous deletion of *p16* exon 2 was demonstrated in the glioblastoma cell lines.

8.3 Mapping the extent of 9p21 deletions in MM cell lines

8.3.1 Selection of marker sequences mapping proximal and distal of *p16* exon 2

To analyse the extent of the *p16* homozygous deletions identified in each of the MM cell lines, fifteen marker sequences mapping centromere distal or proximal of exon 2 of this gene were selected. Thirteen of these marker sequences map to the distal region of 9p21, that is p21.3, and comprise 1063.7, c18.b (Kamb *et al.* 1994a), *p16* exon 3 and c5.1 (Kamb *et al.* 1994a) (listed centromere distal to proximal, all distal of *p16* exon 2), and *p16* exon 1, c5.3, R2.3, R2.7 (Kamb *et al.* 1994a), *p15* exon 2, c1.b (Kamb *et al.* 1994a), D9S171 (Cheng *et al.* 1993), D9S126 (Fountain *et al.* 1993) and, close to the boundary with p21.2, D9S169 (Cheng *et al.* 1993) (also listed centromere distal to proximal, all proximal of *p16* exon 2). The two additional marker sequences comprise the most distal one selected, *TYRP-1*, originally named *TRP1* (Abbott *et al.* 1991), at 9p23, and *IFN A* (Abbott and Povey 1991) at the p21-p22 boundary.

Collectively, therefore, the marker sequences selected extend from the distal region of 9p23, across p22 and p21.3, to the boundary with p21.2, and are present at the greatest density at the most distal region of 9p21, where *p16* maps. The genetic distance from the most distal (*TYRP-1*) to the most proximal (D9S169) selected marker sequence is estimated to be 20cM. The physical distance of this interval has not been determined, however *IFN A* and D11S171 are known to be separated by at least 2Mb (reviewed by Povey *et al.* 1994).

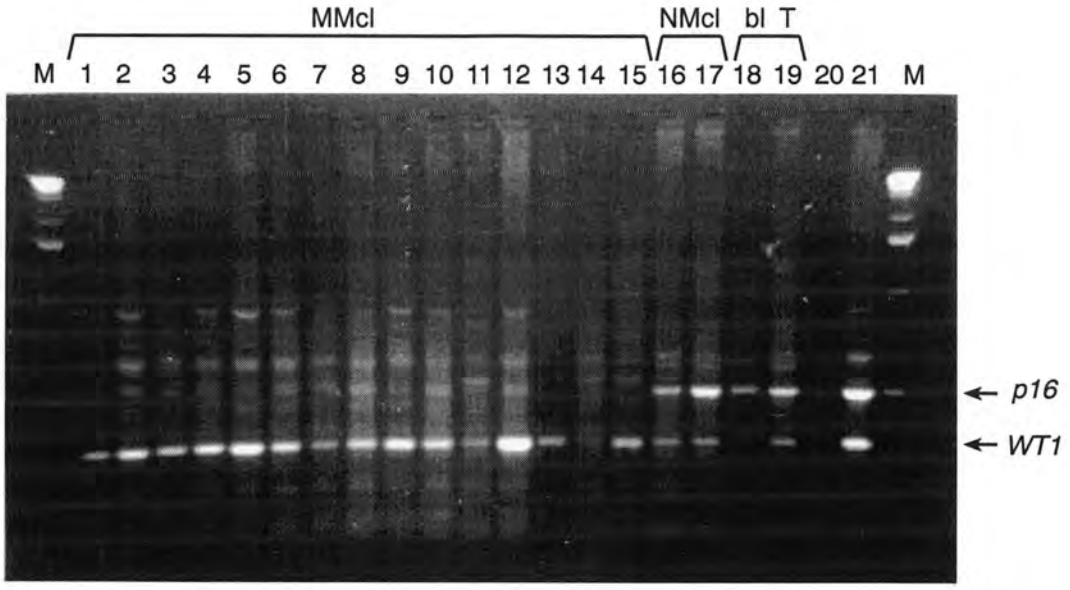


Figure 8.2.1 Homozygous deletion of *p16* in MM cell lines. Multiplex PCR analysis was used to amplify *p16* exon 2 and an internal positive control, *WT1* exon 8. All of the MM cell lines are homozygously deleted for *p16*. Lane 1 - Mero-14; lane 2 - Mero-25; lane 3 - Mero-41; lane 4 - Mero-48a; lane 5 - Mero-48b; lane 6 - Mero-48c; lane 7 - Mero-72; lane 8 - Mero-82; lane 9 - Mero-83; lane 10 - Mero-84; lane 11 - Mero-95; lane 12 - Mero-96; lane 13 - Mero-123; lane 14 - TM2; lane 15 - JU77; lane 16 - NM2; lane 17 - Met5a; lanes 18 and 19 - DC; lane 20- no template, negative control; lane 21 - known wild type, positive control. MMcl - MM cell lines; NMcl - normal mesothelial cell lines; bl - blood; T - MM tumour; M - 1kb size marker.

8.3.2 PCR analysis of marker sequences

DNA from all of the MM cell lines (and from the three glioblastoma cell lines) was PCR amplified for each of the selected 9p marker sequences using oligonucleotides TYRP1F and TYRP1R (*TYRP1*), IFNAF and IFNAR (*IFNA*), G591 and G592 (1063.7), G593 and G594 (c18.b), G857 and G858 (*p16* exon 3), G595 and G596 (c5.1), G599 and G600 (*p16* exon 1), G601 and G602 (c5.3), G603 and G604 (R2.3), G605 and G606 (R2.7), G607 and G608 (*p15* exon 2), G609 and G610 (c1.b), G787 and G788 (D9S171), G729 and G730 (D9S126), G789 and G790 (D9S169). All of these marker sequences were amplified by standard PCR. Additionally, multiplex PCR was used to amplify marker sequences 1063.7 and R2.3, with oligonucleotides C323 and 796/G667 (*WT1* exon 8) as positive control, and R2.7 and c1.b, with oligonucleotides 798/G26 and 801/G26 (*WT1* exon 9) as positive control. Multiplex PCRs could not be successfully established for the remaining marker sequences.

The standard and multiplex PCR amplification patterns generated from each cell line for marker sequences 1063.7, R2.3, R2.7 and c1.b were concordant, indicating that false negative results are not present. Results from some of these PCRs are shown in Figure 8.3.1. False negative results can be generated if a combination of standard and multiplex PCR is not used, as reaction failure in the former and amplification kinetics favouring the positive control in the latter can generate homozygous deletion results from samples that are in fact wild type for the target locus. A lack of false results is further indicated by the amplification patterns generated from each cell line for the remaining marker sequences; these patterns were consistent with those above. Collectively, therefore, this approach indicates that any cell line failing to amplify one or more of the selected marker sequences does so because it is homozygously deleted for that sequence.

All of the MM cell lines (and the glioblastoma cell lines) generated specific PCR products for the most distal and proximal marker sequences, *TYRP1* and D9S169, respectively. For all of the intervening marker sequences, specific PCR products were not generated from each cell line, with the exact pattern of those showing homozygous deletion varying depending on the marker sequence. This indicates that the extent of 9p21 deletion differs between the cell lines.

8.3.3 Conformation that background amplification is non-specific

In many of the PCRs, particularly those that did not generate obvious specific products, a smear of background amplification was observed. It is proposed that this form of amplification is non-specific and is favoured only when oligonucleotides cannot anneal to their true target sequences and, consequently, are available to anneal and amplify inappropriate sequences. The presence of non-specific PCR products may therefore be a further indicator that target sequences are homozygously deleted. To test these proposals, *p16* exon 1 was selected for further analysis. Standard PCR analysis

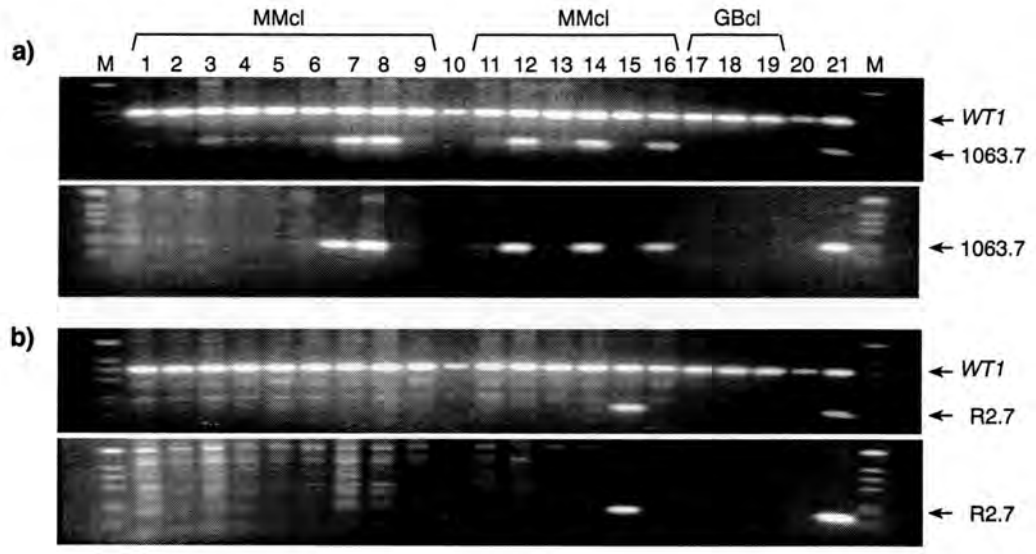


Figure 8.3.1 Comparison of standard and multiplex PCR amplification of 9p21 marker sequences. **a)** marker sequence 1063.7 with (top) or without (bottom) an internal positive control, *WT1* exon 8. **b)** marker sequence R2.7 with (top) or without (bottom) an internal positive control, *WT1* exon 9. For both marker sequences the results between standard and multiplex PCR are concordant. The multiplex PCR negative controls are slightly contaminated with *WT1* products, however the levels of amplification are lower than those observed in the other samples. Lane 1 - Mero-14; lane 2 - Mero-25; lane 3 - Mero-41; lane 4 - Mero-48a; lane 5 - Mero-48b; lane 6 - Mero-48c; lane 7 - Mero-72; lane 8 - Mero-82; lane 9 - Mero-83; lane 10 - no template, negative control; lane 11 - Mero-84; lane 12 - Mero-95; lane 13 - Mero-96; lane 14 - Mero-123; lane 15 - JU77; lane 16 - TM2; lane 17 - U87; lane 18 - U373; lane 19 - A172; lane 20- no template, negative control; lane 21 - known wild type, positive control. MMcl - MM cell lines; GBcl - glioblastoma cell lines; M - 1kb size marker.

of this marker sequence had indicated that it is homozygously deleted in all of the cell lines, and from these PCRs a relatively high level of background amplification was observed. Southern blotting transfer was performed for the PCR products generated with the oligonucleotides for *p16* exon 1, G599 and G600, from DNA from all of the cell lines, Mero-14, -25, -41, -48a, -48b, -48c, -72, -82, -83, -84, -95, -96, -123, JU77 and TM2, and U87, U373 and A172, and a known wild type, C2. As predicted, the smear of background amplification associated with the latter, positive control sample was weaker in comparison to the smears associated with the cell lines. The transferred products were hybridised with the probe oligonucleotide G840, which is specific for *p16* exon 1 as its sequence is complementary to only the internal sequence of this exon. Signal was detected from the positive control sample only, demonstrating that, as previously indicated, *p16* exon 1 PCR products have not been generated from any of the cell lines and that homozygous deletions favour background amplification (Figure 8.3.2). This analysis therefore confirms that the smears of background amplification associated with this marker sequence are comprised solely of non-specific PCR products and suggests that the smears similarly observed in many of the other PCRs are also non-specific.

8.3.4 Formation of a deletion map for 9p21-p23

All of the results from the standard and multiplex PCRs described in 8.3.2 were assembled to form a deletion map of 9p21-p23 for the fifteen MM cell lines and the three glioblastoma cell lines (Table 8.3.1). As described previously, only *TYRP-1* and D9S169 generated specific PCR products from all of the cell lines, and these marker sequences therefore represent the distal and proximal anchor points of the deletion map. The largest deletions present in Mero-41, -48a, -48b, -48c, -83 and -84. For the first four of these cell lines the deletions extend from *TYRP-1* to D9S126, and for the last cell line from *TYRP-1* (*IFN A* not determined) to D9S169. It is not surprising that the deletions present in Mero-48a, -48b and -48c are identical as these three cell lines derive from one individual. The smallest deletions present in Mero-82, -95 and TM2 and extend from 1063.7 to c1.b, which, estimated from the maps detailed by Kamb *et al.* (1994a), is a distance of at least 90kb. The deletions present in Mero-123 and JU77, which extend from *p16* exon 3 to D9S171, and from *IFN A* to R2.7, respectively, may be of a comparable distance, however this can not be confirmed as the distance between c1.b and D9S171, and between *IFN A* and 1063.7, is not known. The extent of each of the above deletions is not inclusive of the appropriate marker sequences named.

In addition to delineating the extent of each deletion, this map reveals a smallest region of overlap (SRO), a distance of approximately 24kb. The distal and proximal boundaries of the SRO are established by , respectively, Mero-123, the only cell line to retain *p16* exon 3, and JU77, the only cell line to retain R2.7. The SRO is therefore virtually limited to the *p16* locus and hence highlights this gene as the critical sequence in each of the 9p21 deletions in the MM cell lines (and glioblastoma cell lines). The map also reveals that in all but one cell line, JU77, the proximal boundary of deletion

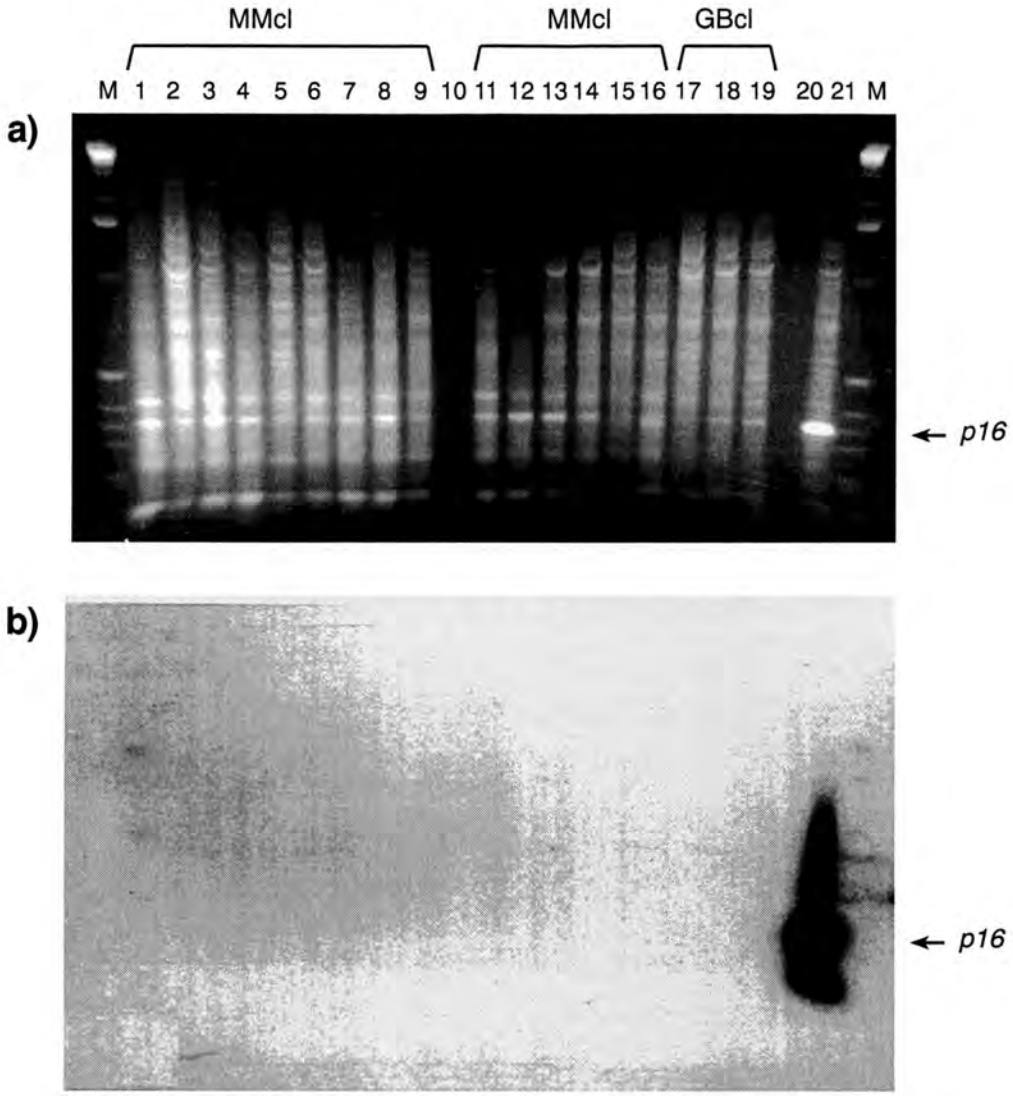
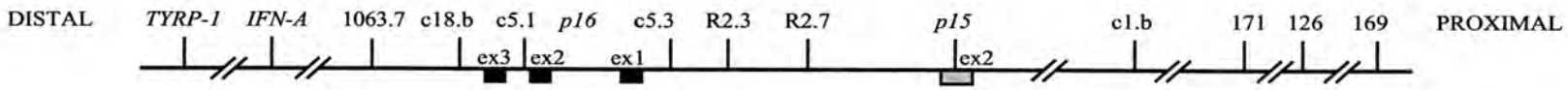


Figure 8.3.2 Non-specific nature of PCR background amplification. **a)** total products from PCR amplification of *p16* exon 1. **b)** Southern blotting and hybridisation of these products with a *p16* exon 1 specific probe detects product in the positive control sample only, demonstrating that all of the other amplification products are non-specific. Lane 1 - Mero-14; lane 2 - Mero-25; lane 3 - Mero-41; lane 4 - Mero-48a; lane 5 - Mero-48b; lane 6 - Mero-48c; lane 7 - Mero-72; lane 8 - Mero-82; lane 9 - Mero-83; lane 10 - no template, negative control; lane 11 - Mero-84; lane 12 - Mero-95; lane 13 - Mero-96; lane 14 - Mero-123; lane 15 - TM2; lane 16 - JU77; lane 17 - U87; lane 18 - U373; lane 19 - A172; lane 20- no template, negative control; lane 21 - known wild type, positive control. MMcl - MM cell lines; GBcl - glioblastoma cell lines; M - 1kb size marker.



mero 14	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+
mero 25	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+
mero 41	+	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
mero 48a	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
mero 48b	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
mero 48c	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
mero 72	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+
mero 82	+	+	+	-	-	-	-	-	-	-	-	-	+	+	+	+
mero 83	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
mero 84	+	?	-	-	-	-	-	-	-	-	-	-	-	-	-	+
mero 95	+	+	+	?	-	-	-	-	-	-	-	-	+	+	+	+
mero 96	+	+	-	-	-	-	-	-	-	-	-	-	-	+	+	+
mero 123	+	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+
JU77	+	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+
TM2	+	+	+	-	-	-	-	-	-	-	-	-	+	+	+	+
U87	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
U373	+	+	-	-	-	-	-	-	-	-	-	-	-	+	+	+
A172	+	+	-	-	-	-	-	-	-	-	-	-	-	+	+	+

SRO approx. 24kb

Table 8.3.1 Deletion map of 9p21-p23 in MM cell lines. Results from PCR amplification of fifteen marker sequences (illustrated along the top) from each of the MM cell lines studied. The positions of the marker sequences are not drawn to scale. The distance from 1063.7 to *p15* exon 2 is approximately 68kb, and from *p15* exon 2 to c1.b at least 22kb (estimated from Kamb *et al.* 1994a). Results from the glioblastoma cell lines U87, U373 and A172 are included. + - present; – - absent, homozygous deletion; ? - not determined.

extends past *p15* exon 2. Therefore in 14 out of 15 of the MM cell lines and in all three of the glioblastoma cell lines the two genes known to map to this region are both deleted.

Furthermore, although all oligonucleotides used in PCRs are designed to amplify only specific products, this map further demonstrates that PCR cross-amplification between the analogous exons of *p16* and *p15*, which share large tracts of identical or near-identical sequence, has not occurred. For exon 1, specificity is indicated by JU77, which is homozygously deleted for this sequence in *p16* but is presumed to retain it in *p15* (as it is proximal of R2.7). For exon 2, although the length of the specific PCR products differ for *p16* and *p15* (509bp and approximately 540bp, respectively), JU77 confirms that these PCRs are completely distinct; in this cell line this sequence is, similar to exon 1, homozygously deleted in *p16* but retained in *p15*. Finally, for exon 3, specificity is indicated by Mero-123 and JU77. The former cell line is presumed to be homozygously deleted for this sequence in *p15* but retains it in *p16*, and the latter cell line displays the opposite pattern, that is this sequence is homozygously deleted in *p16* but presumed to be retained in *p15* (again, as it is proximal of R2.7).

8.4 Homozygous deletion analysis of *p16* and *p15* in primary MM

As the deletion map of 9p21-p23 highlights *p16* as the critical sequence deleted in all fifteen of the MM cell lines analysed, the possible involvement of homozygous deletions of this gene in primary tumours was considered. Furthermore, the co-deletion of *p15* in all but one of the cell lines indicated that analysis of this gene in primary tumours was also appropriate. Eleven human primary MM samples were available for analysis; 2125, 3775-I, 3775-II, 6517, 6517-II, 10563, 12894, 20348, 22754, 23046 and DC. With the exception of the latter, all of the primary MM samples were in the form of wax embedded pleural tissue sections. The DC primary MM was available as an excised portion of peritoneal tumour.

DNA from DC primary MM (and DC blood) was amplified by multiplex PCR as described in 8.2.2, that is for *p16* exon 2 and *WT1* exon 8. DNA from each of the tissue section samples was PCR amplified for *p16*, *p15* and *WT1* using oligonucleotides G386 and G387 (*p16* exon 2), G607 and G608 (*p15* exon 2), C323 and 796/G667 (*WT1* exon 8) and C911 and C912 (*WT1* exon 10). Multiplex PCRs could not be established for these samples as both the quality and quantity of DNA that can be recovered from them is low (it is for this reason also that two positive controls were included).

One or both of the *WT1* exons selected as positive controls failed to amplify from samples 23046 and 22574, however specific PCR products for *p16* exon 2 were generated from each sample and, additionally, for *p15* exon 2 from each of the tissue section samples (Figure 8.2.1 lanes 18 and 19, Figure 8.4.1a) For sample 22574 the intensity of *p15* exon 2 products was extremely low (Figure 8.4.1a lanes 8), suggesting that the template may have derived solely from normal cells and actually be

homozygously deleted in the tumour cells. A mixture of normal and malignant pleural cells is a feature of this type of tissue section. This aspect of possible homozygous deletion of *p15* exon 2 was analysed further in sample 22574 and in three other samples, two displaying a low level of *p15* exon 2 products, 3775-II and 23046, and one displaying approximately equal amounts of *p16* and *p15* products, 2125. To eliminate the possible contribution of DNA from normal cells, microdissections to remove all such cells and leave only tumour cells were performed on these four tissue section samples (work of S. Fleming). DNA from the resulting tumour-specific samples, 22574md, 2775-IImd, 23046md and 2125md, was PCR amplified as before for *p16* exon 2, *p15* exon 2, and *WT1* exons 8 and 10. Similarly to the previous analysis, *WT1* exon 10 failed to amplify from 22574md, however specific PCR products for *p16* exon 2, *p15* exon 2 and *WT1* exon 8 were generated from all four of these tumour-specific samples (Figure 8.4.1b). This analysis demonstrates that, even when the levels of specific PCR products are low, amplification does not derive solely from normal cells and therefore the tumour cells can not be homozygously deleted for either of the *p16* and *p15* sequences tested. Consequently, this analysis indicates that 9p21 homozygous deletions are not present in any of the primary MM samples, including DC and those that were not microdissected.

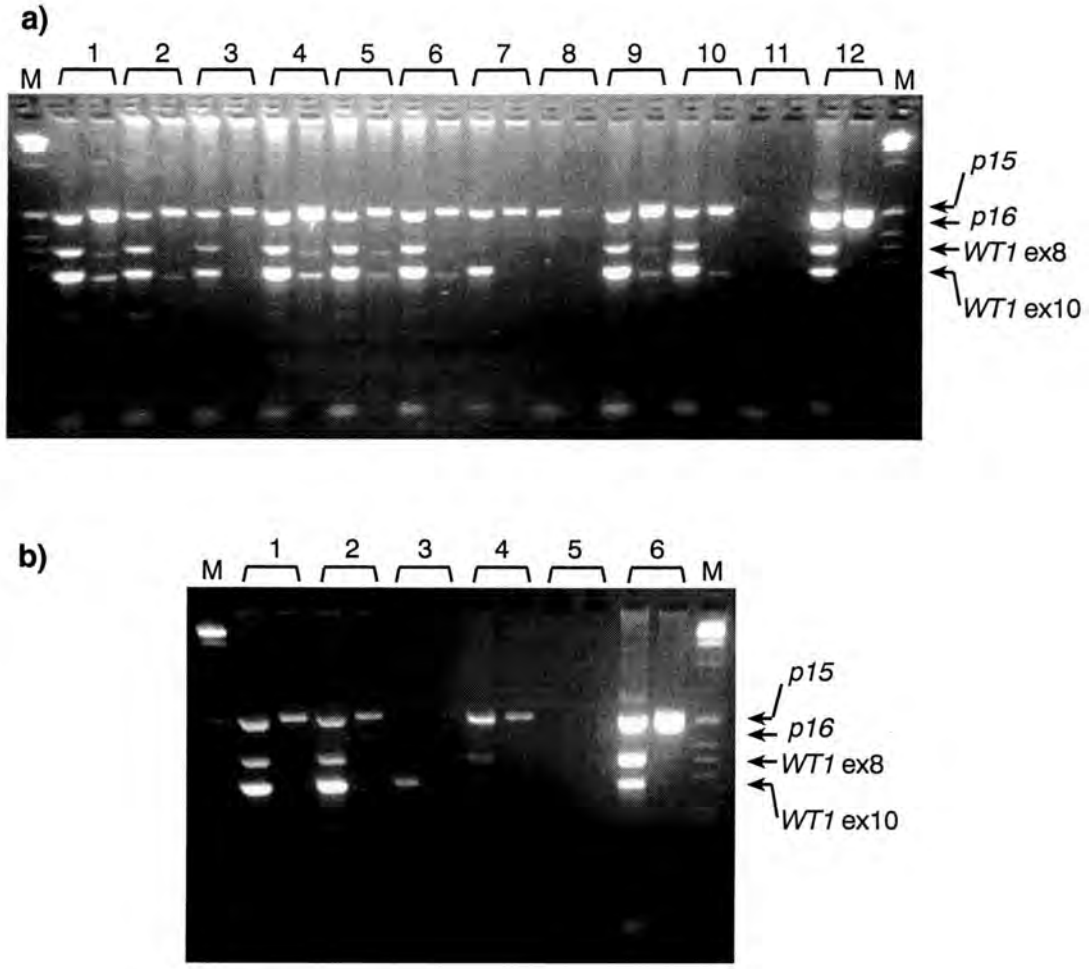


Figure 8.4.1 Homozygous deletion analysis of *p16* and *p15* primary MM. Standard PCR was used to amplify *p16* exon 2, *p15* exon 2 and two positive controls, *WT1* exons 8 and 10. Specific PCR products for *p16* exon 2 and *p15* exon 2 were generated from all of the primary MM [including that in b) lanes 3, very faint], demonstrating a lack of homozygous deletions. For each sample *p16* exon2, *WT1* exon 8 and *WT1* exon 10 were loaded in one lane and *p15* exon 2 in the following lane. **a)** lanes 1 - 10563; lanes 2 - 6517-II; lanes 3 - 6517; lanes 4 - 2125; lanes 5 - 3775-I; lanes 6 - 3775-II; lanes 7 - 23046; lanes 8 - 22574; lanes 9 - 20348; lanes 10 - 12894; lanes 11 - no template, negative control; lanes 12 - known wild type - positive control. **b)** microdissected samples, lanes 1 - 2125md; lanes 2 - 3775-IImd; lanes 3 - 23046md; lanes 4 - 22574md; lanes 5 - no template, negative control; lanes 6 - known wild type - positive control. M - 1kb size marker.

8.5 Discussion

The possible involvement of *p16* homozygous deletions in MM has been investigated by a combination of multiplex and standard PCR analysis of this gene in two sample types, cell lines and primary tumours. Fifteen MM cell lines, 13 of which are Mero cell lines, were analysed for homozygous deletion of *p16* exon 2; such a deletion was identified in each cell line. This feature is remarkable as, although similar analysis has been reported for many panels of tumour cell lines, including 40 MM cell lines reported by Cheng *et al.* (1994), the present cell lines represent the only panel known to display an incidence of *p16* homozygous deletion of 100%. This suggests that in the present cell lines *p16* may be the critical locus that is disrupted. To investigate this possibility the extent of each of the identified deletions was determined by high resolution mapping of 9p23-p21.2. This analysis involved homozygous deletion analysis of fifteen marker sequences that map to this region, including the remaining two exons of *p16*. Nine of these marker sequences map within approximately 40kb distal or proximal of *p16* exon 2, providing a high density of marker sequences in the putative critical region. By amassing of all of the results from the marker sequences a detailed deletion map of this region in the MM cell lines was created, identifying the extent of the largest and smallest deletions, at least 2Mb and at least 90kb, respectively. This deletion map also highlights a SRO of approximately 24kb and positioned directly at the *p16* locus, again indicating the this gene is the critical sequence deleted in the MM cell lines.

Of further interest, the deletion map also indicates that *p15* is co-deleted in all but one of the MM cell lines. This observation suggests that in these cell lines there is a requirement for loss of both *p16* and *p15*. As the proteins encoded by both of these genes are virtually identical in structure and known function, both inhibit CDK4 and CDK6, it is possible that a loss of function of only one will be compensated for by the normal functioning of the other, that is, p16 and p15 may be functionally redundant with respect to each other. If there is a requirement for functional inactivation of both of these proteins this may be achieved, perhaps most easily, by a single mutational event disrupting both genes simultaneously, hence explaining the predominance of homozygous deletion as compared to intragenic mutation of this sequence. Evidence for such a mechanism is indicated by three studies of *p15* reported recently; in two of these studies mutations solely affecting *p15* were found to be not present in a total of 58 tumour cell lines and 131 primary tumours (Otsuki *et al.* 1995, Stone *et al.* 1995), and similarly, in the other study only 3 out of 25 (12%) primary tumours were found to contain such mutations (Okamoto *et al.* 1995). The latter figure is comparable to the observed frequency of intragenic *p16* mutations detected in tumour cell lines and primary tumours (with the exception of melanoma cell lines and oesophageal carcinoma) (see 1.5.8), a similarity that is, perhaps, not surprising if both genes are indeed virtually equivalent, as described.

As analysis of *p16* and *p15* in each of the MM cell lines indicates that these genes are the critical sequences deleted, the potential involvement of homozygous deletion of these genes in primary MM was highlighted. Eleven primary MM tissue sections and four derived tumour cell-specific samples were analysed for homozygous deletions of *p16* exon 2 and *p15* exon 2; for each sample no such deletions were present. This analysis indicates that the pattern of *p16* homozygous deletion in MM is similar to that observed in the majority of other tumour types tested, that is, that although frequent in tumour cell lines, there is a paucity of such deletions in primary tumours. The reasons for this bias are not understood, but it is possible that *in vivo* homozygous deletion of *p16* and *p15* is less evident as an additional level of functional redundancy may exist, and this may prevent such deletions conferring a growth advantage to the cell. Alternatively, the generation of such deletions *in vivo* may be cell lethal unless a specific genetic background is present.

The primary MM in this study were not associated with any of the MM cell lines analysed as, unfortunately, matched tumour and cell line samples were not available. Detailed cytogenetic analysis of the primary tumours that the Mero cell lines derived from is however reported (Hagemeijer *et al.* 1990). This report states that, 'Loss of the short arm of 9 was the most consistent feature of the hypodiploid and hypotetraploid karyotypes: only one case (Me-19) of 24 showed no alteration of chromosome 9, and three cases (Me-41, Me-83, and Me-96) even had apparent nullisomy for 9p.' The stemline karyotype of each of the ten primary tumours associated with all but one (Mero-123) of the thirteen Mero cell lines in the present study show that abnormalities of 9p were present in eight of the tumours and, of these, four had specific deletions of 9p21. The latter four primary tumours derived the cell lines Mero-14, -25, -41 and -96. This analysis therefore suggests that in a proportion of MM homozygous loss of *p16* (and *p15*) may not result simply from *in vitro* culture, but may be of critical importance in tumorigenesis, and hence *p16* may indeed be a true tumour suppressor gene for this tumour type.

In addition to the MM cell lines, three glioblastoma cell lines were similarly analysed for all of the selected 9p marker sequences; in each sample homozygous deletions were identified and each of these extends across both *p16* and *p15*. A lack of homozygous deletion in other tumour cell lines has, however, also been observed; four ovarian carcinoma cell lines, COV362, COV413, COV434 and COV446 (gift from T. Langerak, described by van den Berg-Bakker *et al.* 1993), analysed by multiplex PCR for *p16* exon 2 only, did not display such deletions, in common with one WT cell line (see Chapter 7). In contrast to this perhaps, similar analysis of independent ovarian carcinoma cell lines has identified *p16* homozygous deletions in 2 out of 10 (20%) samples (Chenevix-Trench *et al.* 1994). This difference between two panels of similar tumour cell lines may however simply reflect the small sample size. Moreover, it is of course possible that more subtle *p16* mutations such as intragenic point mutations, or aberrant expression levels, are present in the COV and WT cell lines, and the primary MM analysed. This aspect of *p16* mutations was omitted from this study because, as previously described, these types of mutation are predicted to be infrequent. It is however important

to note that expression of *p16* has been detected in 6 out of 6 normal mesothelial cell lines (J-B Prins, personal communication).

Finally, with the exception of DC, all of the primary MM analysed, and all of the tumours that the MM cell lines derived from, are known to be asbestos induced tumours. Therefore, similar to the findings of Cheng *et al.* (1994), this study provides no evidence for a correlation between 9p21 deletion and MM aetiology.

Chapter 9
Summary

9.1 Summary of the tumour types and genes studied

Two tumour types, Wilms' tumour and malignant mesothelioma, and two genes, *WT1* and *p16*, were selected for analysis in this study. The first tumour type mentioned, WT, is a common paediatric malignancy of the kidney. The survival rate for this tumour is, in general, good as surgical resection and post-operative treatments are highly effective. WT can present in association with GU abnormalities and,, as an extension of this, as a component of WAGR syndrome or DDS. Abnormalities of the short arm of chromosome 11, particularly the region 11p13, are frequently observed in WT individuals. In contrast, the other tumour type analysed in this study, MM, is a rare but increasingly prevalent adult tumour of the pleurae, pericardium and peritoneum. The survival rate for this tumour is poor as surgical resection and post-operative treatments are merely palliative. The majority of cases of MM develop as a result of asbestos exposure and there are no apparent associations with any developmental abnormalities or clinical syndromes. Abnormalities of the short arm of chromosome 9, particularly the region 9p21, have been reported for this tumour.

The first gene mentioned, *WT1*, is expressed in a tissue-specific pattern and is known to contribute to normal development of the urogenital system and the mesothelium. This gene, which maps to 11p13, was cloned in 1990 and the limited amount of *WT1* mutation analysis (in WT) reported at the outset of this study indicated that it is a tumour suppressor gene. Many subsequent reports have confirmed the status of *WT1* as a member of this class of genes. The product of this gene is a nuclear protein, WT1, which functions as a ZF transcription factor. The DNA and protein sequence targets of WT1 are not known. The other gene analysed in this study, *p16*, is expressed in an apparently ubiquitous pattern and is known to contribute to regulation of the cell cycle. This gene, which maps to 9p21, was cloned in 1994 and hence is not as well characterised as *WT1*. Initial reports identified *p16* homozygous deletions in many tumour types and as a result this gene was classified as a tumour suppressor gene. At the outset of this study the bias of *p16* mutations towards *in vitro* samples had not been realised, however subsequent reports of this feature have questioned the initial classification of *p16*. The product of this gene is a nuclear protein, p16, which functions as a CDK inhibitor. CDK4 and CDK6 are known protein targets of p16.

The tumour types WT and MM were selected for analysis as, although distinct in many features, genetic links between the tissues that these tumours derive from are being identified, links which are perhaps a consequence of the developmental pathway these tissues share. It is predicted that the presence of such links may be reflected at the tumorigenic level by the involvement of mutations in commonly expressed genes, two of which, as described, are *WT1* and *p16*. As these latter genes have been classified as tumour suppressor genes and mutations affecting them are reported for either WT or MM, each was considered appropriate for selection as a candidate gene for involvement in both tumour types.

9.2 Summary of results

This study set out to investigate the possible involvement of *WT1* and *p16* in both WT and MM. Analysis of *WT1* did not detect constitutional mutations in individuals with unilateral, bilateral or familial WT, but did detect constitutional and somatic mutations in individuals with WAGR syndrome or DDS. This pattern of results is consistent with the findings reported for many similar studies, that is there is a predominance of *WT1* mutations in individuals with WAGR syndrome or DDS and a paucity of such mutations in individuals with other forms of WT. Considering somatic mutations alone, when WT from individuals with WAGR syndrome or DDS were analysed second hits functionally inactivating the remaining wild type copy of *WT1* were detected. These somatic mutations comprise intragenic deletion in WAGR-WT and LOH in DDS-WT, again a pattern of results consistent with previous reports and confirming that *WT1* is a tumour suppressor gene. This analysis also revealed some unusual features. In one WAGR-WT and one DDS-WT somatic mutations were not detected, and, perhaps more remarkable, in one DDS individual a constitutional mutation was not detected. Although *WT1* was analysed extensively in the latter three samples, it is still possible that mutations in this gene are present but have escaped detection. It is interesting to note however that an apparent lack of *WT1* mutation at the somatic or constitutional level in, respectively, WAGR-WT and DDS has been reported previously (Baird *et al.* 1992a, Baird *et al.* 1992b). In contrast to the above results, analysis of *WT1* in MM cell lines did not detect any mutations considered functionally significant. Additionally, constitutional mutations affecting this gene were similarly found to be not present in individuals from testicular tumour families, although this analysis was more limited than that for WT and MM.

Collectively, the analysis of *WT1* in these tumour types has detected 7 mutations, of which 3 are novel, and demonstrates the involvement of such mutations in tumorigenesis of the kidney, but not in tumorigenesis of the mesothelium or the gonads. Many independent studies have reported similar findings. Furthermore, it is interesting to note that when abnormalities distinct from tumours are considered a pattern opposite to that above is observed, as *WT1* mutations appear to contribute to mesothelial and genital abnormalities but not to renal abnormalities.

Analysis of *p16* in WT and matched cell cultures or cell lines did not detect any mutations. Similar analysis of primary MM and independent MM cell lines did detect mutations but only in the latter type of sample. The incidence of *p16* mutation in the cell lines was 100%, a feature which has not been reported previously for any type of tumour cell line. Additionally, analysis of a limited number of samples detected *p16* mutations in glioblastoma cell lines but not in ovarian carcinoma cell lines and mutations were found to be specific for tumour as compared to normal cell lines. A bias for mutations in tumour cell lines rather than primary tumours is a consistent feature of *p16* and has been reported for many different tumour types. Moreover, this difference between *in vitro* and *in vivo* cells

has raised doubts about the actual importance of *p16* in tumorigenesis. However, the few reports detailing analysis of *p16* in both constitutional and primary tumour samples do demonstrate a mutation pattern consistent with the two hit model for tumorigenesis, suggesting that the initial classification of this gene as a tumour suppressor gene is correct (Hussussian *et al.* 1994, Kamb *et al.* 1994b, Ohta *et al.* 1994) Nonetheless, for the majority of tumour types analysed, the frequency with which *p16* appears to function as this type of gene in primary samples remains low (Cairns *et al.* 1995).

Collectively, analysis of *p16* in these tumour types has detected 15 mutations and demonstrates the involvement of such mutations in MM cell lines but not in primary MM or in any form of WT. As mutations were not detected in the primary samples, this study provides no evidence to support the classification of *p16* as a tumour suppressor gene. However, a report detailing 9p21 deletions in a proportion of the primary tumours that the MM cell lines derived from (Hagemeijer *et al.* 1990) and an intragenic *p16* mutation detected in a single primary MM (Cheng *et al.* 1994) indicate that *p16* may indeed be involved in tumorigenesis of the mesothelium.

This study has therefore detected a contrasting pattern of mutations in WT and MM; *WT1* mutations were detected only in the former tumour type and *p16* mutations only in the latter tumour type. Nine WT and 6 MM cell lines were each analysed for mutations in both genes, and, although it has been reported that mutations affecting *p16* and *RB* are mutually exclusive (Li *et al.* 1994, Otterson *et al.* 1994, Aagaard *et al.* 1995), the number of samples in this study are considered inadequate to propose a similar pattern for *p16* and *WT1*. A further contrast is the type of mutational mechanism associated with each of these genes; analysis of *WT1* indicates a predominance of subtle, intragenic mutations, whereas analysis of *p16* indicates that more gross mutations are favoured. For *WT1*, the small deletion detected somatically in the WAGR-WT is interesting as the predicted protein product may retain sequence-specific DNA binding properties, a feature reported for only one other somatic *WT1* mutation in this form of WT (Park *et al.* 1993c). The *WT1* mutations detected in DDS are of a specific type, predicted to alter critical residues of ZF2 and ZF3, and introduced the proposal that all DDS *WT1* mutations have a dominant negative mode of action. The pattern of mutations detected is consistent with the findings of virtually all reports of *WT1* mutation in this syndrome, and all of these mutation appear compatible with the dominant negative model proposed. Homozygous deletion of *WT1* was not detected. From previous reports it is known that deletion of the entire *WT1* gene can occur as a constitutional, heterozygous event, most obviously in WAGR syndrome, but only rarely is the remaining wild type copy of the gene predicted to suffer a similar type of mutation, and homozygosity is never observed for deletions that extend to adjacent known genes. In contrast, it is exactly this type of mutation that is revealed for *p16* in the MM cell lines. In these cell lines the extent of homozygous deletion differs but a SRO highlights the *p16* locus as the critical deleted region. All of the homozygous deletions extend for at least 90kb and all but one extend to the adjacent and related gene, *p15*. This mutational pattern is consistent with the findings reported for numerous other studies.

and suggests that in many tumour samples loss of both *p16* and *p15* is a requirement for growth advantage. This in turn suggests that p16 and p15 may be functionally redundant.

The consequence of constitutional heterozygous mutation is also different for *WT1* and *p16*. For *WT1*, deletions and intragenic mutations at this level can, in addition to causing predisposition for tumorigenesis, elicit developmental abnormalities. This feature is clearly evident in individuals with WAGR syndrome or DDS. In contrast, this level of *p16* mutation does not appear to elicit any abnormalities and may simply predispose to tumorigenesis, a feature which is perhaps a reflection of the more widespread expression of this gene in comparison to *WT1*.

This study has therefore identified mutations in *WT1* and *p16*, and has highlighted the fact that functional loss of these genes is associated with different tumour types and different mutational mechanisms. Mutation of either of these genes in both WT and MM has not been demonstrated, providing no evidence for a genetic link between the kidney and the mesothelium in tumorigenesis. It is however possible that such a link does exist and this may become apparent if further analysis, similar to that detailed in this study, is pursued.

Bibliography

Aagaard, L., Lukas, J., Bartkova, J., Kjerulff, A-A., Strauss, M., and Bartek, J. (1995). Aberrations of $p16^{Ink4}$ and Retinoblastoma Genes Occur in Distinct Sub-Sets of Human Cancer Cell Lines. *Int. J. Cancer* **61**: 115-120.

Abbott, C., Jackson, I.J., Carritt, B., and Povey, S. (1991). The Human Homolog of the Mouse *Brown* Gene Maps to the Short Arm of Chromosome 9 and Extends the Known Region of Homology with Mouse Chromosome 4. *Genomics* **11**: 471-473.

Abbott, C., and Povey, S. (1991). Development of Human Chromosome-Specific PCR Primers for Characterization of Somatic Cell Hybrids. *Genomics* **9**: 73-77.

Adkins, J.C., and Jaffe, R. (1988). Tumors of the Female Genital Tract in Children. In: *Pediatric Tumors of the Genitourinary Tract*, edited by Broecker, B.H. and Klein, F.A. First edition. Alan R. Liss, Inc., New York.

Akasaka, Y., Kikuchi, H., Nagai, T., Hiraoka, N., Kato, S., and Hata, J-i. (1993). A point mutation found in the *WT1* gene in a sporadic Wilms' tumor without genitourinary abnormalities is identical with the most frequent point mutation in Denys-Drash syndrome. *FEBS Letters* **317**(1,2): 39-43.

Akhtar, M., Knott, E., and Brooks, B. (1977). Extrarenal Wilms' tumour: report of a case and review of the literature. *Cancer* **40**: 3087-3091.

Algar, E.M., Kenney, M.T., Simms, L.A., Smith, S.I., Kida, Y., and Smith, P.J. (1995). Homozygous Intragenic Deletion in the WT1 Gene in a Sporadic Wilms' Tumour Associated With High Levels of Expression of a Truncated Transcript. *Human Mutation* **5**: 221-227.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic Local Alignment Search Tool. *J. Molec. Biol.* **215**: 403-410.

Amin, K.M., Litzky, L.A., Smythe, W.R., Mooney, A.M., Morris, J.M., Mews, D.J.Y., Pass, H.I., Kari, C., Rodeck, U., Rauscher III, F.J., Kaiser, L.R., and Albelda, S.M. (1995). Wilms' Tumor 1 Susceptibility (WT1) Gene Products are Selectively Expressed in Malignant Mesothelioma. *Am. J. Pathol.* **146**(2): 344-356.

Anderson, K.A., Hurley, W.C., Hurley, B.T., and Ohrt, D.W. (1985). Malignant Pleural Mesothelioma Following Radiotherapy in a 16-Year-Old Boy. *Cancer* **56**(2): 273-276.

Antman, K.H., Ruxer, R.L., Aisner, J., and Vawter, G. (1984). Mesothelioma Following Wilms' Tumor in Childhood. *Cancer* **54**: 367-369.

Armstrong, J.F., Pritchard-Jones, K., Bickmore, W.A., Hastie, N.D., and Bard, J.B.L. (1992). The expression of the Wilms' tumour gene, WT1, in the developing mammalian embryo. *Mechanisms of Development* **40**: 85-97.

Aterman, K. (1989). Extrarenal nephroblastomas. *J. Cancer Res. Clinical Oncol.* **115**:409-417.

Ault, J.G., Cole, R.W., Jensen, C.G., Jensen, L.C.W., Bachert, L.A., and Rieder, C.L. (1995). Behavior of Crocidolite Asbestos during Mitosis in Living Vertebrate Lung Epithelial Cells. *Cancer Res.* **55**: 792-798.

Austin, M.B., Fechner, R.E., and Roggli, V.L. (1986). Pleural Malignant Mesothelioma Following Wilms' Tumor. *AJCP* **86**(2): 227-230.

Austruy, E., Cohen-Salmon, M., Antignac, C., Beroud, C., Henry, I., Van Cong, N., Brugieres, L., Junien, C., and Jeanpierre, C. (1993). Isolation of Kidney Complementary DNAs Down-expressed in Wilms' Tumor by a Subtractive Hybridization Approach. *Cancer Res.* **53**: 2888-2894.

Baird, P.N., and Cowell, J.K. (1993). A novel zinc finger mutation in a patient with Denys-Drash syndrome. *Human Molec. Genet.* **2**(12): 2193-2194.

Baird, P.N., Groves, N., Haber, D.A., Housman, D.E., and Cowell, J.K. (1992a). Identification of mutations in the WT1 gene in tumours from patients with the WAGR syndrome. *Oncogene* **7**: 2141-2149.

Baird, P.N., Santos, A., Groves, N., Jadresic, L., and Cowell, J.K. (1992b). Constitutional mutations in the WT1 gene in patients with Denys-Drash syndrome. *Human Molec. Genet.* 1: 301-305.

Ballabio, A., Ranier, J.E., Chamberlain, J.S., Zollo, M., and Caskey, C.T. (1990). Screening for steroid sulfatase (STS) gene deletions by multiplex DNA amplification. *Human Genet.* **84**: 571-573.

Bardeesy, N., Beckwith, J.B., and Pelletier, J. (1995). Clonal Expansion and Attenuated Apoptosis in Wilms' Tumors Are Associated with *p53* Gene Mutations. *Cancer Res.* **55**: 215-219.

Bardeesy, N., Falkoff, D., Petruzzi, M., Nowak, N., Zabel, B., Adam, M., Aguiar, M.C., Grundy, P., Shows, T., and Pelletier, J. (1994a). Anaplastic Wilms' tumour, a subtype displaying poor prognosis, harbours p53 gene mutations. *Nature Genet.* **7**: 91-97.

Bardeesy, N., Zabel, B., Schmitt, K., and Pelletier, J. (1994b). WT1 Mutations Associated with Incomplete Denys-Drash Syndrome Define a Domain Predicted to Behave in a Dominant-Negative Fashion. *Genomics* **21**: 663-665.

Beckwith, J.B., Kiviat, N.B., and Bonadio, J.F. (1990). Nephrogenic Rests, Nephroblastomatosis, And The Pathogenesis Of Wilms' Tumor. *Pediatr. Pathol.* **10**: 1-36.

Benedict, W.F., Murphree, A.L., Banerjee, A., Spina, C.A., Sparkes, M.C., and Sparkes, R.S. (1983). Patient with 13 Chromosome Deletion: Evidence That the Retinoblastoma Gene Is a Recessive Cancer Gene. *Science* **219**: 973-975.

Berg, J.M. (1988). Proposed structure for the zinc-binding domains from transcription factor IIIA and related proteins. *Proc. Natl. Acad. Sci. USA* **85**: 99-102.

Bickmore, W.A., Oghene, K., Little, M.H., Seawright, A., van Heyningen, V., and Hastie, N.D. (1992). Modulation of DNA Binding Specificity by Alternative Splicing of the Wilms Tumor wt1 Gene Transcript. *Science* **257**: 235-237.

Bogan, J.S., and Page, D.C. (1994). Ovary? Testis? - A Mammalian Dilemma. *Cell* **76**: 603-607.

Bonthron, D.T., Morton, C.C., Orkin, S.H., and Collins, T. (1988). Platelet-derived growth factor A chain: Gene structure, chromosomal location, and basis for alternative mRNA splicing. *Proc. Natl. Acad. Sci. USA* **85**: 1492-1496.

Brenner, B., Wildhardt, G., Schneider, S., and Royer-Pokora, B. (1992). RNA polymerase chain reaction detects different levels of four alternatively spliced WT1 transcripts in Wilms' tumors. *Oncogene* **7**: 1431-1433.

Breslow, N.E., and Beckwith, J.B. (1982). Epidemiological features of Wilms' tumor: Results of the National Wilms' Tumor Study. *J. Natl. Cancer Inst.* **68**: 429-436.

Breslow, N., Beckwith, B.J., Ciol, M., and Sharples, K. (1988). Age Distribution of Wilms' Tumor: Report from the National Wilms' Tumor Study. *Cancer Res.* **48**: 1653-1657.

Breslow, N.E., and Langholz, B. (1983). Childhood Cancer Incidence: Geographical And Temporal Variations. *Int. J. Cancer* **32**: 703-716.

Brown, K.W., Shaw, A.P.W., Poirer, V., Tyler, S.J., Berry, P.J., Mott, M.G., and Maitland, N.J. (1989). Loss of chromosome 11p alleles in cultured cells derived from Wilms' tumours. *Br. J. Cancer* **60**: 25-29.

Brown, K.W., Watson, J.E., Poirier, V., Mott, M.G., Berry, P.J., and Maitland, N.J. (1992). Inactivation of the remaining allele of the WT1 gene in a Wilms' tumour from a WAGR patient. *Oncogene* **7**: 763-768.

Brown, K.W., Wilmore, H.P., Watson, J.E., Mott, M.G., Berry, P.J., and Maitland, N.J. (1993). Low Frequency of Mutations in the *WT1* Coding Region in Wilms' Tumor. *Genes, Chromosomes & Cancer* **8**: 74-79.

Browne, D.L., van Heyningen, V., Bickmore, W., and Litt, M. (1993). Dinucleotide repeat polymorphism at the D11S995 locus. *Human Molec. Genet.* **2**(8): 1332.

Browne, K. (1983a). Asbestos-Related Mesothelioma: Epidemiological Evidence for Asbestos as a Promoter. *Arch. of Environmental Health* **38**(5): 261-266.

Browne, K. (1983b). The Epidemiology of Mesothelioma. *J. Soc. Occup. Med.* **33**: 190-194.

Browne, K. (1994). Asbestos-related disease. In: *Occupational Lung Disorders*, edited by Parkes, W.R. Third edition. Butterworth-Heinemann, Oxford.

Bruening, W., Bardeesy, N., Silverman, B.L., Cohn, R.A., Machin, G.A., Aronson, A.J., Housman, D., and Pelletier, J. (1992). Germline intronic and exonic mutations in the Wilms' tumour gene (WT1) affecting urogenital development. *Nature Genet.* **1**: 144-148.

Bruening, W., Gros, P., Sato, T., Stanimir, J., Nakamura, Y., Housman, D., and Pelletier, J. (1993). Analysis of the 11p13 Wilms' Tumor Suppressor Gene (WT1) in Ovarian Tumors. *Cancer Investigation* **11**(4): 393-399.

Bryant, P.J. (1993). Towards the cellular functions of tumour suppressors. *Trends in Cell Biol.* **3**: 31-35.

Buckler, A.J., Pelletier, J., Haber, D.A., Glaser, T., and Housman, D.E. (1991). Isolation, Characterisation, and Expression of the Murine Wilms' Tumor Gene (WT1) During Kidney Development. *Molec. Cell Biol.* **11**(3): 1707-1712.

Buehr, M., Gu, S., and McLaren, A. (1993). Mesonephric contribution to testis differentiation in the fetal mouse. *Development* **117**: 273-281.

Cairney, A.E.L., Andrews, M., Greenberg, M., Smith, D., and Weksberg, R. (1987). Wilms tumor in three patients with Bloom syndrome. *J. Pediatr.* **111**(3): 414-416.

Cairns, P., Mao, L., Merlo, A., Lee, D.J., Schwab, D., Eby, Y., Tokino, K., van der Riet, P., Blaugrund, J.E., and Sidransky, D. (1994). Rates of *p16* (*MTS1*) Mutations in Primary Tumors with 9p Loss. *Science* **265**: 415-416.

Cairns, P., Polascik, T.J., Eby, Y., Tokino, K., Califano, J., Merlo, A., Mao, L., Herath, J., Jenkins, R., Westra, W., Rutter, J.L., Buckler, A., Gabrielson, E., Tockman, M., Cho, K.R., Hedrick, L., Bova, G.S., Asaacs, W., Koch, W., Schwab, D., and Sidransky, D. (1995). Frequency of homozygous deletion at *p16/CDKN2* in primary human tumours. *Nature Genet.* **111**: 210-212.

Caldas, C., Hahn, S.A., da Costa, L.T., Redston, M.S., Schutte, M., Seymour, A.B., Weinstein, C.L., Hruban, R.H., Yeo, C.J., and Kern, S.E. (1994). Frequent somatic mutations and homozygous deletions of the p16 (*MTS1*) gene in pancreatic adenocarcinoma. *Nature Genet.* **8**: 27-32.

Call, K.M., Glaser, T., Ito, C.Y., Buckler, A.J., Pelletier, J., Haber, D.A., Rose, E.A., Kral, A., Yeger, H., Lewis, W.H., Jones, C., and Housman, D.E. (1990). Isolation and Characterization of a Zinc Finger Polypeptide Gene at the Human Chromosome 11 Wilms' Tumor Locus. *Cell* **60**: 509-520.

Cannon-Albright, L.A., Goldgar, D.E., Meyer, L.J., Lewis, C.M., Anderson, D.E., Fountain, J.W., Hegi, M.E., Wiseman, R.W., Petty, E.M., Bale, A.E., Olopade, O.I., Diaz, M.O., Kwiatkowski, D.J., Piepkorn, M.W., Zone, J.J., and Skolnick, M.H. (1992). Assignment of a Locus for Familial Melonoma, MLM, to Chromosome 9p13-p22. *Science* **258**: 1148-1152.

Center, R., Lukeis, R., Dietzsch, E., Gillespie, M., and Garson, O.M. (1993). Molecular Deletion of 9p Sequences in Non-Small Cell Lung Cancer and Malignant Mesothelioma. *Genes, Chromosomes & Cancer* **7**: 47-53.

Chandler, D., El-Naggar, A.K., Brisbay, S., Redline, R.W., and McDonnell, T.J. (1994). Apoptosis and Expression of the *bcl-2* Proto-Oncogene in the Fetal and Adult Human Kidney: Evidence for the Contribution of *bcl-2* Expression to Renal Carcinogenesis. *Human Pathol.* **25**(8): 789-796.

Chao, L.-Y., Huff, V., Tomlinson, G., Riccardi, V.M., Strong, L.C., and Saunders, G.F. (1993). Genetic mosaicism in normal tissues of Wilms' tumour patients. *Nature Genet.* **3**: 127-131.

Chenevix-Trench, G., Kerr, J., Friedlander, M., Hurst, T., Sanderson, B., Coglan, M., Ward, B., Leary, J., and Khoo, S.-K. (1994). Homozygous Deletions on the Short Arm of Chromosome 9 in Ovarian Adenocarcinoma Cell Lines and Loss of Heterozygosity in Sporadic Tumors. *Am. J. Human Genet.* **55**: 143-149.

Cheng, J.Q., Jhanwar, S.C., Klein, W.M., Bell, D.W., Lee, W-C., Altomare, D.A., Nobori, T., Olopade, O.I., Buckler, A.J., and Testa, J.R. (1994). *p16* Alterations and Deletion Mapping of 9p21-p22 in Malignant Mesothelioma. *Cancer Res.* **54**: 5547-5551.

Cheng, J.Q., Jhanwar, S.C., Lu, Y.Y., and Testa, J.R. (1993). Homozygous Deletions within 9p21-p22 Identify a Small Critical Region of Chromosomal Loss in Human Malignant Mesotheliomas. *Cancer Res.* **53**: 4761-4763.

Chomczynski, P., and Sacchi, N. (1987). Single-Step Method of RNA Isolation by Acid Guanidinium Thiocyanate-Phenol-Chloroform Extraction. *Analytical Biochem.* **162**: 156-159.

Christy, B., and Nathans, D. (1989). DNA binding site of the growth factor-inducible protein Zif268. *Proc. Natl. Acad. Sci. USA* **86**: 8737-8741.

Clarkson, P.A., Davies, H.R., Williams, D.M., Chaudhary, R., Hughes, I.A., and Patterson, M.N. (1993). Mutational screening of the Wilms's tumour gene, WT1, in males with genital abnormalities. *J. Med. Genet.* **30**: 767-772.

Clericuzio, C.L. (1993). Clinical Phenotypes and Wilms Tumor. *Med. Pediatr. Oncol.* **21**: 182-187.

Comings, D.E. (1973). A General Theory of Carcinogenesis. *Proc. Natl. Acad. Sci. USA* **70**(12): 3324-3328.

Condie, A., Eeles, R., Borresen, A., Coles, C., Cooper, C., and Prosser, J. (1993). Detection of Point Mutations in the p53 Gene: Comparison of Single-Strand Conformation Polymorphism, Constant Denaturant Gel Electrophoresis, and Hydroxylamine and Osmium Tetroxide Techniques. *Human Mutation* **2**: 58-66.

Coppes, M.J., Bonetta, L., Huang, A., Hoban, P., Chilton-MacNeill, S., Campbell,C.E., Weksberg, R., Yeger, H., Reeve, A.E., and Williams, B.R.G. (1992a). Loss of Heterozygosity Mapping in Wilms Tumor Indicated the Involvement of Three Distinct Regions and a Limited Role for Nondisjunction or Mitotic Recombination. *Genes, Chromosomes & Cancer* **5**: 326-334.

Coppes, M.J., de Kraker, J., van Dijken, P.J. (1989). Bilateral Wilms' tumor: long term survival and some epidemiological features. *J. Clinical Oncol.* **7**: 310-315.

Coppes, M.J., Haber, D.A., and Grundy, P.E. (1994). Genetic Events In The Development Of Wilms' Tumor . *New England J. Med.* **331**(9): 586-590.

Coppes, M.J., Liefers, G.J., Higuchi, M., Zinn, A.B., Balfe, J.W., and Williams, B.R.G. (1992b). Inherited WT1 Mutation in Denys-Drash Syndrome. *Cancer Res.* **52**: 6125-6128.

Coppes, M.J., Liefers, G.J., Paul, P., Yeger, H., and Williams, B.R.G. (1993a). Homozygous somatic WT1 point mutations in sporadic unilateral Wilms tumor. *Proc. Natl. Acad. Sci. USA* **90**: 1416-1419.

Coppes, M.J., Ye, Y., Rackley, R., Zhao, X., Liefers, G.J., Casey, G., and Williams, B.R.G. (1993b). Analysis of *WT1* in Granulosa Cell and Other Sex Cord-Stromal Tumors. *Cancer Res.* **53**: 2712-2714.

Cote, R.J., Jhanwar, S.C., Novick, S., and Pellicer, A. (1991). Genetic Alterations of the p53 Gene Are a Feature of Malignant Mesothelioma. *Cancer Res.* **51**: 5410-5416.

Cotton, R.G.H., Rodrigues, N.R., and Campbell, R.D. (1988). Reactivity of cytosine and thymine in single-base-pair mismatches with hydroxylamine and osmium tetroxide and its application to the study of mutations. *Proc. Natl. Acad. Sci. USA* **85**: 4397-4401.

Counts, J.L., and Goodman, J.I. (1995). Alterations in DNA Methylation May Play a Variety of Roles in Carcinogenesis. *Cell* **83**(1): 13-15.

Cowell, J.K., and Hogg, A. (1992). Genetics and Cytogenetics of Retinoblastoma. *Cancer Genet. Cytogenet.* **64**: 1-11.

Cowell, J.K., Wadey, R.B., Haber, D.A., Call, K.M., Housman, D.E., and Pritchard, J. (1991). Structural rearrangements of the WT1 gene in Wilms' tumour cells. *Oncogene* **6**: 595-599.

Dalla-Fevera, R., Gallo, R.C., Giallongo, A., and Croce, C.M. (1982). Chromosomal Localization of the Human Homolog (*c-sis*) of the Simian Sarcoma Virus *onc* Gene. *Science* **218**: 686-688.

D'Angio, G.J., Breslow, N., Beckwith, B., Evans, A., Baum.Edward, deLorimier, A., Fernbach, D., Hrabovsky, E., Jones, B., Kelalis, P., Othersen, B., Tefft, M., and Thomas, P.R.M. (1989). Treatment of Wilms' Tumor: Results of the Third National Wilms' Tumor Study. *Cancer* **64**(2): 349-360.

Davis, L.M., Stallard, R., Thomas, G.H., Couillin, P., Junien, C., Nowak, N.J., and Shows, T.B. (1988). Two Anonymous DNA Segments Distinguish the Wilms' Tumor and Aniridia Loci. *Science* **241**: 840-842.

Decorte, R., Cuppens, H., Marynen, P., and Cassiman, J. (1990). Rapid Detection of Hypervariable Regions by the Polymerase Chain Reaction Technique. *DNA and Cell Biol.* **9**(6): 461-469.

Delattre, O., Zucman, J., Plougastel, B., Desmaze, C., Melot, T., Peter, M., Kovar, H., Joubert, I., de Jong, P., Rouleau, G., Aurias, A., and Thomas, G. (1992). Gene fusion with an *ETS* DNA-binding domain caused by chromosome translocation in human tumours. *Nature* **359**: 162-165.

Denys, P., Malvaux, P., van den Berghe, H., Tanghe, W., and Proesmans, W. (1967). Association d'un syndrome anatomo-pathologique de pseudohermaphrodisme masculin, d'une tumeur de Wilms, d'une nephropathie parenchymateuse et d'un mosaicisme XX/XY. *Arch. Franc. Ped.* **24**: 729-739.

Devriendt, K., Deloof, E., Moerman, P., Legius, E., Vanhole, C., de Zegher, F., Proesmans, W., and Devlieger, H. (1995). Diaphragmatic Hernia in Denys-Drash Syndrome. *Am. J. Med. Genet.* **57**: 97-101.

Diaz, M.O., Rubin, C.M., Harden, A., Ziemin, S., Larson, R.A., Le Beau, M.M., and Rowley, J.D. (1990). Deletions Of Interferon Genes In Acute Lymphoblastic Leukemia. *New England J. Med.* **322**(2): 77-82.

Douglass, E.C., Wilimas, J.A., Green, A.A., and Look, A.T. (1985). Abnormalities of Chromosomes 1 and 11 in Wilms' Tumor. *Cancer Genet. Cytogenet.* **14**: 331-338.

Drash, A., Sherman, F., Hartmann, W.H., and Blizzard, R.M. (1970). A syndrome of pseudohermaphroditism, Wilms' tumor, hypertension, and degenerative renal disease. *J. Pediatr.* **76**: 585-593.

Dressler, G.R., and Douglass, E.C. (1992). Pax-2 is a DNA-binding protein expressed in embryonic kidney and Wilms tumor. *Proc. Natl. Acad. Sci. USA* **89**: 1179-1183.

Drummond, I.A., Madden, S.L., Rohwer-Nutter, P., Bell, G.I., Sukhatme, V.P., and Rauscher III, F.J. (1992). Repression of the Insulin-Like Growth Factor II Gene by the Wilms Tumor Suppressor WT1. *Science* **257**: 674-678.

Drummond, I.A., Rupprecht, H.D., Rohwer-Nutter, P., Lopez-Guisa, J.M., Madden, S.L., Rauscher III, F.J., and Sukhatme, V.P. (1994). DNA Recognition by Splicing Variants of the Wilms' Tumor Suppressor, WT1. *Molec. Cell Biol.* **14**(6): 3800-3809.

Eccles, M.R., Wallis, L.J., Fidler, A.E., Spurr, N.K., Goodfellow, P.J., and Reeve, A.E. (1992). Expression of the *PAX2* Gene in Human Fetal Kidney and Wilms' Tumor. *Cell Growth & Differentiation* **3**: 279-289.

Einhorn, S., Grander, D., Bjork, O., Brondum-Nielsen, K., and Soderhall, S. (1990). Deletion of α-, β-, and ω-Interferon Genes in Malignant Cells from Children with Acute Lymphocytic Leukemia. *Cancer Res.* **50**: 7781-7785.

Ekblom, P. (1989). Developmentally regulated conversion of mesenchyme to epithelium. *FASEB J.* **3**: 2141-2150.

Eppig, J.J. (1991). Intercommunication Between Mammalian Oocytes and Companion Somatic Cells. *BioEssays* **13**(11): 569-574.

Evans, R.M., and Hollenberg, S.M. (1988). Zinc Fingers: Gilt by Association. *Cell* **52**: 1-3.

Fantes, J.A., Bickmore, W.A., Fletcher, J.M., Ballesta, F., Hanson, I.M., and van Heyningen, V. (1992). Submicroscopic Deletions at the WAGR Locus, Revealed by Nonradioactive In Situ Hybridization. *Am. J. Human Genet.* **51**: 1286-1294.

Fearon, E.R., and Vogelstein, B. (1990). A Genetic Model for Colorectal Tumorigenesis. *Cell* **61**: 759-767.

Fishman, A.P. (1988). *Pulmonary Diseases and Disorders.* Second edition. McGraw-Hill Book Company, New York.

Flejter, W.L., Li, F.P., Antman, K.H., and Testa, J.R. (1989). Recurring Loss Involving Chromosomes 1, 3, and 22 in Malignant Mesothelioma: Possible Sites of Tumor Suppressor Genes. *Genes, Chromosomes & Cancer* **1**: 148-154.

Forrest, S.M., Dahl, H.H., Howells, D.W., Dianzani, I., and Cotton, R.G.H. (1991). Mutation Detection in Phenylketonuria by Using Chemical Cleavage of Mismatch: Importance of Using Probes from Both Normal and Patient Samples. *Am. J. Human Genet.* **49**: 175-183.

Foulkes, N.S., and Sassone-Corsi, P. (1992). More Is Better: Activators and Repressors from the Same Gene. *Cell* **68**: 411-414.

Fountain, J.W., Hudson, T.J., Engelstein, M., Housman, D.E., and Dracopoli, N.C. (1993). Dinucleotide repeat polymorphism at the D9S126 locus (9p21). *Human Molec. Genet.* **2**(6):823.

Fountain, J.W., Karayiorgou, M., Ernstoff, M.S., Kirkwood, J.M., Vlock, D.R., Titus-Ernstoff, L., Bouchard, B., Vijayasaradhi, S., Houghton, A.N., Lahti, J., Kidd, V.J., Housman, D.E., and Dracopoli, N.C. (1992). Homozygous deletions within human chromosome band 9p21 in melanoma. *Proc. Natl. Acad. Sci. USA* **89**: 10557-10561.

Fraizer, G.C., Wu, Y., Hewitt, S.M., Maity, T., Ton, C.C.T., Huff, V., and Saunders, G.F. (1994). Transcriptional Regulation of the Human Wilms' Tumor Gene (WT1). *J. Biol. Chem.* **269**(12): 8892-8900.

Francke, U., Holmes, L.B., Atkins, L., and Riccardi, V.M. (1979). Aniridia-Wilms' tumor association: evidence for specific deletion of 11p13. *Cytogenet. Cell Genet.* **24**: 185-192.

Friedman, A.L., and Finlay, J.L. (1987). The Drash Syndrome Revisited: Diagnosis and Follow-up. *Am. J. Med. Genet. (Suppl.)* **3**: 293-296.

Friend, S.H., Bernards, R., Rogelj, S., Weinberg, R.A., Rapaport, J.M., Albert, D.M., and Dryja, T.P. (1986). A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. *Nature* **323**: 643-646.

Gabrielson, E.W., van der Meeren, A., Reddel, R.R., Reddel, H., Gerwin, B.I., and Harris, C.C. (1992). Human mesothelioma cells and asbestos-exposed mesothelial cells are selectively resistant to amosite toxicity: a possible mechanism for tumor promotion by asbestos. *Carcinogenesis* **13**(8): 1359-1363.

Ganguly, A., and Prockop, D.J. (1990). Detection of single-base mutations by reaction of DNA heteroduplexes with a water-soluble carbodiimide followed by primer extension: application to products from the polymerase chain reaction. *Nucleic Acids Res.* **18**(13): 3933-3939.

Gashler, A.L., Bonthron, D.T., Madden, S.L., Rauscher III, F.J., Collins, T., and Sukhatme, V.P. (1992). Human platelet-derived growth factor A chain is transcriptionally repressed by the Wilms tumor suppressor WT1. *Proc. Natl. Acad. Sci. USA* **89**: 10984-10988.

Gerald, W.L., Gramling, T.S., Sens, D.A., and Garvin, A.J. (1992). Expression of the 11p13 Wilms' Tumor Gene, WT1, Correlates with Histologic Category of Wilms' Tumor. *Am. J. Pathol.* **140**: 1031-1037.

Gerald, W.L., Rosai, J., and Ladanyi, M. (1995). Characterisation of the genomic breakpoint and chimeric transcripts in the *EWS-WT1* gene fusion of desmoplastic small round cell tumor. *Proc. Natl. Acad. Sci. USA* **92**: 1028-1032.

Gerwin, B.I., Lechner, J.F., Reddel, R.R., Roberts, A.B., Robbins, K.C., Gabrielson, E.W., and Harris, C.C. (1987). Comparison of Production of Transforming Growth Factor-*b* and Platelet-derived Growth Factor by Normal Human Mesothelial Cells and Mesothelioma Cell Lines. *Cancer Res.* **47**: 6180-6184.

Gessler, M., Konig, A., Arden, K., Grundy, P., Orkin, S., Sallan, S., Peters, C., Ruyle, S., Mandell, J., Li, F., Cavanee, W., and Bruns, G. (1994). Infrequent Mutation of the *WT1* Gene in 77 Wilms' Tumors. *Human Mutation* **3**: 212-222.

Gessler, M., Konig, A., and Bruns, G.A.P. (1992). The Genomic Organisation and Expression of the WT1 Gene. *Genomics* **12**: 807-813.

Gessler, M., Konig, A., Moore, J., Qualman, S., Arden, K., Cavenee, W., and Bruns, G. (1993). Homozygous Inactivation of WT1 in a Wilms' Tumor Associated With the WAGR Syndrome. *Genes, Chromosomes & Cancer* **7**: 131-136.

Gessler, M., Poustka, A., Cavanee, W., Neve, R.L., Orkin, S.H., and Bruns, G.A.P. (1990). Homozygous deletion in Wilms tumours of a zinc-finger gene identified by chromosome jumping. *Nature* **343**: 774-778.

Giannoukakis, N., Deal, C., Paquette, J., Goodyer, C.G., and Polychronakos, C. (1993). Parental genomic imprinting of the human IGF2 gene. *Nature Genet.* **4**: 98-101.

Gibas, Z., Li, F.P., Antman, K.H., Bernal, S., Stahel, R., and Sandberg, A.A. (1986). Chromosome Changes in Malignant Mesothelioma. *Cancer Genet. & Cytogenet.* **20**: 191-201.

Gillis, A.J.M., Oosterhuis, J.W., Schipper, M.E.I., Barten, E.J., van Berlo, R., van Gurp, R.J.H.L.M., Abraham, M., Saunders, G.F., and Looijenga, L.H.J. (1994). Origin and Biology of a Testicular Wilms' Tumor. *Genes, Chromosomes & Cancer* **11**: 126-135.

Gloudemans, T., Pospiech, I., van der Ven, L.T.M., Lips, C.J.M., Otter, W.D., and Sussenbach, J.S. (1993). An *AvaII* Restriction Fragment Length Polymorphism in the Insulin-like Growth Factor II Gene and the Occurrence of Smooth Muscle Tumors. *Cancer Res.* **53**: 5754-5758.

Golden, W.L., Sudduth, K.W., and Lovell, M.A. (1992). Clonal Evolution of a Wilms' Tumor. *Cancer Genet. Cytogenet.* **60**: 102-104.

Goodyer, P., Dehbi, M., Torban, E., Bruening, W., and Pelletier, J. (1995). Repression of the retinoic acid receptor-α gene by the Wilms' tumor suppressor gene product, wt1. *Oncogene* **10**: 1125-1129.

Greger, V., Debus, N., Lohmann, D., Hopping, W., Passarge, E., and Horsthemke, B. (1994). Frequency and parental origin of hypermethylated RB1 alleles in retinoblastoma. *Human Genet.* **94**: 491-496.

Groves, N., Baird, P.N., Hogg, A., and Cowell, J.K. (1992). A single base pair polymorphism in the WT1 gene detected by single-strand conformation polymorphism analysis. *Human Genet.* **90**: 440-442.

Grubb, G.R., Yun, K., Reeve, A.E., and Eecles, M.R. (1995). Exclusion of the Wilms tumour gene (*WT1*) promoter as a site of frequent mutation in Wilms' Tumour. *Oncogene* **10**: 1677-1681.

Grundy, P.E., Telzerow, P.E., Breslow, N., Moksness, J., Huff, V., and Paterson, M.C. (1994). Loss of Heterozygosity for Chromosome 16q and 1p in Wilms' Tumors Predicts an Adverse Outcome. *Cancer Res.* **54**: 2331-2333.

Grundy, P., Telzerow, P., Paterson, M.C., Haber, D., Berman, B., Li, F., and Garber, J. (1991). Chromosome 11 uniparental isodisomy predisposing to embryonal neoplasms. *The Lancet* (October 26): 1079-1080.

Guan, K-L., Jenkins, C.W., Li, Y., Nichols, M.A., Wu, X., O'Keefe, C.L., Matera, A.G., and Xiong, Y. (1994). Growth suppression by *p18*, a $p16^{INK4/MTS1}$- and $p14^{INK4B/MTS2}$-related CDK6 inhibitor, correlates with wild-type pRb function. *Genes & Development* **8**: 2939-2952.

Guillemot, F., Caspary, T., Tilghman, S.M., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., Anderson, D.J., Joyner, A.L., Rossant, J., and Nagy, A. (1995). Genomic imprinting of *Mash2*, a mouse gene required for trophoblast development. *Nature Genet.* **9**: 235-241.

Gyapay, G., Morissette, J., Vignal, A., Dib, C., Fizames, C., Millasseau, P., Marc, S., Bernardi, G., Lathrop, M., and Weissenbach, J. (1994). The 1993-1994 Genethon human genetic linkage map. *Nature Genet.* **7**: 246-250.

Haber, D.A., Buckler, A.J., Glaser, T., Call, K.M., Pelletier, J., and Sohn, R.L., Douglas, E.C., and Housman, D.E. (1990). An Internal Deletion within an 11p13 Zinc Finger Gene Contributes to the Development of Wilms' Tumor. *Cell* **61**: 1257-1269.

Haber, D.A., Park, S., Maheswaran, S., Englert, C., Re, G.G., Hazen-Martin, D.J., Sens, D.A., and Garvin, A.J. (1993). *WT1*-Mediated Growth Suppression of Wilms Tumor Cells Expressing a *WT1* Splicing Variant. *Science* **262**: 2057-2059.

Haber, D.A., Sohn, R.L., Buckler, A.J., Pelletier, J., Call, K.M., and Housman, D.E. (1991). Alternative splicing and genomic structure of the Wilms tumor gene WT1. *Proc. Natl. Acad. Sci. USA* **88**: 9618-9622.

Haber, D.A., Timmers, H.T.M., Pelletier, J., Sharp, P.A., and Housman, D.E. (1992). A dominant mutation in the Wilms tumor gene WT1 cooperates with the viral oncogene E1A in transformation of primary kidney cells. *Proc. Natl. Acad. Sci. USA* **89**: 6010-6014.

Habib, R., Loirat, C., Gubler, M.C., Niaudet, P., Bensman, A., Levy, M., and Broyer, M. (1985). The nephropathy associated with male pseudohermaphroditism and Wilms' tumor (Drash syndrome): a distinctive glomerular lesion -report of 10 cases. *Clinical Nephrol.* **24**(6): 269-278.

Hagemeijer, A., Versnel, M.A., van Drunen, E., Moret, M., Bouts, M.J., van der Kwast, T.H., and Hoogsteden, H.C. (1990). Cytogenetic Analysis of Malignant Mesothelioma. *Cancer Genet. Cytogenet.* **47**: 1-28.

Hammar, S.P., Bockus, D., Remington, F., Freidman, S., and LaZerte, G. (1989). Familial Mesothelioma: A Report of Two Families. *Human Pathol.* **20**(2): 107-112.

Hannon, G.J., and Beach, D. (1994). $p15^{INK4B}$ is a potential effector of TGF-β-induced cell cycle arrest. *Nature* **371**: 257-261.

Hansteen, I., Hilt, B., Lien, J.T., Skaug, V., and Haugen, A. (1993). Karyotypic Changes in the Preclinical and Subsequent Stages of Malignant Mesothelioma: A Case Report. *Cancer Genet. & Cytogenet.* **70**: 94-98.

Harrington, M.A., Konicek, B., Song, A., Xia, X., Fredericks, W.J., and Rauscher III, F.J. (1993). Inhibition of Colony-stimulating Factor-1 Promoter Activity by the Product of the Wilms' Tumor Locus. *J. Biol. Chem.* **268**(28): 21271-21275.

Hartley, A.L., Birch, J.M., Tricker, K., Wallace, S.A., Kelsey, A.M., Harris, M., and Morris Jones, P.H. (1993). Wilms' Tumor in the Li-Fraumeni Cancer Family Syndrome. *Cancer Genet. Cytogenet.* **67**: 133-135.

He, J., Allen, J.R., Collins, P., Allalunis-Turner, M.J., Godbout, R., Day III, R.S., and James, C.D. (1994). CDK4 Amplification Is an Alternative Mechanism to *p16* Gene Homozygous Deletion in Glioma Cell Lines. *Cancer Res.* **54**: 5804-5807.

Henry, I., Bonaiti-Pellie, C., Chehensse, V., Beldjord, C., Schwartz, C., Utermann, G., and Junien, C. (1991). Uniparental paternal disomy in a genetic cancer-predisposing syndrome. *Nature* **351**: 665-667.

Henry, I., Grandjouan, S., Couillin, P., Barichard, F., Huerre-Jeanpierre, C., Glaser, T., Philip, T., Lenoir, G., Chaussain, J.L., and Junien, C. (1989). Tumor-specific loss of 11p15.5 alleles in del11p13 Wilms tumor and in familial adrenocortical carcinoma. *Proc. Natl. Acad. Sci. USA* **86**: 3247-3251.

Henry, I., Hoovers, J., Barichard, F., Bertheas, M-F., Puech, A., Prieur, F., Gessler, M., Bruns, G., Mannens, M., and Junien, C. (1993). Pericentric Intrachromosomal Insertion Responsible for Recurrence of del(11)(p13p14) in a Family. *Genes, Chromosomes & Cancer* **7**: 57-62.

Herman, J.G., Latif, F., Weng, Y., Lerman, M.I., Zbar, B., Liu, S., Samid, D., Duan, D.R., Gnarra, J.R., Linehan, W.M., and Baylin, S.B. (1994). Silencing of the *VHL* tumor-suppressor gene by DNA methylation in renal carcinoma. *Proc. Natl. Acad. Sci. USA* **91**: 9700-9704.

Herskowitz, I. (1987). Functional inactivation of genes by dominant negative mutations. *Nature* **329**: 219-222.

HGM (1995). Human Gene Mapping 1994, a compendium. Edited by Cuticchia, A.J.. The Johns Hopkins University Press, Baltimore.

Hirsch, A., Brochard, P., de Cremoux, H., Erkan, L., Sebastien, P., di Menza, L., and Bignon, J. (1982). Features of Asbestos-Exposed and Unexposed Mesothelioma. *Am. J. Industrial Dis.* **3**: 413-422.

Hoban, P.R., and Kelsey, A.M. (1991). HinfI polymorphism within the 3' untranslated region of the candidate Wilms tumour gene. *Nucleic Acids Res.* **19**(5):1164.

Hofmann, W., Royer, H., Drechsler, M., Schneider, S., and Royer-Pokora, B. (1993). Characterisation of the transcriptional regulatory region of the human *WT1* gene. *Oncogene* **8**: 3123-3132.

Hovig, E., Smith-Sorensen, B., Brogger, A., and Borresen, A. (1991). Constant denaturant gel electrophoresis, a modification of denaturing gradient gel electrophoresis, in mutation detection. *Mutation Res.* **262**: 63-71.

Huff, V. (1994). Parental origin of *WT1* mutations and mental retardation in WAGR syndrome. *Nature Genet.* **8**:13.

Huff, V., Jaffe, N., Saunders, G.F., Strong, L.C., Villalba, F., and Ruteshouser, E.C. (1995). WT1 Exon Deletion/Insertion Mutations in Wilms Tumor Patients, Associated with Di- and Trinucleotide Repeats and Deletion Hotspot Consensus Sequences. *Am. J. Human Genet.* **56**: 84-90.

Huff, V., Miwa, H., Haber, D.A., Call, K.M., Housman, D., Strong, L.C., and Saunders, G.F. (1991). Evidence for WT1 as a Wilms Tumor (WT) Gene: Intragenic germinal deletion in Bilateral WT. *Am. J. Human Genet.* **48**: 997-1003.

Huff, V., Reeve, A.E., Leppert, M., Strong, L.C., Douglass, E.C., Geiser, C.F., Li, F.P., Meadows, A., Callen, D.F., Lenoir, G., and Saunders, G.F. (1992). Nonlinkage of 16q Markers to Familial Predisposition to Wilms' Tumor. *Cancer Res.* **52**: 6117-6120.

Hunter, T., and Karin, M. (1992). The Regulation of Transcription by Phosphorylation. *Cell* **70**: 375-387.

Hussussian, C.J., Struewing, J.P., Goldstein, A.M., Higgins, P.A.T., Ally, D.S., Sheahan, M.D., Clark, W.H., Tucker, M.A., and Dracopoli, N.C. (1994). Germline p16 mutations in familial melanoma. *Nature Genet.* **8**: 15-21.

Ichimura, K., Schmidt, E.E., Yamaguchi, N., James, C.D., and Collins, V.P. (1994). A Common Region of Homozygous Deletion in Malignant Human Gliomas Lies between the *IFNα/ω* Gene Cluster and the *D9S171* Locus. *Cancer Res.* **54**: 3127-3130.

Ilgren, E.B., and Wagner, J.C. (1991). Background Incidence of Mesothelioma: Animal and Human Evidence. *Regulatory Toxicol. Pharmacol.* **13**: 133-149.

Isobe, M., Emanuel, B.S., Givol, G., Oren, M., and Croce, C.M. (1986). Localisation of gene for human p53 tumour antigen to band 17p13. *Nature* **320**: 84-85.

Jadresic, L., Leake, J., Gordon, I., Dillon, M.J., Grant, D.B., Pritchard, J., Risdon, R.A., and Barratt, T.M. (1990). Clinicopathologic review of twelve children with nephropathy, Wilms' tumor, and genital abnormalities (Drash syndrome). *J. Pediatr.* **117**(5): 717-725.

Jadresic, L., Wadey, R.B., Buckle, B., Barratt, T.M., Mitchell, C.D., and Cowell, J.K. (1991). Molecular analysis of chromosome region 11p13 in patients with Drash syndrome. *Human Genet.* **86**: 497-501.

Jeanpierre, C., Antignac, C., Beroud, C., Lavedan, C., Henry, I., Saunders, G.F., Williams, B., Glaser, T., and Junien, C. (1990). Constitutional and Somatic Deletions of Two Different Regions of Maternal Chromosome 11 in Wilms Tumor. *Genomics* **7**: 434-438.

Jinno, Y., Yun, K., Nishiwaki, K., Kubota, T., Ogawa, O., Reeve, A.E., and Niikawa, N. (1994). Mosaic and polymorphic imprinting of the WT1 gene in humans. *Nature Genet.* **6**: 305-309.

Jones, J.S.P. (1987). Introduction. In: *Pathology of the Mesothelium,* edited by Jones, J.S.P. First edition. Springer-Verlag, Berlin.

Jones, J.S.P., Brachet, E.A., and Butler, E.B. (1987). The Pleura and Its Pathology. In: *Pathology of the Mesothelium,* edited by Jones, J.S.P. First edition. Springer-Verlag, Berlin.

Kamb, A., Gruis, N.A., Weaver-Feldhaus, J., Liu, Q., Harshman, K., Tavtigian, S.V., Stockert, E., Day III, R.S., Johnson, B.E., and Skolnick, M.H. (1994a). A Cell Cycle Regulator Potentially Involved in Genesis of Many Tumor Types. *Science* **264**: 436-440.

Kamb, A., Shattuck-Eidens, D., Eeles, R., Liu, Q., Gruis, N.A., Ding, W., Hussey, C., Tran, T., Miki, Y., Weaver-Feldhaus, J., McClure, M., Aitken, J.F., Anderson, D.E., Bergman, W., Frants, R., Goldgar, D.E., Green, A., MacLennan, R., Martin, N.G., Meyer, L.J., Youl, P., Zone, J.J., Skolnick, M.H., and Cannon-Albright, L.A. (1994b). Analysis of the p16 gene (CDKN2) as a candidate for the chromosome 9p melanoma susceptibility locus. *Nature Genet.* **8**: 22-26.

Kane, M.J., Chahinian, A.P., and Holland, J.F. (1990). Malignant Mesothelioma in Young Adults. *Cancer* **65**(6): 1449-1455.

Kaneko, Y., Kondo, K., Rowley, J.D., Moohr, J.W., and Maurer, H.S. (1983). Further Chromosome Studies on Wilms' Tumor Cells of Patients Without Aniridia. *Cancer Genet. Cytogenet.* **10**: 191-197.

Kaplan, J., Kahn, A., and Chelly, J. (1992). Illegitimate Transcription: Its Use in the Study of Inherited Disease. *Human Mutation* **1**: 357-360.

Keen, J., Lester, D., Inglehearn, C., Curtis, A., and Bhattacharya, S. (1991). Rapid detection of single base mismatches as heteroduplexes on Hydrolink gels. *Trends in Genet.* **7**(1): 5.

Kent, J., Coriat, A-M., Sharpe, P.T., Hastie, N.D., and van Heyningen, V. (1995). The evolution of WT1 sequence and expression pattern in the vertebrates. *Oncogene* **11**: 1781-1792.

Knudson, A.G. (1971). Mutation and Cancer: Statistical Study of Retinoblastoma. *Proc. Natl. Acad. Sci. USA* **68**: 820-823.

Knudson, A.G. (1993). Introduction to the Genetics of Primary Renal Tumors in Children. *Med. Pediat. Oncol.* **21**: 193-198.

Knudson, A.G., Meadows, A.T., Nichols, W.W., and Hill, R. (1976). Chromosomal Deletion And Retinoblastoma. *New England J. Med.* **295**(20): 1120-1123.

Knudson, A.G., and Strong, L.C. (1972). Mutation and Cancer: A Model for Wilms' Tumor of the Kidney. *J. Natl. Cancer Inst.* **48**(2): 313-324.

Konig, A., Jakubiczka, S., Wieacker, P., Schlosser, H.W., and Gessler, M. (1993). Further evidence that imbalance of *WT1* isoforms may be involved in Denys-Drash syndrome. *Human Molec. Genet.* **2**(11): 1967-1968.

Koseki, C., Herzlinger, D., and Al-Awqati, Q. (1992). Apoptosis in Metanephric Development. *J. Cell Biol.* **119**(5): 1327-1333.

Koufos, A., Grundy, P., Morgan, K., Aleck, K.A., Hadro, T., Lampkin, B.C., Kalbakji, A., and Cavenee, W.K. (1989). Familial Wiedemann-Beckwith Syndrome and a Second Wilms Tumor Locus Both Map to 11p15.5. *Am. J. Human Genet.* **44**: 711-719.

Krawczak, M., and Cooper, D.N. (1991). Gene deletions causing human genetic disease: mechanisms of mutagenesis and the role of the local DNA sequence environment. *Human Genet.* **86**: 425-441.

Kreidberg, J.A., Sariola, H., Loring, J.M., Maeda, M., Pelletier, J., Housman, D., and Jaenisch, R. (1993). WT1 Is Required for Early Kidney Development. *Cell* **74**: 679-691.

Kumar, S., Kimberling, W.J., Kenyon, J.B., Smith, R.J.H., Marres, H.A.M., and Cremers, C.W.R.J. (1992). Autosomal dominant branchio-oto-renal syndrome - localization of a disease gene to chromosome 8q by linkage in a Dutch family. *Human Molec. Genet.* **1**(7): 491-495.

Ladanyi, M., and Gerald, W. (1994). Fusion of the *EWS* and *WT1* Genes in the Desmoplastic Small Round Cell Tumor. *Cancer Res.* **54**: 2837-2840.

Larsson, S.H., Charlieu, J., Miyagawa, K., Engelkamp, D., Rassoulzadegan, M., Ross, A., Cuzin, F., van Heyningen, V., and Hastie, N.D. (1995). Subnuclear Localization of WT1 in Splicing or Transcription Factor Domains Is Regulated by Alternative Splicing. *Cell* **81**: 391-401.

Law, M.R., Ward, F.G., Hodson, M.E., and Heard, B.E. (1983). Evidence for longer survival of patients with pleural mesothelioma without asbestos exposure. *Thorax* **38**: 744-746.

Lawler, W. (1991). *Glomerular Pathology*. First edition. Churchill Livingstone, Edinburgh.

Lechner, J.F., Tokiwa, T., LaVeck, M., Benedict, W.F., Banks-Schlegel, S., Yeager Jr., H., Banerjee, A., and Harris, C.C. (1985). Asbestos-associated chromosomal changes in human mesothelial cells. *Proc. Natl. Acad. Sci. USA* **82**: 3884-3888.

Lee, M-H., Reynisdottir, I., and Massague, J. (1995). Cloning of $p57^{KIP2}$, a cyclin-dependent kinase inhibitor with unique domain structure and tissue didtribution. *Genes & Development* **9**: 639-649.

Lee, W., Bookstein, R., Hong, F., Young, L., Shew, J., and Lee, E.YH.P. (1987). Human Retinoblastoma Susceptibility Gene: Cloning, Identification, and Sequence. *Science* **235**: 1394-1399.

Lewis, W.H., Yeger, H., Bonetta, L., Chan, H.S.L., Kang, J., Junien, C., Cowell, J., Jones, C., and Dafoe, L.A. (1988). Homozygous Deletion of a DNA Marker from Chromosome 11p13 in Sporadic Wilms Tumor. *Genomics* **3**: 25-31.

Li, F.P., Fraumeni Jr., J.F., Mulvihill, J.J., Blattner, W.A., Dreyfus, M.G., Tucker, M.A., and Miller, R.W. (1988). A Cancer Family Syndrome in Twenty-four Kindreds. *Cancer Res.* **48**: 5358-5362.

Li, F.P., Lokich, J., Lapey, J., Neptune, W.B., and Wilkins, E.W. (1978). Familial Mesothelioma After Intense Asbestos Exposure at Home. *JAMA* **240**(5): 467.

Li, Y., Nichols, M.A., Shay, J.W., and Xiong, Yue. (1994). Transcriptional Repression of the D-Type Cyclin-dependent Kinase Inhibitor p16 by the Retinoblastoma Susceptibility Gene Product pRb. *Cancer Res.* **54**: 6078-6082.

Little, M.H., Dunn, R., Byrne, J.A., Seawright, A., Smith, P.J., Pritchard- Jones, K., van Heyningen, V., and Hastie, N.D. (1992a). Equivalent expression of paternally and maternally inherited WT1 alleles in normal fetal tissue and Wilms' tumours. *Oncogene* **7**: 635-641.

Little, M., Holmes, G., Bickmore, W., van Heyningen, V., Hastie, N., and Wainwright, B. (1995). DNA binding capacity of the WT1 protein is abolished by Denys-Drash syndrome WT1 point mutations. *Human Molec. Genet.* **4**(3): 351-358.

Little, M.H., Prosser, J., Condie, A., Smith, P.J., van Heyningen, V., and Hastie, N.D. (1992b). Zinc finger point mutations within the WT1 gene in Wilms tumor patients. *Proc. Natl. Acad. Sci. USA* **89**: 4791-4795.

Liu, Q., Neuhausen, S., McClure, M., Frye, C., Weaver-Feldhaus, J., Gruis, N.A., Eddington, K., Allalunis-Turner, M.J., Skolnick, M.H., Fujimura, F.K., and Kamb, A. (1995). CDKN2 (MTS1) tumour suppressor gene mutations in human tumor cell lines. *Oncogene* **10**: 1061-1067.

Looijenga, L.H.J., Abraham, M., Gillis, A.J.M., Saunders, G.F., and Oosterhuis, J.W. (1994). Testicular Germ Cell Tumors of Adults Show Deletions of Chromosomal Bands 11p13 and 11p15.5, but No Abnormalities Within the Zinc-Finger Regions and Exons 2 and 6 of the Wilms' Tumor 1 Gene. *Genes, Chromosomes & Cancer* **9**: 153-160.

Lothe, R.A., Hastie, N., Heimdal, K., Fossa, S.D., Stenwig, A.E., and Borresen, A. (1993). Frequent Loss of 11p13 and 11p15 Loci in Male Germ Cell Tumours. *Genes, Chromosomes & Cancer* **7**: 96-101.

Lu, A., and Hsu, I. (1992). Detection of Single DNA Base Mutations with Mismatch Repair Enzymes. *Genomics* **14**: 249-255.

Lu, Y.Y., Jhanwar, S.C., Cheng, J.Q., and Testa, J.R. (1994). Deletion Mapping of the Short Arm of Chromosome 3 in Human Malignant Mesothelioma. *Genes, Chromosomes & Cancer* **9**: 76-80.

Maelandsmo, G.M., Berner, J-M., Florenes, V.A., Forus, A., Hovig, E., Fodstad, O., and Myklebost, O. (1995). Homozygous deletion frequency and expression of the *CDKN2* gene in human sarcomas - relationship to amplification and mRNA levels of *CDK4* and *CCND1*. *Br. J. Cancer* **72**: 393-398.

Macauley, V.M. (1992). Insulin-like growth factors and cancer. *Br. J. Cancer* **65**: 311-320.

Madden, S.L., Cook, D.M., Morris, J.F., Gashler, A., Sukhatme, V.P., and Rauscher III, F.J. (1991). Transcriptional Repression Mediated by the WT1 Wilms Tumor Gene Product. *Science* **253**: 1550-1553.

Madden, S.L., Cook, D.M., and Rauscher III, F.J. (1993). A structure-function analysis of transcriptional repression mediated by the WT1, Wilms' tumor suppressor protein. *Oncogene* **8**: 1713-1720.

Maheswaran, S., Park, S., Bernard, A., Morris, J.F., Rauscher III, F.J., Hill, D.E., and Haber, D.A. (1993). Physical and functional interaction between WT1 and p53 proteins. *Proc. Natl. Acad. Sci. USA* **90**: 5100-5104.

Malkin, D., Li, F.P., Strong, L.C., Fraumeni Jr., J.F., Nelson, C.E., Kim, D.H., Kassel, J., Gryka, M.A., Bischoff, F.Z., Tainsky, M.A., and Friend, S.H. (1990). Germ Line p53 Mutations in a Familial Syndrome of Breast Cancer, Sarcomas, and Other Neoplasms. *Science* **250**: 1233-1238.

Malkin, D., Sexsmith, E., Yeger, H., Williams, B.R.G., and Coppes, M.J. (1994). Mutations of the *p53* Tumor Suppressor Gene Occur Infrequently in Wilms' Tumor. *Cancer Res.* **54**: 2077-2079.

Mannens, M., Slater, R.M., Heyting, C., Bliek, J., de Kraker, J., Coad, N., de Pagter-Holthuizen, P., and Pearson, P.L. (1988). Molecular nature of genetic changes resulting in loss of heterozygosity of chromosome 11 in Wilms' tumours. *Human Genet.* **81**: 41-48.

Manning, L.S., Whitaker, D., Murch, A.R., Garlepp, M.J., Davis, M.R., Musk, A.W., and Robinson, B.W.S. (1991). Establishment And Characterisation Of Five Human Malignant Mesothelioma Cell Lines Derived From Pleural Effusions. *Int. J. Cancer* **47**: 285-290.

Martha, A.D., Ferrell, R.E., and Saunders, G.F. (1993). Dinucleotide repeat polymorphism in the human aniridia (PAX6) gene. *Human Molec. Genet.* **2**(11): 1982.

Marx, J. (1995). Cell Cycle Inhibitors May Help Brake Growth as Cells Develop. *Science* **267**: 963-964.

Matsuoka, S., Edwards, M.C., Bai, C., Parker, S., Zhang, P., Baldini, A., Harper, J.W., and Elledge, S.J. (1995). $p57^{KIP2}$, a structurally distinct member of the $p21^{CIP1}$ Cdk inhibitor family, is a candidate tumor suppressor gene. *Genes & Development* **9**: 650-662.

Maw, M.A., Grundy, P.E., Millow, L.J., Eccles, M.R., Dunn, R.S., Smith, P.J., Feinberg, A.P., Law, D.J., Peterson, M.C., Telzerow, P.E., Callen, D.F., Thompson, A.D., Richards, R.I., and Reeve, A.E. (1992). A Third Wilms' Tumor Locus on Chromosome 16q. *Cancer Res.* **52**: 3094-3098.

McDonald, A.D., and McDonald, J.C. (1986). Epidemiology of Malignant Mesothelioma. In: *Asbestos Related Malignancy*, edited by Antman, K. and Aisner, J. Grune & Stratton, Inc., Orlando.

McDowell, H., Howard, P., Martin, J., Hart, C., and Crampton, J. (1989). Chromosome 1 Studies in Wilms' Tumor. *Cancer Genet. Cytogenet.* **43**: 203-209.

McGuinness, M.P., and Griswold, M.D. (1994). Interactions between Sertoli cells and germ cells in the testis. *Seminars In Developmental Biology* **5**: 61-66.

Mehta, M.P., Bastin, K.T., and Wiersma, S.R. (1991). Treatment of Wilms' Tumour. *Drugs* **42**: 766-780.

Melocoton, T.L., Salusky, I.B., Hall, T.R., Cohen, A.H., Ehrlich, R.M., and Fine, R.N. (1991). A Case Report of Drash Syndrome in a 46,XX Female. *Am. J. Kidney Dis.* **XVIII**: 503-508.

Meloni, A.M., Stephenson, C.F., Li, F.P., and Sandberg, A.A. (1992). del(6q) as a Possible Primary Change in Malignant Mesothelioma. *Cancer Genet. Cytogenet.* **59**: 57-61,

Merlo, A., Herman, J.G., Mao, L., Lee, D.J., Gabrielson, E., Burger, P.C., Baylin, S.B., and Sidransky, D. (1995). 5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor *p16/CDKN2/MTS1* in human cancers. *Nature Med.* **1**(7): 686-692.

Metcalf, R.A., Welsh, J.A., Bennett, W.P., Seddon, M.B., Lehman, T.A., Pelin, K., Linnainmaa, K., Tammilehto, L., Mattson, K., Gerwin, B.I., and Harris, C.C. (1992). p53 and Kirsten-*ras* Mutations in Human Mesothelioma Cell Lines. *Cancer Res.* **52**: 2610-2615.

Michaely, P., and Bennett, V. (1992). The ANK repeat: a ubiquitous motif involved in macromolecular recognition. *Trends in Cell Biol.* **2**: 127-129.

Miller, R.W., Fraumeni, J.F., and Manning, M.D. (1964). Association of Wilms' tumor with aniridia, hemihypertrophy and other congenital abnormalities. *New England J. Med.* **270**: 922-927.

Mitchell, P.J., and Tjian, R. (1989). Transcriptional Regulation in Mammalian Cells by Sequence-Specific DNA Binding Proteins. *Science* **245**: 371-378.

Miwa, H., Beran, M., and Saunders, G.F. (1992a). Expression of the Wilms' Tumor Gene (WT1) in Human Leukemias. *Leukemia* **6**: 405-409.

Miwa, H., Tomlinson, G.E., Timmons, C.F., Huff, V., Cohn, S.L., Strong, L.C., and Saunders, G.F. (1992b). RNA Expression of the WT1 Gene in Wilms' Tumors in Relation to Histology. *J. Natl. Cancer Inst.* **84**(3): 181-187.

Miyagawa, K., Kent, J., Schedl, A., van Heyningen, V., and Hastie, N.D. (1994). Wilms' tumour - a case of disrupted development. *J. Cell Science (Suppl.)* **18**: 1-5.

Miyagi, T., Ahuja, H., Kubota, T., Kubonishi, I., Koeffler, H.P., and Miyoshi, I. (1993). Expression of the Candidate Wilms' Tumor Gene, *WT1*, in Human Leukemia Cells. *Leukemia* **7**(7): 970-977.

Moore, K.L., and Persaud, T.V.N. (1993). *The Developing Human, Clinically Oriented Embryology.* Fifth edition. W. B. Saunders Company, Philadelphia.

Moorthy, A.V., Chesney, R.W., and Lubinsky, M. (1987). Chronic Renal Failure and XY Gonadal Dysgenesis: "Frasier" Syndrome - A Commentary on Reported Cases. *Am. J. Med. Genet. (Suppl.)* **3**: 297-302.

Morgan, D.O. (1995). Principles of CDK regulation. *Nature* **374**: 131-134.

Mori, T., Miura, K., Aoki, T.,Nishihira, T., Mori, S., and Nakamura, Y. (1994). Frequent Somatic Mutation of the *MTS1/CDKNI* (Multiple Tumor Suppressor/Cyclin-dependent Kinase 4 Inhibitor) Gene in Esophageal Squamous Cell Carcinoma. *Cancer Res.* **54**: 3396-3397.

Morris, J.F., Madden, S.L., Tournay, O.E., Cook, D.M., Sukhatme, V.P., and Rauscher III, F.J. (1991). Characterisation of the zinc finger protein encoded by the WT1 Wilms' tumor locus. *Oncogene* **6**: 2339-2348.

Moulton, T., Crenshaw, T., Hao, Y., Moosikasuwan, J., Lin, N., Dembitzer, F., Hensle, T., Wiess, L., McMorrow, L., Loew, T., Kraus, W., Gerald, W., and Tycko, B. (1994). Epigenetic lesions at the *H19* locus in Wilms' tumour patients. *Nature Genet.* **7**: 440-447.

Muller, H.J. (1932). Further Studies On The Nature And Causes Of Gene Mutations. *Proceedings of the Sixth International Congress of Genetics*: 213-255.

Mundlos, S., Pelletier, J., Darveau, A., Bachmann, M., Winterpacht, A., and Zabel, B. (1993). Nuclear localization of the protein encoded by the Wilms' tumor gene *WT1* in embryonic and adult tissues. *Development* **119**: 1329-1341.

Myers, R.M., Fischer, S.G., Lerman, L.S., and Maniatis, T. (1985a). Nearly all single base substitutions in DNA fragments joined to a GC-clamp can be detected by denaturing gradient gel electrophoresis. *Nucleic Acids Res.* **13**(9): 3131-3145.

Myers, R.M., Larin, Z., and Maniatis, T. (1985b). Detection of Single Base Substitutions by Ribonuclease Cleavage at Mismatches in RNA:DNA Duplexes. *Science* **230**: 1242-1246.

Nakagama, H., Heinrich, G., Pelletier, J., and Housman, D.E. (1995). Sequence and Structural Requirements for High-Affinity DNA Binding by the WT1 Gene Product. *Molec. Cell Biol.* **15**(3): 1489-1498.

Netter, F.H. (1979). *Kidneys, Ureters, and Urinary Bladder, The CIBA Collection of Medical Illustrations.* Volume 6, third edition. CIBA, USA.

Nobori, T., Miura, K., Wu, D.J., Lois, A., Takabayashi, K., and Carson, D.A. (1994). Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. *Nature* **368**: 753-756.

Nordenskjold, A., Friedman, E., and Anvret, M. (1994). WT1 mutations in patients with Denys-Drash syndrome: a novel mutation in exon 8 and paternal allele origin. *Human Genet.* **93**: 115-120.

Ogawa, O., Eccles, M.R., Szeto, J., McNoe, L.A., Yun, K., Maw, M.A., Smith, P.J., and Reeve, A.E. (1993a). Relaxation of insulin-like growth factor II gene imprinting implicated in Wilms' tumour. *Nature* **362**: 749-751.

Ogawa, O., Eccles, M.R., Yun, K., Mueller, R.F., Holdaway, M.D.D., and Reeve, A.E. (1993b). A novel insertional mutation at the third zinc finger coding region of the WT1 gene in Denys-Drash syndrome. *Human Molec. Genet.* **2**: 203-204.

Ohlsson, R., Nystrom, A., Pfeifer-Ohlsson, S., Tohonen, V., Hedborg, F., Schofield, P., Flam, F., and Ekstrom, T.J. (1993). IGF2 is parentally imprinted during human embryogenesis and in the Beckwith-Wiedemann syndrome. *Nature Genet.* **4**: 94-97.

Ohta, M., Nagai, H., Shimizu, M., Rasio, D., Berd, D., Mastrangelo, M., Singh, A.D., Sheilds, J.A., Sheilds, C.L., Croce, C.M., and Huebner, K. (1994). Rarity of Somatic and Germline Mutations of the Cyclin-dependent Kinase 4 Inhibitor Gene, *CDK4I*, in Melanoma. *Cancer Res.* **54**: 5269-5272.

Okamoto, A., Hussain, S.P., Hagiwara, K., Spillare, E.A., Rusin, M.R., Demetrick, D.J., Serrano, M., Hannon, G.J., Shiseki, M., Zariwala, M., Xiong, Y., Beach, D.H., Yokota, J., and Harris, C.C. (1995). Mutations in the $p16^{INK4}$ */MTS1/CDKN2*, $p15^{INK4B}$ */MTS2*, and *p18* Genes in Primary and Metastatic Lung Cancer. *Cancer Res.* **55**: 1448-1451.

Olopade, O.I., Buchhagen, D.L., Malik, K., Sherman, J., Nobori, T., Bader, S., Nau, M.M., Gazdar, A.F., Minna, J.D., and Diaz, M.O. (1993). Homozygous Loss of the Interferon Genes Defines the Critical Region on 9p That Is Deleted in Lung Cancers. *Cancer Res.* **53**: 2410-2415.

Olsen, S. (1984). *Tumors of the Kidney and Urinary Tract, Color Atlas and Textbook.* First edition. Munksgaard, Copenhagen.

Orita, M., Iwahana, H., Kanazawa, H., Hayashi, K., and Sekiya, T. (1989). Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proc. Natl. Acad. Sci. USA* **86**: 2766-2770.

Otsuki, T., Clark, H.M., Wellmann, A., Jaffe, E.S., and Raffeld, M. (1995). Involvement of *CDKN2* (*$p16^{INK4}$/MTS1*) and *$p15^{INK4B}$/MTS2* in Human Leukemias and Lymphomas. *Cancer Res.* **55**: 1436-1440.

Otterson, G.A., Kratzke, R.A., Coxon, A., Kim, Y.W., and Kaye, F.J. (1994). Absence of $p16^{INK4}$ protein is restricted to the subset of lung cancer lines that retains wildtype RB. *Oncogene* **9**: 3375-3378.

Pal, N., Wadey, R.B., Buckle, B., Yeomans, E., Pritchard, J., and Cowell, J.K. (1990). Preferential loss of maternal alleles in sporadic Wilms' tumour. *Oncogene* **5**: 1665-1668.

Park, S., Bernard, A., Bove, K.E., Sens, D.A., Hazen-Martin, D.J., Garvin, A.J., and Haber, D.A. (1993a). Inactivation of *WT1* in nephrogenic rests, genetic precursors to Wilms' tumor. *Nature Genet.* **5**: 363-367.

Park, S., Schalling, M., Bernard, A., Maheswaran, S., Shipley, G.C., Roberts, D., Fletcher, J., Shipman, R., Rheinwald, J., Demetri, G., Griffin, J., Minden, M., Housman, D.E., and Haber, D.A. (1993b). The Wilms tumour gene WT1 is expressed in murine mesoderm-derived tissues and mutated in a human mesothelioma. *Nature Genet.* **4**: 415-420.

Park, S., Tomlinson, G., Nisen, P., and Haber, D.A. (1993c). Altered *trans*-Activational Properties of a Mutated *WT1* Gene Product in a WAGR-associated Wilms' Tumor. *Cancer Res.* **53**: 4757-4760.

Pavletich, N.P., and Pabo, C.O. (1991). Zinc Finger-DNA Recognition: Crystal Structure of a Zif268-DNA Complex at 2.1A. *Science* **252**: 809-817.

Pelletier, J., Bruening, W., Kashtan, C.E., Mauer, S.M., Manivel, J.C., Striegel, J.E.; Houghton, D.C., Junien, C., Habib, R., Fouser, L., Fine, R.N., Silverman, B.L., Haber, D.A., and Housman, D. (1991a). Germline Mutations in the Wilms' Tumor Suppressor Gene Are Associated with Abnormal Urogenital Development in Denys-Drash Syndrome. *Cell* **67**: 437-447.

Pelletier, J., Bruening, W., Li, F.P., Haber, D.A., Glaser, T., and Housman, D.E. (1991b). WT1 mutations contribute to abnormal genital system development and hereditary Wilms' tumour. *Nature* **353**: 431-434.

Pelletier, J., Schalling, M., Buckler, A.J., Rogers, A., Haber, D.A., and Housman, D. (1991c). Expression of the Wilms' tumor gene WT1 in the murine urogenital system. *Genes & Development* **5**: 1345-1356.

Perlman, M., Levin, M., and Wittels, B. (1975). Syndrome Of Fetal Gigantism, Renal Hamartomas, And Nephroblastomatosis With Wilms' Tumor. *Cancer* **35**(4): 1212-1217.

Perry, D.J., and Carrell, R.W. (1992). Hydrolink gels: a rapid and simple approach to the detection of DNA mutations in thromboembolic disease. *J. Clin. Pathol.* **45**: 158-160.

Peterson, J.T., Greenberg, S.D., and Buffler, P.A. (1984). Non-Asbestos-Related Malignant Mesothelioma. *Cancer* **54**: 951-960.

Ponce-Castaneda, M.V., Lee, M-H., Latres, E., Polyak, K., Lacombe, L., Montgomery, K., Mathew, S., Krauter, K., Sheinfeld, J., Massague, J., and Cordon-Cardo, C. (1995). $p27^{Kip1}$: Chromosomal Mapping to 12p12-12p13.1 and Absence of Mutations in Human Tumors. *Cancer Res.* **55**: 1211-1214.

Ponder, B. (1988). Gene losses in human tumours. *Nature* **335**: 400-402.

Popescu, N.C., Chahinian, A.P., and DiPaolo, J.A. (1988). Nonrandom Chromosome Alterations in Human Malignant Mesothelioma. *Cancer Res.* **48**: 142-147.

Poulat, F., Morin, D., Konig, A., Brun, P., Giltay, J., Sultan, C., Dumas, R., Gessler, M., and Berta, P. (1993). Distinct molecular origins for Denys-Drash and Frasier syndromes. *Human Genet.* **91**: 285-286.

Povey, S., Armour, J., Farndon, P., Haines, J.L., Knowles, M., Olopade, F., Pilz, A., White, J.A., Utah Genome Center Genetic Marker and Mapping Group, and Kwiatkowski, D.J. (1994). Report on the Third International Workshop on Chromosome 9. *Ann. Human Genet.* **58**: 177-250.

Pritchard-Jones, K. and Fleming, S. (1991). Cell types expressing the Wilms' tumour gene (WT1) in Wilms' tumours: implications for tumour histogenesis. *Oncogene* **6**: 2211-2220.

Pritchard-Jones, K., Fleming, S., Davidson, D., Bickmore, W., Porteous, D., Gosden, C., Bard, J., Buckler, A., Pelletier, J., Housman, D., van Heyningen, V., and Hastie, N. (1990). The candidate Wilms' tumour gene is involved in genitourinary development. *Nature* **346**: 194-197.

Pritchard-Jones, K., and Hastie, N.D. (1990). Wilms' tumour as a paradigm for the relationship of cancer to development. *Cancer Surveys* **9**(3): 555-578.

Pritchard-Jones, K., Renshaw, J., and King-Underwood, L. (1994). The Wilms tumour (WT1) gene is mutated in a secondary leukaemia in a WAGR patient. *Human Molec. Genet.* **3**(9): 1633-1637.

Promega (1991). Promega Protocols and Applications Guide. Second edition. Promega, USA.

Prosser, J. (1993). Detecting single-base mutations. *Trends in Biotechnol.* **11**: 238-246.

Radice, P., Pilotti, S., De Benedetti, V., Mondini, P., Miozzo, M., Luksch, R., Fossati Bellani, F., Della Porta, G., and Pierotti, M.A. (1993). Homozygous intragenic loss of the WT1 locus in a sporadic intralobar Wilms' tumor. *Int. J. Cancer* **55**: 174-176.

Rainier, S., Johnson, L.A., Dobry, C.J., Ping, A.J., Grundy, P.E., and Feinberg, A.P. (1993). Relaxation of imprinted genes in human cancer. *Nature* **362**: 747-749.

Ranade, K., Hussussian, C.J., Sikorski, R.S., Varmus, H.E., Goldstein, A.M., Tucker, M.A., Serrano, M., Hannon, G.J., Beach, D., and Dracopoli, N.C. (1995). Mutations associated with familial melanoma impair $p16^{INK4}$ function. *Nature Genet.* **10**: 114-116.

Rauscher III, F.J., Benjamin, L.E., Fredericks, W.J., and Morris, J.F. (1994). Novel Oncogenic Mutations in the *WT1* Wilms' Tumor Suppressor Gene: A t(11;22) Fuses the Ewing's Sarcoma Gene, *EWS1*, to *WT1* in Desmoplastic Small Round Cell Tumor. *Cold Spring Harbor Symposia on Quantitative Biology* **LIX**: 137-146.

Rauscher, III, F.J., Morris, J.F., Tournay, O.E., Cook, D.M., and Curran, T. (1990). Binding of the Wilms' Tumor Locus Zinc Finger Protein to the EGR-1 Consensus Sequence. *Science* **250**: 1259-1262.

Reddy, J.C., Morris, J.C., Wang, J., English, M.A., Haber, D.A., Shi, Y., and Licht, J.D. (1995). WT1-mediated Transcriptional Activation Is Inhibited by Dominant Negative Mutant Proteins. *J. Biol. Chem.* **270**(18): 10878-10884.

Redfield, R.J. (1994). Male mutation rates and the cost of sex for females. *Nature* **369**: 145-147.

Reeve, A.E., Eccles, M.R., Wilkins, R.J., Bell, G.I., and Millow, L.J. (1985). Expression of insulin-like growth factor-II transcripts in Wilms' tumour. *Nature* **317**: 258-260.

Reeve, A.E., Sih, S.A., Raizis, A.M., and Feinberg, A.P. (1989). Loss of Allelic Heterozygosity at a Second Locus on Chromosome 11 in Sporadic Wilms' Tumor Cells. *Molec. Cell. Biol.* **9**: 1799-1803.

Risberg, B., Nickels, J., and Wagermark, J. (1980). Familial Clustering of Malignant Mesothelioma. *Cancer* **45**(9): 2422-2427.

Rose, E.A., Glaser, T., Jones, C., Smith, C.L., Lewis, W.H., Call, K.M., Minden, M., Champagne, E., Benetta, L., Yeger, H., and Housman, D.E. (1990). Complete Physical Map of the WAGR Region of 11p13 Localizes a Candidate Wilms' Tumor Gene. *Cell* **60**: 495-508.

Rouleau, G.A., Merel, P., Lutchman, M., Sanson, M., Zucman, J., Marineau, C., Hoang-Xuan, K., Demczuk, S., Desmaze, C., Plougastel, B., Pulst, S.M., Lenoir, G., Bijlsmal, E., Fashold, R., Dumanski, J., de Jong, P., Parry, D., Eldrige, R., Aurias, A., Delattre, O., and Thomas, G. (1993). Alteration in a new gene encoding a putative membrane-organizing protein causes neuro-fibromatosis type 2. *Nature* **363**: 515-5521.

Rupprecht, H.D., Drummond, I.A., Madden, S.L., Rauscher III, F.J., and Sukhatme, V.P. (1994). The Wilms' Tumor Suppressor Gene WT1 Is Negatively Autoregulated. *J. Biol. Chem.* **269**(8): 6198-6206.

Ryan, G., Steele-Perkins, V., Morris, J.F., Rauscher III, F.J., and Dressler, G.R. (1995). Repression of *PAX2* by *WT1* during normal kidney development. *Development* **121**: 867-875.

Rysavy, F.R., Bishop, M.J., Gibbs, G.P., and Williams, G.W. (1992). The UK Human Genome Mapping Project online computing service. *Computer Applications in the Biosciences* **8**(2): 149-154.

Sager, R. (1989). Tumor Suppressor Genes: The Puzzle and the Promise. *Science* **246**: 1406-1412.

Sakai, A., Tadokoro, K., Yanagisawa, H., Nagafuchi, S., Hoshikawa, N., Suzuki, T., Kohsaka, T., Hasegawa, T., Nakahori, Y., and Yamada, M. (1993). A novel mutation of the WT1 gene (a tumour suppressor gene for Wilms' tumor) in a patient with Denys-Drash syndrome. *Human Molec. Genet.* **2**(11): 1969-1970.

Saleeba, J.A., Ramus, S.J., and Cotton, R.G.H. (1992). Complete Mutation Detection Using Unlabeled Chemical Cleavage. *Human Mutation* **1**: 63-69.

Santos, A., Osorio-Almeida, L., Baird, P.N., Silva, J.M., Boavida, M.G., and Cowell, J. (1993). Insertional inactivation of the WT1 gene in tumour cells from a patient with WAGR syndrome. *Human Genet.* **92**: 83-86.

Schneider, S., Wildhardt, G., Ludwig, R., and Royer-Pokora, B. (1993). Exon skipping due to a mutation in a donor splice site in the *WT1* gene is associated with Wilms' tumor and severe genital malformations. *Human Genet.* **91**:nb599-604.

Schroeder, W.T., Chao, L.-Y., Dao, D.D., Strong, L.C., Pathak, S., Riccardi, V., Lewis, W.H., and Saunders, G.F. (1987). Nonrandom Loss of Maternal Chromosome 11 Alleles in Wilms Tumors. *Am. J. Human Genet.* **40**: 413-420.

Scott, J., Cowell, J., Robertson, M.E., Priestley, L.M., Wadey, R., Hopkins, B., Pritchard, J., Bell, G.I., Rall, L.B., Graham, C.F., and Knott, T.J. (1985). Insulin-like growth factor-II gene expression in Wilms' tumour and embryonic tissues. *Nature* **317**: 260-262.

Sekido, Y., Pass, H.I., Bader, S., Mew, D.J.Y., Christman, M.F., Gazdar, A.F., and Minna, J.D. (1995). Neurofibromatosis Type 2 (*NF2*) Gene Is Somatically Mutated in Mesothelioma but not in Lung Cancer. *Cancer Res.* **55**: 1227-1231.

Serrano, M., Hannon, G.J., and Beach, D. (1993). A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature* **366**: 704-707.

Sharma, P.M., Bowman, M., Madden, S.L., Rauscher III, F.J., and Sukumar, S. (1994). RNA editing in the Wilms' tumor susceptibility gene, WT1. *Genes & Development* **8**: 720-731.

Sharma, P.M., Yang, X., Bowman, M., Roberts, V., and Sukumar, S. (1992). Molecular Cloning of Rat Wilms' Tumor Complementary DNA and a Study of Messenger RNA Expression in the Urogenital System and the Brain. *Cancer Res.* **52**: 6407-6412.

Sincock, A., and Seabright, M. (1975). Induction of chromosome changes in Chinese hamster cells by exposure to asbestos fibres. *Nature* **257**: 56-58.

Skapek, S.X., Rhee, J., Spicer, D.B., and Lassar, A.B. (1995). Inhibition of Myogenic Differentiation in Proliferating Myoblasts by Cyclin D1-Dependent Kinase. *Science* **267**: 1022-1024.

Slater, R.M., and Mannens, M.M.A.M. (1992). Cytogenetics and Molecular Genetics of Wilms' Tumor of Childhood. *Cancer Genet. Cytogenet.* **61**: 111-121.

Sotelo-Avila, C. (1990). Nephroblastoma and other pediatric renal cancers. In: *Tumours and Tumour-Like Conditions of the Kidneys and Ureters*, edited by Eble, J.N. First edition. Churchill Livingstone, New York.

Sotelo-Avila, C., Gonzales-Crussi, F., and Fowler, J.W. (1980). Complete and incomplete forms of Beckwith-Wiedemann syndrome: Their oncogenic potential. *J. Pediatr.* **96**(1): 47-50.

Sotos, J.F., Dodge, P.R., Muirhead, D., Crawford, J.D., Talbot, N.B. (1964). Cerebral Gigantism In Childhood: A Syndrome of Excessively Rapid Growth with Acromegalic Features and a Nonprogressive Neurologic Disorder. *New England J. Med.* **271**(3): 109-116.

Spruck III, C.H., Gonzales-Zulueta, M., Shibata, A., Simoneau, A.R., Lin, M-F., Gonzales, F., Tsai, Y.C., and Jones, P.A. (1994). p16 gene in uncultured tumours. *Nature* **370**: 183-184.

Steenman, M.J.C., Rainier, S., Dobry, C.J., Grundy, P., Horon, I.L., and Feinberg, A.P. (1994). Loss of imprinting of *IGF2* is linked to reduced expression and abnormal methylation of *H19* in Wilms' tumour. *Nature Genet.* **7**: 433-439.

Steinmetz, M., Uematsu, Y., and Lindahl, K.F. (1987). Hotspots of homologous recombination in mammalian genomes. *Trends in Genet.* **3**: 7-10.

Stock, R.J., Fu, Y.S., and Carter, J.R. (1979). Malignant Peritoneal Mesothelioma Following Radiotherapy For Seminoma Of The Testis. *Cancer* **44**: 914-919.

Stone, S., Dayananth, P., Jiang, P., Weaver-Feldhaus, J.M., Tavitgian, S.V., Cannon-Albright, L., and Kamb, A. (1995). Genomic structure, expression and mutational analysis of the P15 (MTS2) gene. *Oncogene* **11**: 987-991.

Stuart, E.T., and Gruss, P. (1995). PAX Genes - What's New in Developmental Biology and Cancer. *Human Molec. Genet.* **4**: 1717-1720.

Sukhatme, V.P., Cao, X., Chang, L.C., Tsai-Morris, C.-H., Stamenkovich, D., Ferreira, P.C.P., Cohen, D.R., Edwards, S.A., Shows, T.B., Curran, T., Le Beau, M.M., and Adamson, E.D. (1988). A Zinc Finger-Encoding Gene Coregulated with c-fos during Growth and Differentiation, and after Cellular Depolarization. *Cell* **53**: 37-43.

Sun, Y., Hildesheim, A., Lanier, A.E.P., Cao, K-T., Raab-Traub, N., and Yang, C-S. (1995). No point mutation but decreased expression of the p16/MTS1 tumor suppressor gene in nasopharyngeal carcinomas. *Oncogene* **10**: 785-788.

Suzuki, Y. (1980). Pathology of Human Malignant Mesothelioma. *Seminars in Oncol.* **8**(3): 268-282.

Swan, D.C., McBride, O.W., Robbins, K.C., Keithley, D.A., Reddy, E.P., and Aaronson, S.A. (1982). Chromosomal mapping of the simian sarcoma virus *onc* gene analogue in human cells. *Proc. Natl. Acad. Sci.* **79**: 4691-4695.

Tadokoro, K., Fujii, H., Inoue, T., and Yamada, M. (1991). Polymerase chain reaction (PCR) for detection of ApaI polymorphism at the insulin like growth factor II gene (IGF2). *Nucleic Acids Res.* **19**(24): 6967.

Tadokoro, K., Fujii, H., Ohshima, A., Kakizawa, Y., Shimizu, K., Sakai, A., Sumiyoshi, K., Inoue, T., Hayashi, Y., and Yamada, M. (1992a). Intragenic homozygous deletion of the WT1 gene in Wilms' tumor. *Oncogene* **7**: 1215-1221.

Tadokoro, K., Oki, N., Fujii, H., Ohshima, A., Inoue, T., and Yamada, M. (1992b). Genomic Organisation of the Human WT1 Gene. *Jpn. J. Cancer Res.* **83**: 1198-1203.

Tadokoro, K., Oki, N., Sakai, A., Fujii, H., Ohshima, A., Nagafuchi, S., Inoue, T., and Yamada, M. (1993). PCR detection of 9 polymorphisms in the WT1 gene. *Human Molec. Genet.* **2**(12): 2205-2206.

Taguchi, T., Jhanwar, S.C., Siegfried, J.M., Keller, S.M., and Testa, J.R. (1993). Recurrent Deletions of Specific Chromosomal Sites in 1p, 3p, 6q, and 9p in Human Malignant Mesothelioma. *Cancer Res.* **53**: 4349-4355.

Tam, S.W., Shay, J.W., and Pagano, M. (1994). Differential Expression and Cell Cycle Regulation of the Cyclin-dependent Kinase 4 Inhibitor $p16^{INK4}$. *Cancer Res.* **54**: 5816-5820.

Telerman, A., Dodemont, H., Degraef, C., Galand, P., Bauwens, S., van Oostveldt, P., and Amson, R.B. (1992). Identification of the cellular protein encoded by the human Wilms' tumor (WT1) gene. *Oncogene* **7**: 2545-2548.

Thomas, N.W. (1987). Embryology and Structure of the Mesothelium. In: *Pathology of the Mesothelium,* edited by Jones, J.S.P. First edition. Springer-Verlag, Berlin.

Tiainen, M., Tammilehto, L., Mattson, K., and Knuutila, S. (1988). Nonrandom Chromosomal Abnormalities in Malignant Pleural Mesothelioma. *Cancer Genet. Cytogenet.* **33**: 251-274.

Tiainen, M., Tammilehto, L., Rautonen, J., Tuomi, T., Mattson, K., and Knuutila, S. (1989). Chromosomal abnormalities and their correlations with asbestos exposure and survival in patients with mesothelioma. *Br. J. Cancer* **60**: 618-626.

Ton, C.C.T., Hirvonen, H., Miwa, H., Weil, M.M., Monaghan, P., Jordan, T., van Heyningen, V., Hastie, N.D., Meijers-Heijboer, H., Drechsler, M., Royer-Pokora, B., Collins, F., Swaroop, A., Strong, L.C., and Saunders, G.F. (1991). Positional Cloning and Characterization of a Paired Box- and Homeobox-Containing Gene from the Aniridia Region. *Cell* **67**: 1059-1074.

Trofatter, J.A., MacCollin, M.M., Rutter, J.L., Murrell, J.R., Duyao, M.P., Parry, D.M., Eldridge, R., Kley, N., Menon, A.G., Pulaski, K., Haase, V.H., Ambrose, C.M., Munroe, D., Bove, C., Haines, J.L., Martuza, R.L., MacDonald, M.E., Seizinger, B.R., Short, M.P., Buckler, A.J., and Gusella, J.F. (1993). A Novel Moesin-, Ezrin-, Radixin-like Gene Is a Candidate for the Neurofibromatosis 2 Tumor Suppressor. *Cell* **72**: 791-800.

Tsuda, M., Sakiyama, T., Kitagawa, T., Watanabe, S., Watanabe, T., Takahashi, S., Kawaguchi, H., and Ito, K. (1993). Molecular Analysis of Two Japanese Cases of Denys-Drash Syndrome. *J. Inher. Metab. Dis.* **16**: 876-880.

Ueki, K., Rubio, M-P., Ramesh, V., Correa, K.M., Rutter, J.L., von Deimling, A., Buckler, A.J., Gusella, J.F., and Louis, D.N. (1994). *MTS1/CDKN2* gene mutations are rare in primary human astrocytomas with allelic loss of chromosome 9p. *Human Molec. Genet.* **3**(10): 1841-1845.

van de Graaff, K.M. (1995). *Human Anatomy*. First edition. Wm. C. Brown Publishers, Dubuque.

van den Berg-Bakker, C.A.M., Hagemeijer, A., Franken-Postma, E.M., Smit, V.T.H.B.M., Kuppen, P.J.K., van Ravenswaay Claasen, H.H., Cornelisse, C.J., and Schrier, P.I. (1993). Establishment And Characterization Of 7 Ovarian Carcinoma Cell Lines And One Granulosa Tumor Cell Line: Growth Features And Cytogenetics. *Int. J. Cancer* **53**: 613-620.

van Heyningen, V. (1994). One gene - four syndromes. *Nature* **367**: 319-320.

van Heyningen, V., Bickmore, W.A., Seawright, A., Fletcher, J.M., Maule, J., Fekete, G., Gessler, M., Bruns, G.A.P., Heurre-Jeanpierre, C., Junien, C., Williams, B.R.G., and Nastie, N.D. (1990). Role for the Wilms' tumor gene in genital development? *Proc. Natl. Acad. Sci. USA* **87**: 5383-5386.

Varanasi, R., Bardeesy, N., Ghahremani, M., Petruzzi, M-J., Nowak, N., Adam, M.A., Grundy, P., Shows, T.B., and Pelletier, J. (1994). Fine structure analysis of the *WT1* gene in sporadic Wilms tumors. *Proc. Natl. Acad. Sci. USA* **91**: 3554-3558.

Versnel, M.A., Bouts, M.J., Hoogsteden, H.C., van der Kwast, T.H., Delahaye, M., and Hagemeijer, A. (1989a). Establishment Of Human Malignant Mesothelioma Cell Lines. *Int. J. Cancer* **44**: 256-260.

Versnel, M.A., Hagemeijer, A., Bouts, M.J., van der Kwast, T.H., and Hoogsteden, H.C. (1988). Expression of *c-sis* (PDGF B-chain) and PDGF A-chain genes in ten human malignant mesothelioma cell lines derived from primary and metastatic tumors. *Oncogene* **2**: 601-605.

Versnel, M.A., Hoogsteden, H.C., Hagemeijer, A., Bouts, M.J., van der Kwast, T.H., Delahaye, M., Schaart, G., and Ramaekers, F.C.S. (1989b). Characterization of Three Human Malignant Mesothelioma Cell Lines. *Cancer Genet. Cytogenet.* **42**: 115-128.

Vianna, N.J., and Polan, A.K. (1978). Non-Occupational Exposure To Asbestos And Malignant Mesothelioma In Females. *The Lancet* (May 20): 1061-1063.

Viel, A., Giannini, F., Tumiotto, L., Sopracordevole, F., Visentin, M.C., and Boiocchi, M. (1992). Chromosomal localisation of two putative 11p oncosuppressor genes involved in human ovarian tumours. *Br. J. Cancer* **66**: 1030-1036.

Wadey, R.B., Pal, N., Buckle, B., Yeomans, E., Pritchard, J., and Cowell, J.K. (1990). Loss of heterozygosity in Wilms' tumour involves two distinct regions of chromosome 11. *Oncogene* **5**: 901-907.

Wagner, E. (1870). Das Tuberkelahnliche Lymphadenom. *Arch. Klin. Exp. Ohren Nasen Kehlkopfheilkd* **2**: 497.

Wagner, J.C., Sleggs, C.A., and Marchand, P. (1960). Diffuse Pleural Mesothelioma And Asbestos Exposure In The North Western Cape Province. *Brit. J. Industr. Med.* **17**: 260-271.

Walker, C., Rutten, F., Yuan, X., Pass, H., Mew, D.M., and Everitt, J. (1994). Wilms' Tumor Suppressor Gene Expression in Rat and Human Mesothelioma. *Cancer Res.* **54**: 3101-3106.

Walker, D.G., Duan, W., Popovic, E.A., Kaye, A.H., Tomlinson, F.H., and Lavin, M. (1995). Homozygous Deletions of the Multiple Tumor Suppressor Gene 1 in the Progression of Human Astrocytomas. *Cancer Res.* **55**: 20-23.

Wang, Z.Y., Madden, S.L., Deuel, T.F., and Rauscher III, F.J. (1992). The Wilms' Tumor Gene Product, WT1, Represses Transcription of the Platelet-derived Growth Factor A-chain Gene. *J. Biol. Chem.* **267**: 21999-22002.

Wang, Z.-Y., Qiu, Q.-Q., and Deuel, T.F. (1993a). The Wilms' Tumor Gene Product WT1 Activates or Suppresses Transcription through Separate Functional Domains. *J. Biol. Chem.* **268**(13): 9172-9175.

Wang, Z.-Y., Qiu, Q.-Q., Enger, K.T., and Deuel, T.F. (1993b). A second transcriptionally active DNA-binding site for the Wilms tumor gene product, WT1. *Proc. Natl. Acad. Sci. USA* **90**: 8896-8900.

Wang, Z.-Y., Qiu, Q.-Q., Huang, J., Gurrieri, M., and Deuel, T.F. (1995). Products of alternatively spliced transcripts of the Wilms' tumor suppressor gene, wt1, have altered DNA binding specificity and regulate transcription in different ways. *Oncogene* **10**: 415-422.

Wang-Wuu, S., Soukup, S., Bove, K., Gotwals, B., and Lampkin, B. (1990). Chromosome Analysis of 31 Wilms' Tumors. *Cancer Res.* **50**: 2786-2793.

Wartell, R.M., Hosseini, S.H., and Moran Jr., C.P. (1990). Detecting base pair substitutions in DNA fragments by temperature-gradient gel electrophoresis. *Nucleic Acids Res.* **18**(9): 2699-2705.

Webb, J. (1995). Tragic asbestos error will kill thousands. *New Scientist* **1968**: 4.

Weinberg, R.A. (1995). The Retinoblastoma Protein and Cell Cycle Control. *Cell* **81**: 323-330.

Werner, H., Re, G.G., Drummond, I.A., Sukhatme, V.P., Rauscher III, F.J., Sens, D.A., Garvin, A.J., LeRoith, D., and Roberts Jr., C.T. (1993). Increased expression of the insulin-like growth factor I receptor gene, IGF1R, in Wilms tumor is correlated with modulation of IGF1R promoter activity by the WT1 Wilms tumor gene product. *Proc. Natl. Acad. Sci. USA* **90**: 5828-5832.

Williams, J.C., Brown, K.W., Mott, M.G., and Maitland, N.J. (1989). Maternal Allele Loss In Wilms' Tumour. *The Lancet* (February 4) 283-284,

Wilmore, H.P., White, G.F.J., Howell, R.T., and Brown, K.W. (1994). Germline and Somatic Abnormalities of Chromosome 7 in Wilms' Tumor. *Cancer Genet. Cytogenet.* **77**: 93-98.

Wilms, M. (1899). Die Mischgeschwulste der Nieren. Aurthur Georgi, Leipzig: 1-90.

Winship, P.R. (1989). An improved method for directly sequencing PCR amplified material using dimethyl sulphoxide. *Nucleic Acids Res.* **17**: 1266.

Wright, D.K., and Manos, M.M. (1990). Sample Preparation From Paraffin-Embedded Tissues. In: *PCR Protocols: A Guide to Methods and Applications*, Anonymous Academic Press, Inc.,: 153-158.

Xiong, Y., Hannon, G.J., Zhang, H., Casso, D., Kobayashi, R., and Beach, D. (1993). p21 is a universal inhibitor of cyclin kinases. *Nature* **366**: 701-704.

Xu, L., Sgroi, D., Sterner, C.J., Beauchamp, R.L., Pinney, D.M., Keel, S., Ueki, K., Rutter, J.L., Buckler, A.J., Louis, D.N., Gusella, J.F., and Ramesh, V. (1994). Mutational Analysis of *CDKN2* (*MTS1*/p16^{ink4}) in Human Breast Carcinomas. *Cancer Res.* **54**: 5262-5264.

Yang, A.S., Shen, J., Zingg, J., Mi, S., and Jones, P.A. (1995). *HhaI* and *HpaII* DNA methyltransferases bind DNA mismatches, methylate uracil and block DNA repair. *Nucleic Acids Res.* **23**(8): 1380-1387.

Yeger, H., Cullinane, C., Flenniken, A., Chilton-MacNeill, S., Campbell, C., Huang, A., Bonetta, L., Coppes, M.J., Thorner, P., and Williams, B.R.G. (1992). Coordinate Expression of Wilms' Tumor Genes Correlates with Wilms' Tumor Phenotypes. *Cell Growth & Differentiation* **3**: 855-864.

Zeiger, M.A., Gnarra, J.R., Zbar, B., Linehan, W.M., and Pass, H.I. (1994). Loss of Heterozygosity on the Short Arm of Chromosome 3 in Mesothelioma Cell Lines and Solid Tumors. *Genes, Chromosomes & Cancer* **11**: 15-20.

Zhang, S-Y., Klein-Szanto, A.J.P., Sauter, E.R., Shafarenko, M., Mitsunaga, S., Nobori, T., Carson, D.A., Ridge, J.A., and Goodrow, T.L. (1994). Higher Frequency of Alterations in the *p16/CDKN2* Gene in Squamous Cell Carcinoma Cell Lines Than in Primary Tumors of the Head and Neck. *Cancer Res.* **54**: 5050-5053.

Zhang, Y., and Tycko, B. (1992). Monoallelic expression of the human *H19* gene. *Nature Genet.* **1**: 40-44.

Appendices

Appendix A

Known cDNA sequence of each gene studied

Part I - *WT1*

1 GTTCAAGGCA GCGCCCACAC CCGGGGGCTC TCCGCAACCC GACCGCCTGT

51 CCGCTCCCCC ACTTCCCGCC CTCCCTCCCA CCTACTCATT CACCCACCCA

101 CCCACCCAGA GCCGGGACGG CAGCCCAGGC GCCCGGGCCC CGCCGTCTCC

151 TCGCCGCGAT CCTGGACTTC CTCTTGCTGC AGGACCCGGC TTCCACGTGT

201 GTCCCGGAGC CGGCGTCTCA GCACACGCTC CGCTCCGGGC CTGGGTGCCT

251 ACAGCAGCCA GAGCAGCAGG GAGTCCGGGA CCCGGGCGGC ATCTGGGCCA

301 AGTTAGGCGC CGCCGAGGCC AGCGCTGAAC GTCTCCAGGG CCGGAGGAGC

351 CGCGGGGCGT CCGGGTCTGA GCCTCAGCAA **ATG**GGCTCCG ACGTGCGGGA

401 CCTGAACGCG CTGCTGCCCG CCGTCCCCTC CCTGGGTGGC GGCGGCGGCT

451 GTGCCCTGCC TGTGAGCGGC GCGGCGCAGT GGGCGCCGGT GCTGGACTTT

501 GCGCCCCCGG GCGCTTCGGC TTACGGGTCG TTGGGCGGCC CCGCGCCGCC

551 ACCGGCTCCG CCGCCACCCC CGCCGCCGCC GCCTCACTCC TTCATCAAAC

601 AGGAGCCGAG CTGGGGCGGC GCGGAGCCGC ACGAGGAGCA GTGCCTGAGC

651 GCCTTCACTG TCCACTTTTC CGGCCAGTTC ACTGGCACAG CCGGAGCCTG

701 TCGCTACGGG CCCTTCGGTC CTCCTCCGCC CAGCCAGGCG TCATCCGGCC

751 AGGCCAGGAT GTTTCCTAAC GCGCCCTACC TGCCCAGCTG CCTCGAGAGC

801 CAGCCCGCTA TTCGCAATCA GG↓GTTACAGC ACGGTCACCT TCGACGGGAC

851 GCCCAGCTAC GGTCACACGC CCTCGCACCA TGCGGCGCAG TTCCCCAACC

901 ACTCATTCAA GCATGAGGAT CCCATGGGCC AGCAGGGCTC GCTGG↓GTGAG

951 CAGCAGTACT CGGTGCCGCC CCCGGTCTAT GGCTGCCACA CCCCCACCGA

1001 CAGCTGCACC GGCAGCCAGG CTTTGCTGCT GAGGACGCCC TACAGCAG↓TG

1051 ACAATTTATA CCAAATGACA TCCCAGCTTG AATGCATGAC CTGGAATCAG

1101 ATGAACTTAG GAGCCACCTT AAAGGG↓AGTT GCTGCTGGGA GCTCCAGCTC

1151 AGTGAAATGG ACAGAAGGGC AGAGCAA↓CCA CAGCACAGGG TACGAGAGCG

1201 ATAACCACAC AACGCCCATC CTCTGCGGAG CCCAATACAG AATACACACG

1251 CACGGTGTCT TCAGAGGCAT TCAG↓GATGTG CGACGTGTGC CTGGAGTAGC

1301 CCCGACTCTT GTACGGTCGG CATCTGAGAC CAGTGAGAAA CGCCCCTTCA

1351 TGTGTGCTTA CCCAGGCTGC AATAAGAGAT ATTTTAAGCT GTCCCACTTA

1401 CAGATGCACA GCAGGAAGCA CACTG↓GTGAG AAACCATACC AGTGTGACTT

1451 CAAGGACTGT GAACGAAGGT TTTCTCGTTC AGACCAGCTC AAAAGACACC

1501 AAAGGAGACA TACAG↓GTGTG AAACCATTCC AGTGTAAAAC TTGTCAGCGA

1551 AAGTTCTCCC GGTCCGACCA CCTGAAGACC CACACCAGGA CTCATACAGG

1601 TAAAACAA↓GT GAAAAGCCCT TCAGCTGTCG GTGGCCAAGT TGTCAGAAAA

1651 AGTTTGCCCG GTCAGATGAA TTAGTCCGCC ATCACAACAT GCATCAGAGA

1701 AACATGACCA AACTCCAGCT GGCGCTT**TGA** GGGGTCTCCC TCGGGGACCG

1751 TTCAGTGTCC CAGGCAGCAC AGTGTGTGAA CTGCTTTCAA GTCTGACTCT

1801 CCACTCCTCC TCACTAAAAA GGAAACTTCA GTTGATCTTC TTCATCCAAC

1851 TTCCAAGACA AGATACCGGT GCTTCTGGAA ACTACCAGGT GTGCCTGGAA

1901 GAGTTGGTCT CTGCCCTGCC TACTTTTAGT TGACTCACAG GCCCTGGAGA

1951 AGCAGCTAAC AATGTCTGGT TAGTTAAAAG CCCATTGCCA TTTGGTCTGG

2001 ATTTTCTACT GTAAGAAGAG CCATAGCTGA TCATGTCCCC CTGACCCTTC

2051 CCTTCTTTTT TTATGCTCGT TTTCGCTGGG GATGGAATTA TTGTACCATT

2101 TTCTATCATG GAATATTTAT AGGCCAGGGC ATGTGTATGT GTCTGCTAAT

2151 GTAAACTTTG TCATGGTTTC CATTTACTAA CAGCAACAGC AAGAAATAAA

2201 TCAGAGAGCA AGGCATCGGG GGTGAATCTT GTCTAACATT CCCGAGGTCA

2251 GCCAGGCTGC TAACCTGGAA AGCAGGATGT AGTTCTGCCA GGCAACTTTT

2301 AAAGCTCATG CATTTCAAGC AGCTGAAGAA AGAATCAGAA CTAACCAGTA

2351 CCTCTGTATA GAAATCTAAA AGAATTTTAC CATTCAGTTA ATTCAATGTG

2401 AACACTGGCA CACTGCTCTT AAGAAACTAT GAAGATCTGA GATTTTTTTG

2451 TGTATGTTTT TGACTCTTTT GAGTGGTAAT CATATGTGTC TTTATAGATG

2501 TACATACCTC CTTGCACAAA TGGAGGGGAA TTCATTTTCA TCACTGGGAC

2551 TGTCCTTAGT GTATAAAAAC CATGCTGGTA TATGGCTTCA AGTTGTAAAA

2601 ATGAAAGTGA CTTTAAAAGA AAATAGGGGA TGGTCCAGGA TCTCCACTGA

2651 TAAGACTGTT TTTAAGTAAC TTAAGGACCT TTGGGTCTAC AAGTATATGT

2701 GAAAAAAATG AGACTTACTG GGTGAGGAAA TCCATTGTTT AAAGATGGTC

2751 GTGTGTGTGT GTGTGTGTGT GTGTGTGTTG TGTTGTGTTT TGTTTTTTAA

2801 GGGAGGGAAT TTATTATTTA CCGTTGCTTG AAATTACTGT GTAAATATAT

2851 GTCTGATAAT GATTTGCTCT TTGACAACTA AAATTAGGAC TGTATAAGTA

2901 CTAGATGCAT CACTGGGTGT TGATCTTACA AGATATTGAT GATAACACTT

2951 AAAATTGTAA CCTGCATTTT TCACTTTGCT CTCAATTAAA GTCTATTCAA

3001 AAGGAAAAAA AAAAAAAAAA

↓ - exon junctions

Nucleotides underlined and in bold - initiation and stop codons

Nucleotides underlined only - alternative splice I (51bp) and alternative splice II (9bp)

Part II - *p16*

```
   1  CGGAGAGGGG GAGAACAGAC AACGGGCGGC GGGGAGCAGC ATGGAGCCGG
  51  CGGCGGGGAG CAGCATGGAG CCTTCGGCTG ACTGGCTGGC CACGGCCGCG
 101  GCCCGGGGTC GGGTAGAGGA GGTGCGGGCG CTGCTGGAGG CGGGGGCGCT
 151  GCCCAACGCA CCGAATAGTT ACGGTCGGAG GCCGATCCAG ↓GTCATGATGA
 201  TGGGCAGCGC CCGAGTGGCG GAGCTGCTGC TGCTCCACGG CGCGGAGCCC
 251  AACTGCGCCG ACCCCGCCAC TCTCACCCGA CCCGTGCACG ACGCTGCCCG
 301  GGAGGGCTTC CTGGACACGC TGGTGGTGCT GCACCGGGCC GGGGCGCGGC
 351  TGGACGTGCG CGATGCCTGG GGCCGTCTGC CCGTGGACCT GGCTGAGGAG
 401  CTGGGCCATC GCGATGTCGC ACGGTACCTG CGCGCGGCTG CGGGGGGCAC
 451  CAGAGGCAGT AACCATGCCC GCATAGATGC CGCGGAAGGT CCCTCAG↓ACA
 501  TCCCCGATTG AAAGAACCAG AGAGGCTCTG AGAAACCTCG GGAAACTTAG
 551  ATCATCAGTC ACCGAAGGTC CTACAGGGCC ACAACTGCCC CCGCCACAAC
 601  CCACCCCGCT TTCGTAGTTT TCATTTAGAA AATAGAGCTT TTAAAAATGT
 651  CCTGCCTTTT AACGTAGATA TAAGCCTTCC CCCACTACCG TAAATGTCCA
 701  TTTATATCAT TTTTTATATA TTCTTATAAA AATGTAAAAA AGAAAAACAC
 751  CGCTTCTGCC TTTTCACTGT GTTGGAGTTT TCTGGAGTGA GCACTCACGC
 801  CCTAAGCGCA CATTCATGTG GGCATTTCTT GCGAGCCTCG CAGCCTCCGG
 851  AAGCTGTCGA CTTCATGACA AGCATTTTGT GAACTAGGGA AGCTCAGGGG
 901  GGTTACTGGC TTCTCTTGAG TCACACTGCT AGCAAATGGC AGAACCAAAG
 951  CTCAAATAAA AATAAAATAA TTTTCATTCA TTCACTCAAA AAAAAAAAAA
1001  AAAAAA
```

↓ - exon junctions

Nucleotides underlined and in bold - initiation and stop codons

The initiation codon highlighted corresponds to Serrano *et al.* (1993). This has been revised by Hannon and Beach (1994), who have extended the N terminus of p16 by eight residues (no DNA sequence reported). The 40bp of sequence now available 5' to the highlighted ATG do not contain an initiation codon, and the extent of exon 1 remains unknown.

Part III - *p15*

```
  1  GAGGACTCCG CGACGGTCCG CACCCTGCGG CCAGAGCGGC TTTGAGCTCG
 51  GCTGCTTCCG CGCTAGGCGC TTTTTCCCAG AAGCAATCCA GGCGCGCCCG
101  CTGGTTCTTG AGCGCCAGGA AAAGCCCGGA GCTAACGACC GGCCGCTCGG
151  CACTGCACGG GGCCCCAAGC CGCAGAAGAA GGACGACGGG AGGGTAATGA
201  AGCTGAGCCC AGGTCTCCTA GGAAGGAGAG AGTGCGCCGG AGCAGCGTGG
251  GAAAGAAGGG AAGAGTGTCG TTAAGTTTAC GGCCAACGGT GGATTATCCG
301  GGCCGCTGCG CGTCTGGGGG CTGCGGAATG CGCGAGGAGA ACAAGGGCAT
351  GCCCAGTGGG GGCGGCAGCG ATGAGGGTCT GGCCACGCCG GCGCGGGGAC
401  TAGTGGAGAA GGTGCGACAC TCCTGGGAAG CCGGCGCGGA TCCCAACGGA
451  GTCAACCGTT TCGGGAGGCG CGCGATCCAG ↓GTCATGATGA TGGGCAGCGC
501  CCGCGTGGCG GAGCTGCTGC TGCTCCACGG CGCGGAGCCC AACTGCGCAG
551  ACCCTGCCAC TCTCACCCGA CCGGTGCATG ATGCTGCCCG GGAGGGCTTC
601  CTGGACACGC TGGTGGTGCT GCACCGGGCC GGGGCGCGGC TGGACGTGCG
651  CGATGCCTGG GGTCGTCTGC CCGTGGACTT GGCCGAGGAG CGGGGCCACC
701  GCGACGTTGC AGGGTACCTG CGCACAGCCA CGGGGGACTG ACGCCAGGTT
751  CCCCAGCCGC CCACAACGAC TTTATTTTCT TACCCAA↓TTT CCCACCCCCA
801  CCCACCTAAT TCGATGAAGG CTGCCAACGG GGAGCGG
```

↓ - exon junctions

Nucleotides underlined and in bold - initiation and stop codons

Appendix B

Oligonucleotides used in this study

Part I - Sequence information

Oligonucleotides for *WT1*

to amplify DNA:

for	location	name	sequence (5' to 3')	length, T_M	orient.	origin
exon 1	exon 1	C145	GGG TCT GAG CCT CAG CAA ATG	21mer 66	S	KW
		F518	repeat of C145			
	intron 1	C146	GGG CGC TCC CCG GCC TAC	18mer 66	AS	KW
		F519	repeat of C146			
exon 1	exon 1	F516	CGA GGA GCA GTG CCT GAG CG	20mer 68	S	PP
	intron 1	F517	GCG GAG AGT CCC TGG CGC	18mer 64	AS	PP
exon 2	intron 1	C147	AAG CTT GCG AGA GCA CCG CTG	21mer 68	S	KW
	intron 2	C148	TAA TTT GCT GTG GGT TAG GAA TTC	24mer 66	AS	KW
exon 3	intron 2	C149	GGC TCA GGA TCT CGT GTC TC	20mer 64	S	KW
	intron 3	C150	CCA AGT CCG CCG GCT CAT G	19mer 64	AS	KW
exon 4	intron 3	C486	AAA CAG TTG TGT ATT ATT TTG TGG	24mer 62	S	ML
	intron 4	C152	ACT TTC TTC ATA AGT TCT AAG CAC	24mer 64	AS	KW
exon 5	intron 4	C153	CAG ATC CAT GCA TGC TCC ATT C	22mer 66	S	KW
	intron 5	C154	CTC TTG CAT TGC CCC AGG TG	20mer 64	AS	KW
exon 6	intron 5	C178	AAG CTT CAC TGA CCC TTT TTC CCT TC	26mer 76	S	KW
	intron 6	C177	GAA TTC CAA AGA GTC CAT CAG TAA GG	26mer 74	AS	KW
exon 7	intron 6	C155	GAC CTA CGT GAA TGA TCA CAT G	22mer 64	S	KW
	intron 7	945	ACA ACA CCT GGA TCA AGA CCT	21mer 62	AS	ML
		E194	repeat of 945			
exon 8	intron 7	C323	CCT TTA ATG AGA TCC CCT TTT CC	23mer 66	S	ML
	intron 8	796	GGG GAA ATG TGG GGT GTT TCC	21mer 66	AS	ML
		G667	repeat of 796			
exon 9	intron 8	798	TGC AGA CAT TGC AGG CAT GGC AGG	24mer 76	S	ML
		G26	repeat of 798			
	intron 9	801	GCA CTA TTC CTT CTC TCA ACT GAG	24mer 70	AS	ML
		G25	repeat of 801			
exon 10	intron 9	C911	ACT TCA CTC GGG CCT TGA TAG	21mer 64	S	KW
	exon 10	C912	GTG GAG AGT CAG ACT TGA AAG	21mer 62	AS	KW

to amplify cDNA:

for	location	name	sequence (5' to 3')	length, T_M	orient.	origin
5'TD	exon 1	D609	CAA ACA GGA GCC GAG CTG G	19mer 62	S	KW
nest -1	exon 6	D610	GCA CAT CCT GAA TGC CTC TG	20mer 62	AS	KW
5'TD	exon 1	B920	AGC AGT GCC TGA GCG CCT T	19mer 62	S	PP
nest -2	exon 6	B918	CCG TGC GTG TGT ATT CTG TA	20mer 62	AS	PP
5'TD	exon 1	M3897	biotinylated B920	19mer 62	S	KW
nest -2	exon 6	M3896	biotinylated B918	20mer 62	AS	KW

for	location	name	sequence (5' to 3')	length, T_M	orient.	origin
3'ZF	exon 5	C582	AAA TGG ACA GAA GGG CAG AGC	21mer 64	S	KW
nest -1	exon 10	C583	TTG GAA GTT GGA TGA AGA AGA TC	23mer 64	AS	KW
3'ZF	exon 6	B919	GAG AGC GAT AAC CAC ACA AC	20mer 60	S	PP
nest -2	exon 10	B917	ACT TGA AAG CAG TTC ACA CA	20mer 56	AS	PP
3'ZF'	exon 6	M3895	biotinylated B919	20mer 60	S	KW
nest -2	exon 10	M3898	biotinylated B917	20mer 56	AS	KW

to amplify polymorphisms:

for	location	name	sequence (5' to 3')	length, T_M	orient.	origin
Hinf I	intron 3	G186	CAC TCG CTC AGC TGT CTT CGG	21mer 68	S	PP
RFLP	intron 3	G187	CAA GGA CCC AGA CGC AGA G	19mer 62	AS	PP
TaqI/ApaI	intron 5	G188	GTT GTC CTT TTC AGC ATT GC	20mer 58	S	PP
RFLP	intron5	G189	GCA GGG TTT CAT CCT CGG	18mer 58	AS	PP
Taq I	intron 7	G190	CTC ACA CTG CTA AGG ACC C	19mer 60	S	PP
RFLP	intron 7	G191	CTC CTG ACC TTG GGT GAT C	19mer 60	AS	PP
Hha I	intron 9	G192	ACA ATA TTT CGA TCC TTA AAG CCC	24mer 66	S	PP
RFLP	intron 9	G193	GTC CCC GAG GGA GAC CCC	18mer 64	AS	PP
(GT)n	exon 10	716	AAT GAG ACT TAC TGG GTG AGG	21mer 62	S	ML
micros.	exon 10	718	TTA CAC AGT AAT TTC AAG CAA CGG	24mer 66	AS	ML
Hinf I	exon 10	D157	GCC TGG AAG AGT TGG TCT CT	20mer 62	S	PP
RFLP	exon 10	D158	ACA CAG TAA TTT CAA GCA ACG G	22mer 62	AS	PP

other oligonucleotides:

for	location	name	sequence (5' to 3')	length, T_M	orient.	origin
	exon 1	H193	CTG TCC ACT TTT CCG GCC AG	20mer 64	S	KW
	exon 1/2	434	CAA TCA GGG TTA CAG CAC	18mer 54	S	ML
		F25	repeat of 434			
	exon 5/6	B700	GTA CCC TGT GCT GTG GTT GCT C	22mer 70	AS	ML
		E976	repeat of B700			
	exon 6	H195	CAT CCT CTG CGG AGC CCA	18mer 60	S	KW
	exon 7	792	GCT GTC CCA CTT ACA GAT GC	20mer 62	S	ML
	exon 8	B451	GTG TGA CTT CAA GGA CTG TG	20mer 60	S	ML

Oligonucleotides for other 11p sequences

for	location	name	sequence (5' to 3')	length, T_M	orient.	origin
VNTR	Ha-*ras*	G517	GAG CTA GCA GGG CAT GCC GC	20mer 68	S	PP
		G518	AGC ACG GTG TGG AAG GAG CC	20mer 66	AS	PP
Apa I RFLP	*IGF2*	G16	CTT GGA CTT TGA GTC AAA TTG G	21mer 62	S	PP
		G17	CCT CCT TTG GTC TTA CTG GG	20mer 62	AS	PP
Ava II RFLP	*IGF2*	G20	AGC ACA GCA GCA TCT TCA AAC	21mer 62	S	PP
		G21	TTG TTG CTA TTT TCG GAT GGC	21mer 60	AS	PP
D11S1312 micros.		E460	CTC CTG GAG GTA GGG AGT	18mer 58	S	WB
		E461	ACA ACA CCG TTT TTG CTA A	19mer 52	AS	WB
D11S995 micros.		E221	GAT TTT CCC ACA TCT ATA ATT ATA	24mer 60	S	PP
		E222	CTC TCA GAG AAA CTG AAC CAA	21mer 60	AS	PP
$(GT)_n$ micros.	*PAX6*	G276	ATG CCA CAT CTT CAG TAC	18mer 44	S	PP
		F825	TTG GAA TGG CAT TCA GTG AC	20mer 58	AS	AB

Oligonucleotides for *p16*

for	location	name	sequence (5' to 3')	length, T_M	orient.	origin
exon 1	exon 1	G599	GAA GAA AGA GGA GGG GCT G	19mer 60	S	PP
	exon 1	G600	GCG CTA CCT GAT TCC AAT TC	20mer 60	AS	PP
exon 2	intron 2	G386	GGA AAT TGG AAA CTG GAA GC	20mer 58	S	PP
	intron 2	G387	TCT GAG CTT TGG AAG CTC T	19mer 56	AS	PP
exon 3	exon 3	G857	CGC TTT CGT AGT TTT CAT TTA G	22mer 60	S	KW
	exon 3	G858	CCA TTT GCT AGC AGT GTG AC	20mer 60	AS	KW
	exon 1	G840	GCT GCC CAA CGC ACC GAA TAG	21mer 68	S	KW

Oligonucleotides for other 9p sequences

for	location	name	sequence (5' to 3')	length, T_M	orient.	origin
TYRP-1		TRRP1F	GGA TAG TGT GAA GAT CTT TGG CAT G	25mer 72	S	PP
		TYRP1R	ACA GTG GCA AAC ACA GGC AAT ATC C	25mer 74	AS	PP
IFN-A		IFNAF	GGA TTG AAA ACT GGT TCA ACA TGG C	25mer 72	S	PP
		IFNAR	TAC TAG TGC CTG CAC AGG TAT ACA C	25mer 74	AS	PP
1063.7		G591	CCG TTT CAG CTT CTC ATC AC	20mer 60	S	PP
		G592	CCG ACT GTC CCA TTG TGA TT	20mer 60	AS	PP

for	location	name	sequence (5' to 3')	length, T_M	orient.	origin
c18.b		G593	CAA AGA CTT TAT GGA TGG GG	20mer 58	S	PP
		G594	TCC ATT TCT CTG CTT GCT C	19mer 56	AS	PP
c5.1		G595	GAA GTC TTG GTC CTG ATG TC	20mer 60	S	PP
		G596	CTC TTC TGC ACA ACT CAA CT	20mer 58	AS	PP
c5.3		G601	GTG GTA GAA CTA GGA CAG GG	20mer 62	S	PP
		G602	CTG TGT TAA GCC TTC ATA GA	20mer 56	AS	PP
R2.3		G603	GAA AAT GAA ACT GTA CCC ATT G	22mer 60	S	PP
		G604	GGG ACA CAC ATT AAA TAC ACT	21mer 58	AS	PP
R2.7		G605	GAG AAC AGG TTT TGG GCA G	19mer 58	S	PP
		G606	AAC TAG ACC TAG GGA TAA GG	20mer 58	AS	PP
p15	intron 1	G607	TGA GTT TAA CCT GAA GGT GG	20mer 58	S	PP
	intron 2	G608	GGG TGG GAA ATT GGG TAA G	19mer 58	AS	PP
c1.b		G609	AAG CTT TCC CAC AAA CTG GC	20mer 60	S	PP
		G610	AAT GCC TTG GCA TAA GGG AC	20mer 60	AS	PP
D9S126		G729	ATT GAA ACT CTG CTG AAT TTT CTG	24mer 64	S	PP
		G730	CAA CTC CTC TTG GGA ACT GC	20mer 62	AS	PP
D9S171		G787	AGC TAA GTG AAC CTC ATC TCT GTC T	25mer 72	S	PP
		G788	ACC CTA GCA CTG ATG GTA TAG TCT	24mer 70	AS	PP
D9S169		G789	AGA GAC AGA TCC AGA TCC CA	20mer 60	S	PP
		G790	TAA CAA CTC ACT GAT TAT TTA AGG C	25mer 66	AS	PP

All T_M are in ^{o}C; orient. - orientation; S - sense; AS - antisense. Origin KW - self; ML - M. Little; WB - W. Bickmore; AB - A. Brown; PP - published paper, the appropriate paper for each oligonucleotide is detailed within the main body of text (except for B920, B918, B919 and B917, which are from Brown *et al.* 1992).

Part II - Annealing temperatures, PCR programme profiles and lengths of specific PCR products

Oligonucleotides for *WT1*

Oligonucleotide pair	T_A	PCR programme profile	specific PCR product length
C145/F518 C146/F519	60	standard + formamide	482
F516 F517	69	standard + formamide	249
C147 C148	60	standard + formamide	260
C149 C150	59	standard	268
C486 C152	57	standard	244
C153 C154	60	standard	176
C178 C177	58	standard	230
C155 945/E194	57	standard	350
C323 796/G667	58	standard	352
798/G26 801/G25	63	standard	349
C911 C912	57	standard	260
D609 D610	54	10 cycles 1' ext., 10 cycles 1' 30''ext., 10 cycles 2' ext.	686
B920 B918	54	10 cycles 1' ext., 10 cycles 1' 30''ext., 10 cycles 2' ext + DMSO	568/619
M3897 M3896	as B920 and B918		
C582 C583	62-57	touchdown and 10 cycles 1' ext., 10 cycles 1' 30''ext., 10 cycles 2' ext	702
B919 B917	51	10 cycles 1' ext., 10 cycles 1' 30''ext., 10 cycles 2' ext.	590/599
M3895 M3898	as B919 and B917		
G186 G187	62	standard	171
G188 G189	59	standard	405
G190 G191	60	standard	520
G192 G193	55	standard	340

Oligonucleotide pair	T_A	PCR programme profile	specific PCR product length
716 718	57	standard	138 - 146 (microsatellite)
D157 D158	58	standard	952

Oligonucleotides for other 11p sequences

Oligonucleotide pair	T_A	PCR programme profile	specific PCR product length
G517 G518	61	3 minute ext. + DMSO	860 - 2600 (VNTR)
G16 G17	55	standard	236
G20 G21	49	standard	485
E221 E222	65-50	touchdown	123 - 139 (microsatellite)
G276	47	standard and also	
F825	55-50	touchdown and also	
	47-42	touchdown	180 (no amplification)

Oligonucleotides for *p16*

Oligonucleotide pair	T_A	PCR programme profile	specific PCR product length
G599 G600	55	standard + DMSO	~350
G386 G387	51	standard + DMSO	509
G857 G858	55	standard	~330

Oligonucleotides for other 9p sequences

Oligonucleotide pair	T_A	PCR programme profile	specific PCR product length
TYRP1F			
TYRP1R	55-50	touchdown	439
IFNAF			
IFNAR	50	standard	178
G591			
G592	55-50	touchdown	~200
G593			
G594	55-50	touchdown	~180
G595			
G596	55-50	touchdown	~140
G601			
G602	55-50	touchdown	~160
G603			
G604	55-50	touchdown	~300
G605			
G606	55-50	touchdown	~160
G607			
G608	51	standard + DMSO	~540
G609			
G610	55-50	touchdown	~140
G729			
G730	55	standard	~250 (microsatellite)
G787			
G788	65	standard	~160 (microsatellite)
G789			
G790	55	standard	~180 (microsatellite)

Multiplex PCRs

Oligonucleotide pair	T_A	PCR programme profile	specific PCR product length
G386 - G387 with C323 - 796/G667	51	10 cycles 1' ext., 10 cycles 1' 30"ext., 10 cycles 2' ext + DMSO	509 with 352
G591 - G592 with C323 - 796/G667	55-50	touchdown	~200 with 352
G603 - G604 with C323 - 796/G667	55-50	touchdown	~300 with 352
G605 - G606 with 798/G26 - 801/G25	64-56	touchdown	~160 with 349
G609 - G610 with 798/G26 - 801/G25	68-60	touchdown	~140 with 349

All T_A are in ^{o}C; specific PCR product lengths are in bp; ext. - extension; ' - minute; ''- second.

Appendix C
Published papers

Fantes, J.A., Oghene, K., Boyle, S., Danes, S., Fletcher, J.M., Bruford, E.A., Williamson, K., Seawright, A., Schedl, A., Hanson, I., Zehetner, G., Bhogal, R., Lehrach, H., Gregory, S., Williams, J., Little, P.F.R., Sellar, G.C., Hoovers, J., Mannens, M., Weissenbach, J., Junien, C., van Heyningen, V., and Bickmore, W.A. (1995). A High-Resolution Integrated Physical, Cytogenetic, and Genetic Map of Human Chromosome 11: Distal p13 to Proximal p15.1. *Genomics* **25**: 447-461.

Langerak, A.W., Williamson, K.A., Miyagawa, K., Hagemeijer, A., Versnel, M.A., and Hastie, N.D. (1995). Expression of the Wilms' Tumor Gene *WT1* in Human Malignant Mesothelioma Cell Lines and Relationship to Platelet-Derived Growth Factor A and Insulin-Like Growth Factor 2 Expression. *Genes, Chromosomes & Cancer* **12**: 87-96.

Little, M.H., Williamson, K.A., Mannens, M., Kelsey, A., Gosden, C., Hastie, N.D., and van Heyningen, V. (1993). Evidence that WT1 mutations in Denys-Drash syndrome patients may act in a dominant-negative fashion. *Human Molec. Genet.* **2**(3): 259-264.

Webb, N.J.A., Lewis, M.A., Williamson, K., van Heyningen, V., Bruce, J., Lendon, M., and Postlethwaite, R.J. (1995). Do children with diffuse mesangial sclerosis in association with mutations of the Wilms' tumour suppressor gene (WT1) require bilateral nephrectomy? *Pediatr. Nephrol.* **9**: 252-253.

Williamson, K.A., and van Heyningen, V. (1994). Towards an understanding of Wilms' tumour. *Int. J. Exp. Pathol.* **75**: 147-155.

GENOMICS 25, 447–461 (1995)

A High-Resolution Integrated Physical, Cytogenetic, and Genetic Map of Human Chromosome 11: Distal p13 to Proximal p15.1

JUDY A. FANTES, KATHY OGHENE, SHELAGH BOYLE, SARAH DANES, JUDY M. FLETCHER, ELSPETH A. BRUFORD, KATHY WILLIAMSON, ANNE SEAWRIGHT, ANDREAS SCHEDL, ISABEL HANSON, GÜNTHER ZEHETNER,* RANJIT BHOGAL,* HANS LEHRACH,* SIMON GREGORY,† JON WILLIAMS,† PETER F. R. LITTLE,† GRANT C. SELLAR,‡ JAN HOOVERS,§ MARCEL MANNENS,§ JEAN WEISSENBACH,¶ CLAUDINE JUNIEN,‖ VERONICA VAN HEYNINGEN, AND WENDY A. BICKMORE[1]

*MRC Human Genetics Unit, Western General Hospital, Edinburgh EH4 2XU, Scotland; *Genome Analysis Laboratory, ICRF 44 Lincoln's Inn Fields, London, WC2A 3PX, England; †Wolfson Laboratories, Department of Biochemistry, Imperial College of Science and Technology, London, SW7 2AY, England; ‡Department of Genetics and Biotechnology Institute, Lincoln Place Gate, Trinity College, Dublin 2, Ireland; §Institute of Human Genetics, University of Amsterdam, Amsterdam Medical Centre, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands;* ¶Généthon, 1 Rue de l'Internationale, 91002 Evry, France; and ‖*INSERM Unite 383, Hôpital Necker-Enfants Malades, 149–161 Rue de Sevres, 75743 Paris, France*

Received June 28, 1994; revised September 27, 1994

We describe a detailed physical map of human chromosome 11, extending from the distal part of p13 through the entirety of p14 to proximal p15.1. The primary level of mapping is based on chromosome breakpoints that divide the region into 20 intervals. At higher resolution YACs cover approximately 12 Mb of the region, and in many places overlapping cosmids are ordered in contiguous arrays. The map incorporates 18 known genes, including precise localization of the GTF2H1 gene encoding the 62-kDa subunit of TFIIH. We have also localized four expressed sequences of unknown function. The physical map incorporates genetic markers that allow relationships between physical and genetic distance to be examined, and similarly includes markers from a radiation hybrid map of 11. The cytogenetic location of cosmids has been examined on high-resolution banded chromosomes by fluorescence *in situ* hybridization, and FLpter values have been determined. The map therefore fully integrates physical, genic, genetic, and cytogenetic information and should provide a robust framework for the rapid and accurate assignment of new markers at a high level of resolution in this region of 11p.

INTRODUCTION

The short arm of human chromosome 11 is well studied and mapped, due to its involvement in the Wilms tumor, aniridia, genitourinary anomalies, mental retardation (WAGR) syndrome and Beckwith–Weidemann syndrome (BWS). Several physical maps of 11p13 and 11p15 have been published (Compton *et al.*, 1988; Gessler and Bruns, 1989; Rose *et al.*, 1990; Redeker *et al.*, 1994). There are also good genetic maps and a radiation hybrid map of chromosome 11 (Gyapay *et al.*, 1994; Litt *et al.*, 1993; James *et al.*, 1994). A problem in comparing information from these maps has been that they use different sets of DNA markers and landmarks in their construction and the information is expressed in different formats (for example, in kb, cM, or cR). This means that it is difficult to relate information from one source with that from another and to integrate a marker mapped in one particular way, such as by genetic mapping, into a map derived in a different form, such as an FLpter map (Evans, 1993).

We have tackled this problem by constructing a detailed and integrated map of the distal region of 11p13, extending in a telomeric direction to reach the proximal part of 11p15.1. This region encompasses the G band 11p14, an example of a cytogenetic region that is not often well studied and whose DNA content remains somewhat of a mystery. The map is integrated at the physical, genetic, and cytogenetic levels. To do this we have used, as our primary mapping landmarks, a set of lymphoblastoid cell lines carrying mainly WAGR-derived chromosome breakpoints. These breakpoints divide the region into 20 intervals, and in several cases the intervals can be subdivided by further breakpoints. The breakpoints chosen to define interval boundaries are those where the aberrant chromosome 11 has been segregated from the normal homologue in somatic cell hybrids so that mapping can be by both PCR and FISH. Within this framework we have ordered anonymous DNA markers, cosmids, YACs, microsatellite markers,

[1] To whom correspondence should be addressed at MRC Human Genetics Unit, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, Scotland. Telephone: (44) 31-3322471. Fax: (44) 31-3432620.

0888-7543/95 $6.00

genes, and other expressed sequences, generated both in our own laboratory and in the laboratories of many other groups (Compton *et al.*, 1988; Gessler and Bruns, 1989; Tanigami *et al.*, 1992; Litt *et al.*, 1993; Smith *et al.*, 1993; Gyapay *et al.*, 1994; James *et al.*, 1994). Mapping has been primarily by PCR on hybrid DNAs and YACs and by fluorescence *in situ* hybridization (FISH) to metaphase chromosomes from breakpoint cell lines. Markers in the same interval were ordered either by physical mapping on cloned and genomic DNAs or by interphase FISH. Genetic markers were mapped by PCR on hybrid DNAs and YACS and the PCR primers were also used to derive cosmids by hybridization, allowing them to be mapped by FISH. In some instances, cosmids were derived from YACs, both by library construction from YAC DNA and by hybridization of isolated YAC DNA to cosmid libraries. Arrays of overlapping cosmids were identified by fingerprinting (Heding *et al.*, 1992). The FLpter value for a representative cosmid from each interval, when available, was also ascertained (Lichter *et al.*, 1990).

The map is estimated to extend over 15 Mb of genomic DNA and includes 237 cosmids, 220 of which are arrayed in 31 contigs, each of which contains up to 32 members. Approximately 12 Mb of the region is cloned in YACs. The map covers 13% of the physical metaphase length of chromosome 11 and contains 18 known genes, 4 expressed sequence-tagged sites (ESTs), and at least 15 CpG islands. These are unevenly distributed across the region in a manner consistent with other studies that have indicated that genes cluster in the R band regions of the genome (Gessler and Bruns, 1989; Craig and Bickmore, 1994). There are >50 STSs in the map including 16 microsatellite markers covering at least 15 cM of genetic distance (Couillin *et al.*, 1994). The map also covers 375 cR (one-third of the entire p arm) of the radiation hybrid map of chromosome 11, established by James *et al.* (1994) using an X-ray dose of 9000 rads. Finally, the map spans a region where the conserved region of synteny with the mouse genome changes from chromosome 2 to chromosome 7.

The many layers of this map are internally consistent and therefore rapidly highlight any discrepancies and errors in the placement of new markers. The integration of genetic and physical data has pinpointed the physical location of genes that might play a role in Type I Usher syndrome and familial hyperinsulinism (Keats *et al.*, 1994; Glaser *et al.*, 1994).

MATERIALS AND METHODS

Cell lines and hybrids. Cell lines, and the corresponding hybrids where available, the phenotype and karyotype of the individuals from which they were established, and the estimated physical extent of deletions are all summarized in Table 1.

Pulsed-field gel electrophoresis. High-molecular-weight mammalian DNAs were prepared, digested, and resolved, as previously described (Bickmore *et al.*, 1993). YAC plugs were processed as described by Fantes *et al.* (1995).

PCR. Most primers and cycling conditions have been described elsewhere (Miwa *et al.*, 1993; Smith *et al.*, 1993; Gyapay *et al.*, 1994; James *et al.*, 1994; Sellar *et al.*, 1994a) or are available in GDB. In addition, primers C809 (5′-GCAAGCAGTATTCAATTTCTG-3′) and C810 (5′-CTCGCACAGTACAATCAGTGC-3′) were from introns 1 and 2, respectively, of the FSHB gene (Watkins *et al.*, 1987) and give a 300-bp product. B960 (5′-CTATCTTCCCCTTTTAATGGTC-3′) and C908 (5′-AAGGACGCAGACTTGTACACG-3′) were designed from BDNF sequence published by Maisonpierre *et al.* (1991) and generate a 500-bp product. These reactions were carried out in the presence of 1 m*M* Mg^{2+} and cycled at 94°C, 1 min, 55°C, 1 min, and 72°C, 1 min for 30 cycles. Last, G78 (5′-GTGGCCATGATGCTTACAGG) and G77 (5′-AGTCTCCAATGTGCAAGTAC) were designed from the 3′ untranslated region of the GTF2H1 gene (GenBank Accession No. M95809). These primers amplify a 165-bp human product in the presence of 2.5 m*M* Mg^{2+}.

Microsatellite analysis of WAGR deletions. The extent of chromosome 11 deletions was examined by PCR with microsatellite markers on DNAs from the deleted individual and their unaffected parents. Primers were fluoresceinated and the PCR products analyzed on an ALF automated sequencer (Pharmacia) with a 6% hydrolink (Long Ranger, AT Biochem), 7 *M* urea gel made up and run in 0.6% TBE. Alternatively, primers were labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase and the products examined on a manual sequencing gel. Deletions manifested as regions of apparent homozygosity/hemizygosity (Bruford *et al.*, manuscript in preparation).

Cosmid and YAC isolation and overlap analysis. Random-prime labeled plasmid-derived probes were hybridized to gridded chromosome 11 cosmid library 107 supplied by the ICRF RLDB (Zehetner and Lehrach, 1994). The names of these cosmids have the prefix cICRFc107-, but this is usually omitted, especially in figures. Oligonucleotides were end-labeled and hybridized to filters at 50°C. YAC-derived probes were obtained by PCR amplification of catch-linked gel-purified YAC DNA, in the presence of [α-^{32}P]dCTP. Probes containing repeated DNA sequences were preannealed in the presence of 2.5 μg human CotI DNA (Gibco) before adding to the hybridization mix.

To generate cosmid libraries from YAC DNA itself, spheroplasts were lysed in NDS (10 m*M* Tris–HCl, 0.5 *M* EDTA, 1% lauryl sarcosine, pH 9.5) and incubated at 55°C for 4 h with 0.5 mg/ml proteinase K. After extraction and ethanol precipitation, the DNA was resuspended in TE, RNased, reextracted, and dialyzed at 4°C against TE. It was then digested partially with serial dilutions of *Sau*3AI. DNA cut to the optimal size range was dephosphorylated and ligated to Lawrist 4 cosmid arms, at the *Bam*HI site. Cosmids containing human DNA were selected by hybridization to total human DNA. All cosmids were subject to fingerprint analysis to identify overlapping clones (Heding *et al.*, 1992).

YACs were obtained from CEPH or were isolated by screening the ICI (Anand *et al.*, 1990) and ICRF YAC libraries 900 and 901 by PCR analysis or hybridization to gridded filters. All YAC clones are denoted by the prefix y.

FISH. Cosmid DNAs were nick-translated with biotin-16– or digoxigenin-11–dUTP (Langer-Safer *et al.*, 1982). Isolated YAC DNAs were labeled with biotin after isolation from pulsed-field gels and ligation to catch-link oligonucleotides (Fantes *et al.*, 1995). Single-color FISH of biotin-labeled probes to metaphase chromosomes from 11p breakpoint cell lines was as described in Fantes *et al.* (1992).

FLpter values (Lichter *et al.*, 1990) were obtained for a representative cosmid in each map interval, where available. Digital images were obtained with the MRC BioRad confocal microscope from at least 20 chromosomes with a length between 8 and 12 μm. Fractional length measurements were made with BioRad software, and mean FLpter values and 95% confidence intervals were calculated (Table 2).

Cosmids were localized on prometaphase chromosomes produced by early S phase synchronization with methotrexate (10^{-7} *M*) of PHA-stimulated peripheral blood cultures followed by release with 5×10^{-3} *M* bromodeoxyuridine (BrdU). Hybridization signals were detected by successive layers of avidin–Texas red, biotinylated anti-avidin, and avidin–Texas red. A final incubation with FITC-conjugated anti-BrdU antibody gave a replication G-banding pattern (Craig and Bickmore, 1994). The Texas red cosmid signal and the FITC banding pattern were visualized simultaneously through a

Chroma Pinkel No. 1 filter set fitted to a Zeiss Axioplan microscope, with a Photometrics CCD camera and Digital Scientific software.

The order of cosmids within a map interval was determined by multicolor FISH to interphase nuclei from G1 serum-depleted fibroblasts (Trask *et al.*, 1991). Two cosmids, labeled with either biotin or digoxigenin, were hybridized simultaneously with a reference cosmid, labeled with both biotin and digoxigenin. Wherever possible the analysis was repeated using reference cosmids from adjacent map intervals both telomeric and centromeric of the region under study. Hybridization signals were detected with successive layers of FITC-conjugated sheep anti-digoxigenin, FITC-conjugated anti-sheep antibody plus avidin–Texas red, biotinylated anti-avidin, and avidin–Texas red. The Pinkel No. 1 filter set was used to visualize the three cosmids simultaneously as red, green, and orange hybridization signals, respectively, and their order was determined in ~50 nuclei. The cosmids used were chosen to be equidistant from each other and neither too far apart nor too close to each other. Only signals that were lying approximately in a straight line to each other were scored.

YACs were labeled with biotin and used as FISH probes as described by Fantes *et al.* (1995).

RESULTS

The region under study is divided up into 20 intervals defined by chromosome breakpoints. The most centromere-proximal interval is interval I, and the most telomeric is interval XX. The detailed mapping of each interval is described in detail below.

Interval I

This interval is defined at the centromeric end by the T-ALL translocation (Boehm *et al.*, 1991) and at the telomeric end by the deletion breakpoint of ANNA and is approximately 300 kb in size. The T-ALL breakpoint is 5′ of the RBTNL1 gene, transcribed from the most centromere-proximal CpG island of Fig. 1. Distal to this is a 550-kb *Not*I fragment that is bissected by the ANNA breakpoint (Bickmore *et al.*, 1989). Located on this fragment, in interval I, are the D11S48 and D11S102 loci (contained on the same 120-kb *Bss*HII fragment), the D11S184 and D11S1301 loci, and the gene(s) encoding the CD59/MIC11 antigens (Bickmore *et al.*, 1993). Although the relative order of CD59/MIC11, D11S48, D11S102, and D11S1301 has not been established here, James *et al.* (1994) have placed D11S1301 distal of RBTNL1 and CD59. c65-6/6 (D11S102) has an FLpter value of 0.256 (Table 2).

Interval II

The boundaries of this interval, of approximately 400 kb in size, are the deletion breakpoint of ANNA and the POR translocation (Fig. 1). D11S376 is on the 550-kb *Not*I fragment but distal of the ANNA breakpoint (van Heyningen and Jones, 1993). PCR analysis also places the microsatellite D11S1776 (James *et al.*, 1994) and D11S672 (Miwa *et al.*, 1993) in this interval. FISH analysis with D11S672 (FLpter value of 0.251) shows that this locus is proximal of the submicroscopic deletion in the WAGR patient NAHAS. Similarly, in ALSTA, a submicroscopic deletion associated with Wilms tumor and aniridia alone (Table 1), there is FISH signal on both chromosomes 11, but one of the signals is consistently weaker than the other, suggesting that D11S672 spans the ALSTA proximal breakpoint. Hence, the ALSTA deletion extends more proximally than that of NAHAS (Fig. 1). Both ALSTA and NAHAS are heterozygous, and hence not deleted, for D11S1776. We do not know whether the POR breakpoint is within the small *Not*I fragment immediately distal to the 550-kb fragment or on the next one, since the breakpoint is a minimum of 350 kb and a maximum of 600 kb proximal to D11S294 (Bickmore *et al.*, 1989) (Fig. 1).

Interval III

This small (200–300 kb) interval is defined by the POR translocation and the TRAKE deletion breakpoints (Fig. 1). D11S307 and D11S311 loci (van Heyningen and Jones, 1993) are both located on a 300-kb *Not*I fragment (Gessler and Bruns, 1989), but only D11S307 is present on y23IC9 (see Interval IV). The distal end of this *Not*I fragment has been cloned (LFC5), but we do not know whether it is deleted in TRAKE (Gessler *et al.*, 1990). The EST D11S1938E (Adams *et al.*, 1993; James *et al.*, 1994) maps to this interval by PCR analysis on hybrid DNAs and, like D11S311, appears to be proximal to y23IC9.

Interval IV

This is a 350-kb interval defined by the proximal deletion breakpoints of TRAKE and PAZO, at the centromeric end of which is a 160-kb *Not*I fragment harboring D11S299 (Gessler and Bruns, 1989; van Heyningen and Jones, 1993). Distal to this a 500-kb *Not*I fragment is disrupted by the PAZO breakpoint (van Heyningen *et al.*, 1990). The centromeric end of this fragment has been isolated as a *Not*I end clone (D11S2133), which is probably equivalent to LFC1 (Gessler *et al.*, 1990). D11S294 and D11S303 map to the same 90-kb *Bss*HII fragment as D11S2133 (Bickmore *et al.*, 1989; Gessler and Bruns, 1989), and the distal end of this *Bss*HII fragment (D11S305) has also been cloned (Gessler *et al.*, 1990). D11S417 and D11S321 are <200 kb distal to these probes (Compton *et al.*, 1990; Gessler and Bruns, 1989). Cosmid ICRFc-107G1135 at the D11S417 locus has an FLpter value of 0.239 (Table 2) and is present on both chromosomes 11 in PAZO but absent from the previously uncharacterized deletion chromosomes of PKPA, NAHAS, and ALSTA (Table 1, Fig. 1). PKPA has a proximal deletion end point centromeric of D11S102 (interval I). The most distal interval IV marker is HD42, a *Bss*HII jumping clone 250 kb centromeric of WT1 (Royer Pokora *et al.*, 1991), equivalent to LFT1 (Gessler *et al.*, 1990).

Primers for D11S2133 were used to isolate y23IC9 (375 kb), which also contains D11S294 and D11S307. This YAC therefore spans the proximal TRAKE breakpoint (Fig. 1). D11S311 did not hybridize to y23IC9, and restriction mapping suggests that y23IC9 ends within the 300-kb genomic *Not*I fragment containing D11S307 and D11S311 (Fig. 1). Neither D11S-1776 nor HD42 are contained on y23IC9. FISH con-

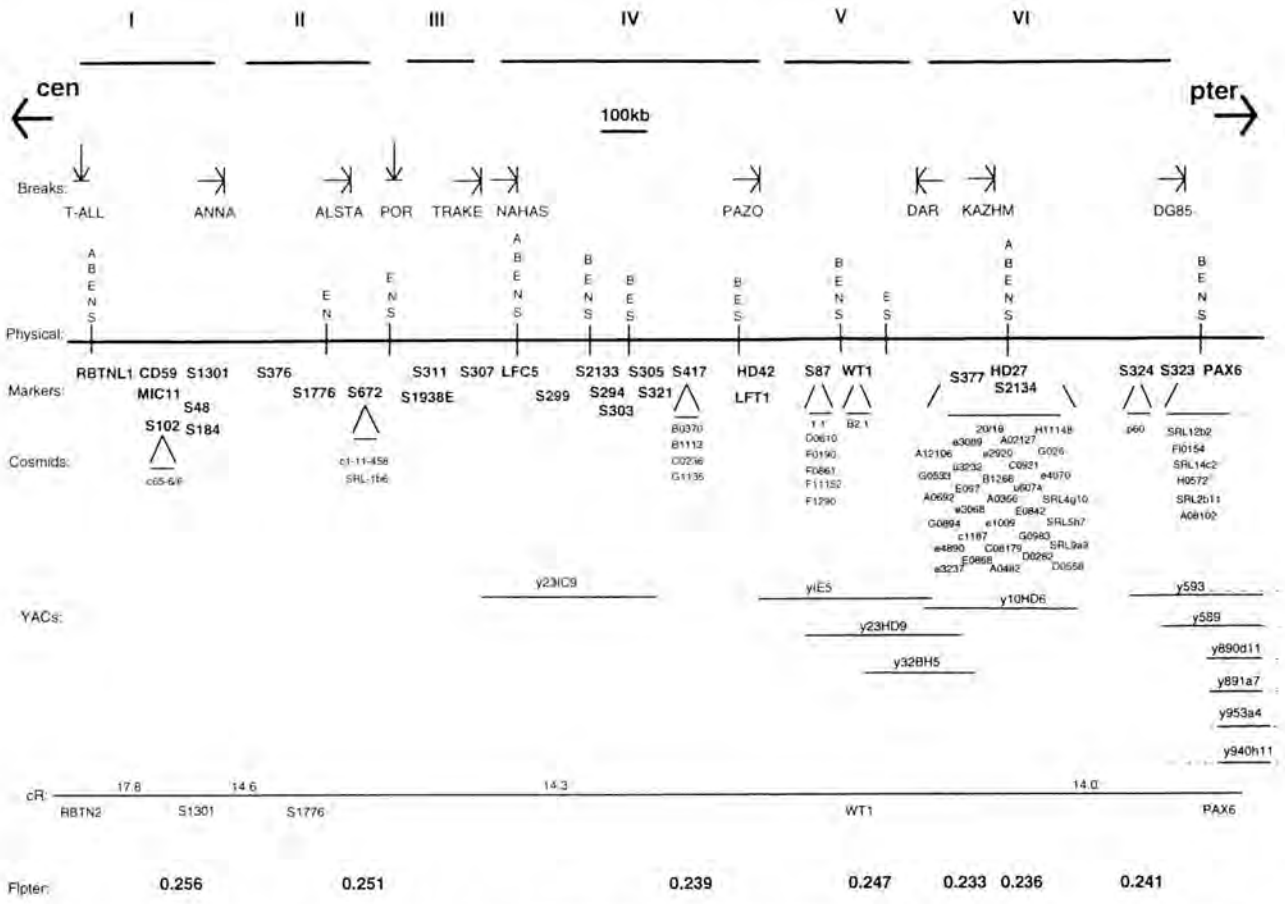


FIG. 1. Integrated map of the distal region of 11p13 (intervals I to VI). The positions of chromosome breakpoints that define map intervals are shown relative to the physical map of the chromosome determined by genomic and YAC PFGE analysis (scale bar = 100 kb). The orientation of the map relative to the centromere is shown. Vertical arrows indicate translocation breakpoints. Horizontal arrows pointing right or left mark the proximal and distal extent of deletions, respectively. The positions of known genes and markers are shown, as are ordered cosmids at these loci. The prefix is omitted from the names of cosmids from the ICRF RLDB due to space constraints. The extent of YAC clones covering the region is shown. Markers in common with the map described by James *et al.* (1994) are shown together with their separation on the radiation hybrid map (cR). Finally, the FLpter values for representative cosmids of each interval are indicated (Table 2). Some restriction enzyme sites on the genomic map, principally those indicating the presence of CpG islands, are included: A, *Asc*I; B, *Bss*HII; E, *Eag*I; N, *Not*I; S, *Sac*II.

firmed that this YAC is nonchimeric. Some signal was present on the NAHAS deletion chromosome, but there was no detectable hybridization to the TRAKE or ALSTA deletion chromosomes (Figs. 2a and 2b).

Interval V

The most proximal marker in this 450-kb interval, bounded by the PAZO and DAR deletion breakpoints, is D11S87 (Lewis *et al.*, 1988). This is 87 kb from the WT1 gene, which lies on a 375-kb *Not*I fragment and is represented by cB2.1 (Fantes *et al.*, 1992). The FLpter value of cB2.1 (0.247) suggests a more centromeric location than D11S417 (interval IV) and serves to illustrate the unreliability of determining order by FISH on metaphase chromosomes for markers so close together (~250 kb apart).

WT1-specific primers were used to isolate YACs y1IE5 (450 kb), y23HD9 (275 kb), and y32BH5 (250 kb). y1IE5 is nonchimeric by FISH and contains D11S87 and HD42. Hence, it must cross the PAZO breakpoint, although no FISH signal was detectable on these chromosomes. Restriction mapping of the WT1 YACs indicates that they all include the CpG island 100 kb distal of the 5′ end of WT1 (Fig. 1).

Interval VI

This interval is 400 kb in size and bounded by the distal DAR breakpoint and the proximal breakpoint in the deletional translocation of DG85 (Compton *et al.*, 1988). D11S377 is on the same 375-kb *Not*I fragment as WT1, and the distal end of this fragment is present in clone HD27 (Royer Pokora *et al.*, 1991). None of the WT1 YACs contain D11S377, although FISH signal from yIE5 is visible in DAR. The next most distal *Not*I fragment (325 kb) contains D11S324 and D11S323 (Compton *et al.*, 1988). The interval is subdivided by the proximal deletion breakpoint of KAZHM, which must be contained within the set of overlapping cosmids covering the D11S377 and D11S2134 loci (Fig. 1), since FISH shows that c20/18/D11S377 (FLpter =

TABLE 1

Derivation of Cell Lines

Parental cell line	Hybrid	Karyotype (11)	Estimated size of deletion (Mb)	Phenotype	Reference
ALSTA		del p13 (s)	2.5	A, W	
ANNA	ANX6.14	del p12 p14.1	4.0	A	Seawright *et al.* (1988)
BAN	BAN68	del p12p14	~15	A, G, R, W	Couillin *et al.* (1989)
BID	BID7	del p12p14.3		A, G + cardiomyopathy	Couillin *et al.* (1989)
GM4613	POR4/POR11	t(2;11)(p11;p13)		G, P	Seawright *et al.* (1988)
DAR	DAR15.14	del p12p13	~10	G, W	Couillin *et al.* (1989)
DG85	DGR7/DG R30	t(11;22)(p13;q12.2)del p13 (s)		A	Compton *et al.* (1988)
GOTY	GOX 1.5	del p12 p14	~12	A, R, W	Seawright *et al.* (1988)
KAZHM		del p13p14.1 (s)	2.3	A	
LEV	LEV14.13.6/LEV 22.1	t ins (14;11)(q13;p12p14)		Retinopathy	Couillin *et al.* (1989)
MARGA	MAX 13	del p13	7	A, W	Seawright *et al.* (1988)
NAHAS		del p13 (s)	1.8	A, G, R, W	
NYMI	NYX 3.1	del p12p15.1	>20	A, R	Seawright *et al.* (1988)
PAZO	PAX 1	del p13 (s)	1.1	A, G	van Heyningen *et al.* (1990)
PKPA		del p13p14.1		Pt	Hanson *et al.* (1994a)
RIWAR	RIX 1	del p12p14.3	~17	A, R	Seawright *et al.* (1988)
SATO	SAX 3.10	del p12p13	7	A, R, W	Seawright *et al.* (1988)
SGL		inv p13 (s)		A	Fukushima *et al.* (1993)
SIMO	SMXB1/SMX B2	t(4;11)(q22;p13)		A	Seawright *et al.* (1988)
SOA	SOA54	del p13p14	12	A, R, W	Couillin *et al.* (1989)
T-ALL	8511-36/8511-7	t(11;14)(p13;q11)		T-ALL	Boehm *et al.* (1991)
TRAKE	TRX	del p13p14.2	8	A, G, R	van Heyningen and Jones (1993)

Note. The table shows the relationship between cell lines and corresponding somatic cell hybrids where the abnormal chromosome 11 has been segregated from the normal homologue. The karyotype of the chromosome 11 abnormality, defined in molecular terms, is indicated together with an estimate of the physical size of deletions. (s) indicates submicroscopic deletion, defined solely by molecular analysis. The phenotype of the individual from which the line was derived is described. A, aniridia. G, genitourinary anomalies such as cryptorchidism, hypospadias. P, Potters facies, urethral and ureteral atresia, and cryptorchidism. Pt, Peter's anomaly. R, Mental retardation. W, Wilms tumor.

0.233) is on both KAZHM chromosomes 11, whereas ICRFc107H11148/D11S2134 (FLpter = 0.236), 55 kb more distal, is deleted. y10HD6 (230 kb) hybridizes to D11S377 and HD27 but not to probes for WT1 or D11S324. The 32 cosmids shown in Fig. 1, which constitute the largest cosmid contig on our map, were isolated with y10HD6 and probes therein.

Interval VII

This 100-kb interval, defined by the DG85 and SATO breakpoints (Fig. 3), has been intensively studied since it is the location of the aniridia gene PAX6 (Ton *et al.*, 1991) contained on cFAT5 (FLpter = 0.244) and cFAT10 (Fantes *et al.*, 1992). Cosmids ICRFc107G-11117, A1280, and A11151 hybridize to the 3′ untranslated region of PAX6. y589 (310 kb), y591 (260 kb), and y593 (420 kb) were isolated with PAX6-specific primers (Fantes *et al.*, 1995). y593 and y589 extend both centromeric and telomeric of PAX6 and extend past D11S323 and D11S324. Restriction analysis and FISH suggest that D11S324 is more proximal than D11S323. y591 does not contain the full 5′ end of PAX6 and extends 3′ of the gene. A cosmid library made from this YAC and screening of a chromosome 11 library with y591 produced a series of overlapping cosmids covering over 150 kb of DNA from intervals VI, VII, and VIII (Fig. 3). ICRFc107C1170 (D11S2135) and 591/21 show FISH signal only on a proportion of SATO deletion chromosomes and at reduced intensity, suggesting that these cosmids cross the distal SATO breakpoint.

Interval VIII

This interval lies between the SATO deletion breakpoint and the translocation breakpoint in SIMO (Table 1). This interval also contains the distal inversion breakpoint of SGL. All of the PAX6-derived YACs cross the SATO breakpoint, and y591 and y593, but not y589, cross over the SIMO translocation. This is thought to be 100–200 kb from the *Not*I site at the proximal end of the 1400-kb fragment harboring PAX6 and is marked by the D11S310 locus (Gessler *et al.*, 1989a; Fantes *et al.*, 1995). FISH showed that the most distal members of the overlapping cosmids extending from PAX6 to ICRFc107F02121 (D11S2136/FLpter = 0.241) and c591/8 are still proximal to the SIMO breakpoint.

Interval IX

This interval (of approximately 200 kb) is bounded by the SIMO translocation and by the distal deletion breakpoints of PAZO and NAHAS. Some of the cosmids isolated with y591 are in this interval (Fig. 3). One of them, c591/1 (FLpter = 0.242), distal to the endpoints of y589 and y593, was used to identify a polymorphic

TABLE 2

FLpter Measurements in 11p13 to 11p15.1

Interval	Cosmid	Locus	Cytogenetic location (11)	FLpter Mean	95% confidence limits
I	c65-6/6	D11S102		0.256	0.247–0.264
II	N/A				
III	c1-11-458	D11S672	p13	0.251	0.241–0.260
IV	clCRFc107G1135	D11S417	p13	0.239	0.230–0.248
V	cB2.1	WT1	p13	0.247	0.243–0.251
VI	c20/18	D11S377		0.233	0.226–0.239
	clCRFc107H11148	D11S2134	p13	0.236	0.228–0.244
	cp60	D11S324		0.241	0.233–0.248
VII	FAT5	PAX6	p13	0.244	0.237–0.250
VIII	clCRFc107F02121	D11S2136	p13	0.241	0.232–0.250
IX	591/1	D11S995	p13	0.242	0.235–0.249
X	clCRFc107E08153	D11S112	p13/p14	0.236	0.231–0.242
XI	ZF17	D11S781	p13/p14	0.232	0.225–0.239
XII	clCRFc107E0499	FSHB	p13/p14	0.219	0.214–0.225
	clCRFc107C08160	D11S151	p14.1	0.223	0.219–0.228
	clCRFc107G09161	KCNA4	p14.1	0.222	0.210–0.234
XIII	clCRFc107D1029	BDNF	p14.1	0.199	0.189–0.209
XIV	N/A				
XV	c1-11-585	D11S739		0.187	0.176–0.198
	clCRFc107F1238	HVBS1	p14.2	0.194	0.185–0.203
	clCRFc107G0122	D11S904		0.193	0.185–0.200
XVI	N/A				
XVII	c1-11-536	D11S713		0.178	0.171–0.185
XVIII	N/A				
XIX	cSRL-7d7	D11S865	p14.3	0.172	0.167–0.177
	clCRFc107C0720	D11S1359		0.158	0.152–0.165
	clCRFc107BO798	D11S2137	p15.1	0.152	0.145–0.158
	clCRFc107E0873	D11S1308		0.135	0.129–0.140
	c1-11-514	D11S592	p15.1	0.151	0.146–0.156
	c1-11-443	D11S664		0.149	0.144–0.154
	clCRFc107H0229	D11S21	p15.1	0.147	0.142–0.152
XX	c1-11-242	D11S455		0.135	0.130–0.140
	c1-11-564	D11S729		0.125	0.119–0.130
	cHSAA	SAA	p15.1	0.141	0.133–0.149
	clCRFc107E048	TPH		0.125	0.119–0.132

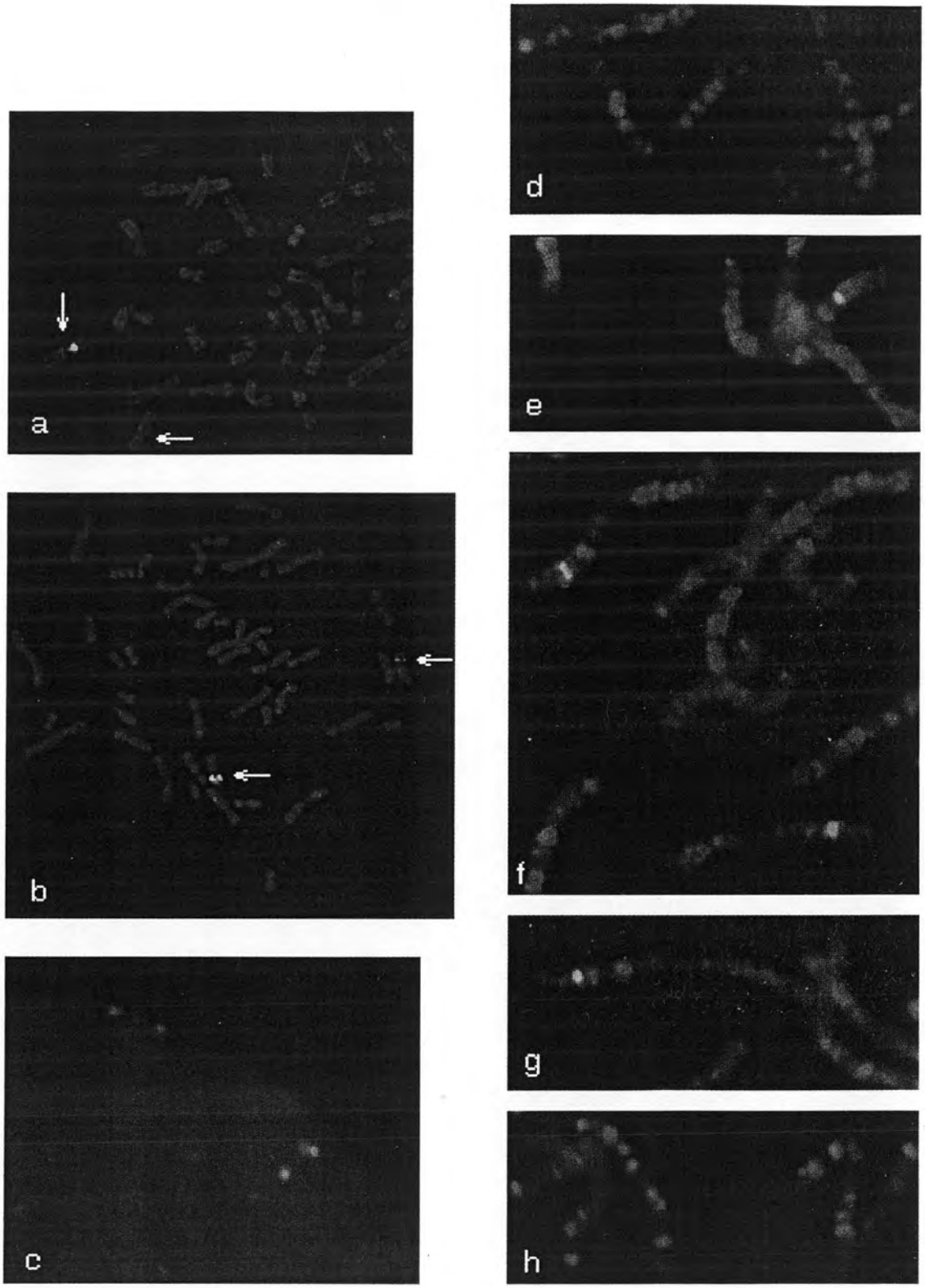
Note. For each interval of the map, the mean FLpter measurement and 95% confidence limits (Lichter *et al.*, 1990) of at least one representative cosmid are shown. N/A, none available.

CA_n repeat D11S995 that maps 3 cM proximal to D11S929 (interval XV), but 2 cM distal of D11S914, a marker that physically is in interval X (Browne *et al.*, 1993). Hence there is a discrepancy between the physical and the genetic mapping data. The position of D11S995 on the radiation hybrid map (James *et al.*, 1994) confirms its physical location proximal of D11S914.

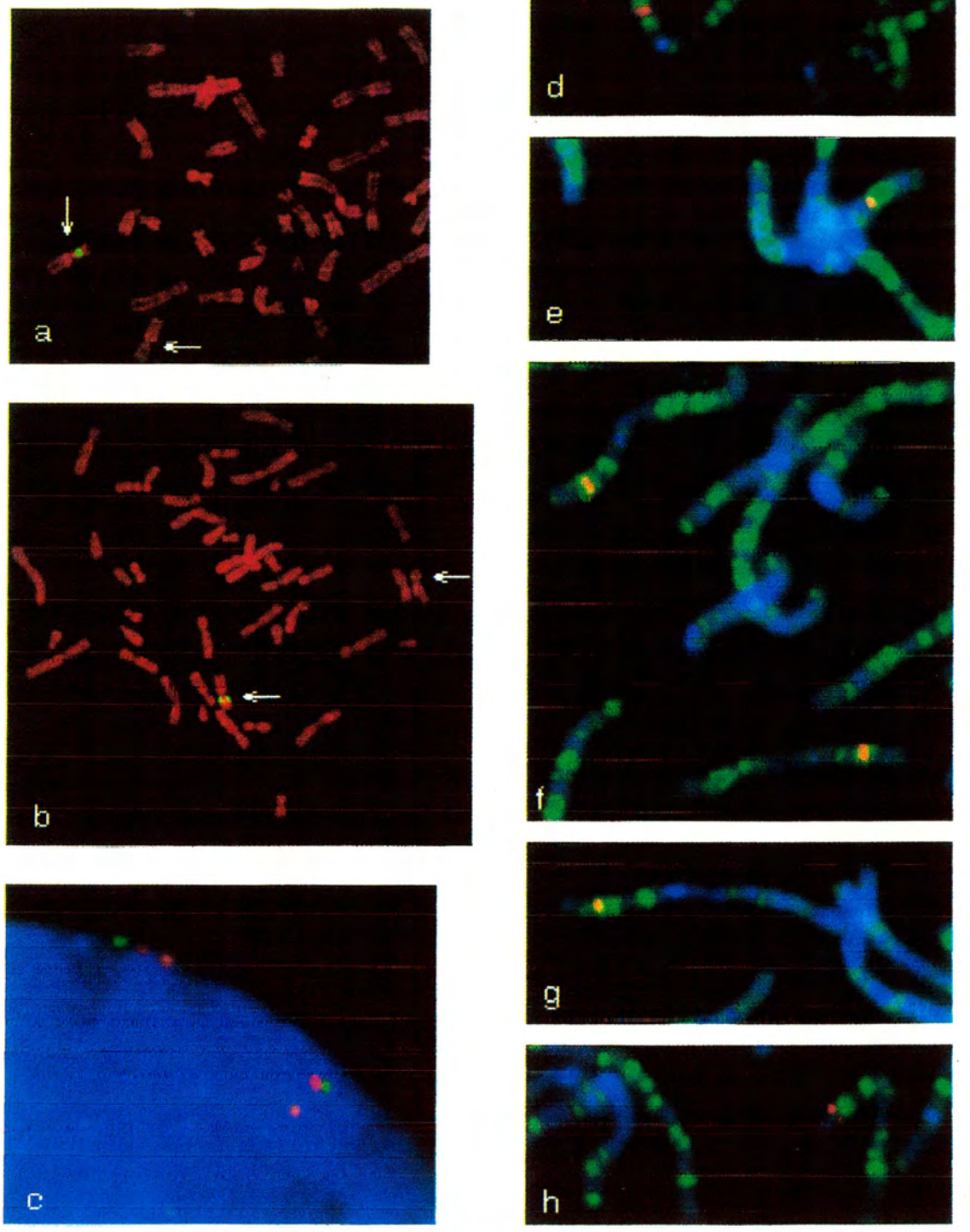
Interval X

This interval is defined by the distal deletion breakpoints of PAZO/NAHAS and DG85/ALSTA, an

FIG. 2. FISH analyses. Biotinylated y23Ic9 DNA, detected with avidin–FITC (green) on propidium iodide-stained chromosomes (red) from ALSTA (**a**) and NAHAS (**b**). Chromosomes 11 are indicated by the arrows. Note the complete absence of FISH signal on one of the ALSTA homologues and the reduced FISH signal detectable on the deletion chromosome from NAHAS. (**c**) Interphase FISH with interval X cosmids cICRFc107A04160/D11S317 (green) and cICRFc107E08153/D11S112 (red), together with the more distal interval XI ZF17/D11S781 (orange). G1 nuclei were counterstained with DAPI. Seventy-eight percent of the nuclei produce the preferred order cen–D11S317–D11S112–D11S781. In 12% of cases the order of D11S317 and D11S112 was inverted, and D11S781 and D11S112 were inverted in 9% of nuclei. (**d**) FISH with biotinylated (red) cICRFc107E08153 (D11S112) from interval X on long DAPI-stained metaphase chromosomes with a late replication (G) banding pattern detected with anti-BrdU–FITC (green). The signal lies at the distal boundary of 11p13, with no visible overlap into 11p14.1, which would appear as yellow signal. (**e**) FISH with cICRFc107D1029 (BDNF) from interval XIII. Detection was as in (**d**), but the FISH signal appears orange/yellow as it overlaps the late-replicating (green) band 11p14.1. (**f**) FISH with cICRFc107F1238 (HVBS1) from interval XV. Two chromosome 11 homologues are shown, and in each case signal is in the middle of 11p14 in the small subband 11p14.2. (**g**) FISH with cSRL7d7 (D11S865) from interval XIX. Signal (orange/yellow) is in distal 11p14 in subband p14.3. (**h**) FISH with ICRFc107B0798 (D11S2137) from interval XIX, but distal of D11S865 (Fig. 5). Signal is in 11p15.1 between the late-replicating p14.3 and p15.2 subbands. Note that the red color of the signal indicates that this cosmid is in an early-replicating compartment.



estimated distance of 500 kb. Genetically, the order of microsatellite markers in this interval is cen–D11S914–1 cM–D11S1322/D11S1312 (Gyapay *et al.*, 1994). The radiation hybrid map suggests the order cen–D11S1322–D11S914, with D11S1322 close to D11S995 (James *et al.*, 1994). In addition, this interval contains D11S317, linked to D11S914 by overlapping cosmids (Fig. 3) and D11S112. Multicolor interphase FISH, with reference cosmids both centromeric and telomeric of this group, confirms the physical order cen–FAT5–D11S317–D11S112–ZF17–tel (Fig. 2c). Analysis of these markers in YACs that encompass the region (Fig. 3) confirms this order. yICRFy900FO9162 (850 kb),


a
b
c
d
e
f
g
h

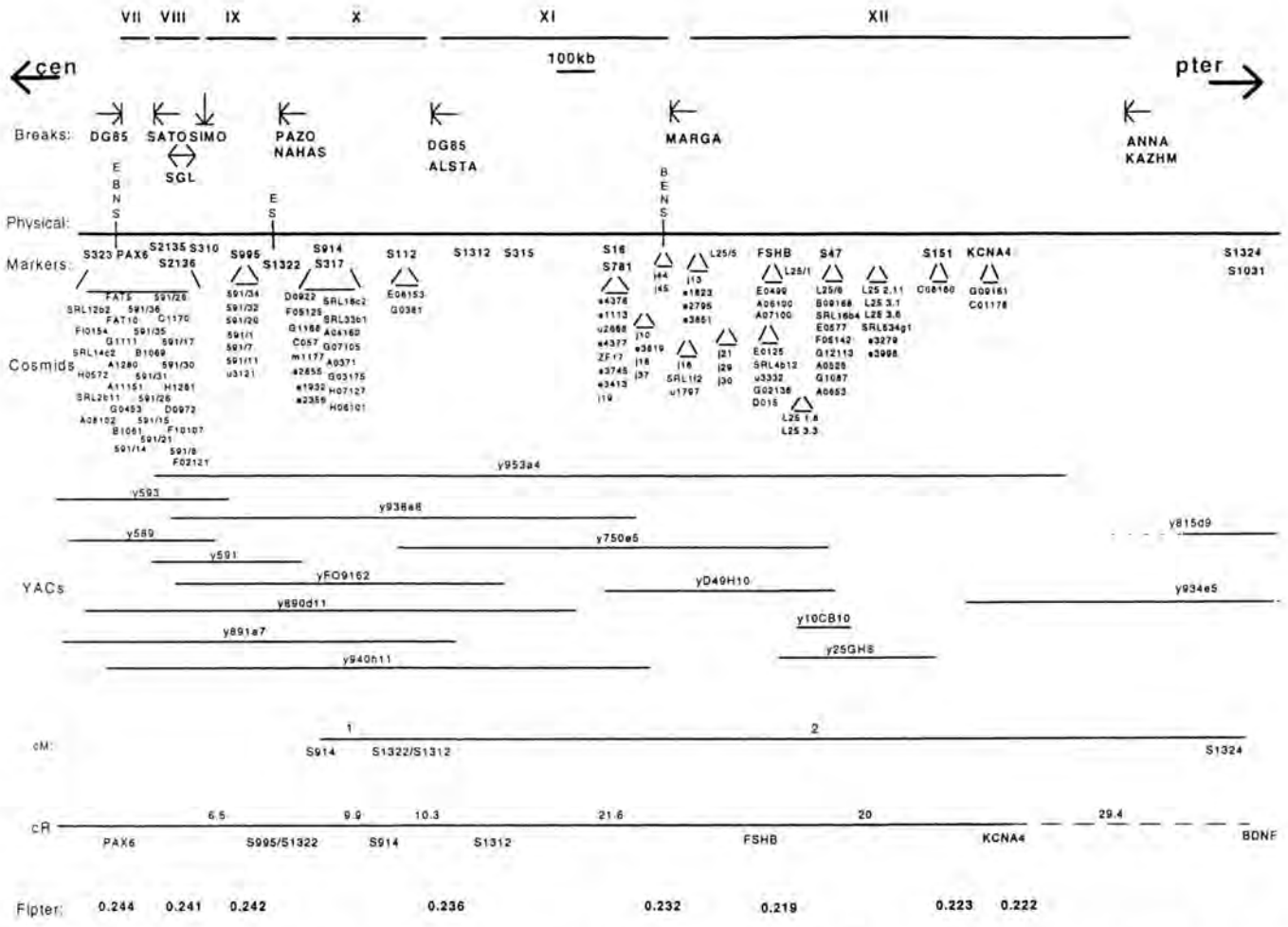


FIG. 3. Integrated map of 11p13–p14 boundary (intervals VII to XII). As in Fig. 1. The position of chromosome breakpoints, cosmids, and YACs are shown relative to the physical map of the chromosome (scale bar = 100 kb). The double-headed arrow of SGL denotes an inversion breakpoint. In addition to the radiation hybrid distances (cR), the relationship of the physical map to genetic distances (cM) is also shown. Note the inversion of D11S914 and D11S1322 in the genetic map vs the physical and radiation hybrid maps.

isolated with D11S112, is nonchimeric and contains all of the interval X markers. The proximal end of this YAC extends approximately 250 kb into the 400-kb *Sac*II fragment containing PAX6, since it hybridizes to cFO2121, but it does not extend as far as the PAX6 gene (Fig. 3). Distally it includes D11S1312 from interval XI, and FISH suggests that a small part of yFO9162 crosses the ALSTA breakpoint, implying that this breakpoint is approximately half way through the 1000-kb *Sac*II fragment (Fig. 3). Two apparently nonchimeric YACs isolated with D11S1322 were analyzed in detail. y890d11 (1000 kb) contains PAX6 and D11S1312. y891a7 (1500 kb) also includes the PAX6 gene; however, its maximal distal extent is D11S914. Of the D11S914-containing YACs, y938a8 (1400 kb) extends from cF02121 (interval VIII) to D11S16 in interval XI, but not as far as FSHB. y940h11 (1600 kb) has a similar distal extent but also includes PAX6. y953a4 (>1600 kb) extends from PAX6 up to interval XII to include the KCNA4 gene (Gessler *et al.*, 1992) (Fig. 3). FISH on high-resolution banded chromosomes places D11S112 at the distal extent of, but still within, 11p13, although on some chromosomes hybridization was to the p13/p14 boundary (Fig. 2d).

Interval XI

The DG85/ALSTA and MARGA breakpoints flank this interval (approximately 500 kb in size). At the proximal end it contains the microsatellite D11S1312. More distally it contains the contiguous D11S16 and D11S781 (FLpter = 0.232) loci (Heding *et al.*, 1992) that are present on YAC yD49H10 (650 kb). D11S315 (Gessler and Bruns, 1989) is located between the DG85 breakpoint and D11S16. yD49H10 contains the genomic CpG island immediately distal of D11S16 and extends 420 kb past this to include the FSHB gene, but not the more telomeric locus D11S47. These latter markers are on a 3100-kb genomic *Not*I fragment whose large size and apparent dearth of CpG islands is indicative of G band DNA (Gessler and Bruns, 1989; Craig and Bickmore, 1994). This, together with the cytogenetic location of D11S112 (Fig. 2d), suggests that

the interval X–XI region spans the transition from 11p13 to p14.1.

Interval XII

This interval is flanked by the distal deletion breakpoints of MARGA and ANNA/KAZHM. Toward its proximal end is the FSHB gene (FLpter = 0.219), ~400 kb distal of the telomeric *Not*I site of Fig. 3. y10CB10 (100 kb) and y25GH6 (300 kb) contain both FSHB and D11S47. y25GH6 was shown to be nonchimeric by FISH. D11S151 maps distal to D11S47 and outside the extent of y25GH6. The potassium channel gene KCNA4 has also been mapped 200–600 kb distal of FSHB (Gessler *et al.*, 1992). Cosmids were isolated from the FSHB and KCNA4 genes, D11S47, D11S151, and y25GH6 (Fig. 3). D11S151 and KCNA4 cosmids have FLpter values of 0.223 and 0.222, respectively (Table 2), and appear to map to 11p14.1. Interphase FISH gives the order cen–D11S151–KCNA4–tel and suggests that D11S151 and KCNA4 are closely apposed. Although FISH with the KCNA4 cosmid cICRFc107GO9161 shows signal in the appropriate place on the 11p, there is also signal on 11q. While Gessler *et al.* (1992) place KCNA4 in 11p14, Philipson *et al.* (1993) have mapped it to 11q13–q14, and Wymore *et al.* (1994) also detect some FISH signal in this location. These results suggest that there may be two related copies of this gene, one on each arm of chromosome 11. FSHB, D11S47, D11S151, and KCNA4, but not markers from interval XIII, are all contained on the large YAC y953a4 (Fig. 3).

Interval XIII

This interval is bounded by the ANNA/KAZHM and GOTY deletion breakpoints and is further subdivided by the PKPA deletion and the LEV insertional translocation. The only microsatellite in this interval is D11S1324 (Couillin *et al.*, 1994). James *et al.* (1994) place D11S1324 proximal to KCNA4, but our map indicates that it is more distal. D11S1324 containing YACs y815d9 (1000 kb) and y934e5 (1100 kb) both include D11S1031 (Smith *et al.*, 1993), but the relative order of these loci is not known. y934e5 (1100 kb) also contains KCNA4, and since KCNA4 is maximally 1000 kb distal of the *Not*I site centromeric of FSHB (Gessler and Bruns, 1989; Gessler *et al.*, 1992), D11S1324 and D11S1031 must also be on that 3100-kb *Not*I fragment. y934e5 is nonchimeric and shows some FISH signal on KAZHM and ANNA deletion chromosomes as expected from the markers that it contains. No signal could be seen on the PKPA deletion. In LEV, all of the signal was on the part of 11 translocated to chromosome 14 (Fig. 4).

The only interval XIII marker on the telomeric side of PKPA/LEV is the BDNF gene (Hanson *et al.*, 1992). A cosmid containing BDNF (cICRFc107D1029) has a mean FLpter value of 0.199 (Table 2) and an 11p14.1 location on high-resolution banded chromosomes (Fig. 2e). y886h12 (800 kb) and y739e5 (1300 kb) contain BDNF as well as markers from more distal intervals. Neither of these YACs contains the more proximal interval XIII markers; thus, there is a gap in our physical map at this point. y886h12 is nonchimeric by FISH and apparently absent from the GOTY deletion chromosome, even though HSPCAL3 is present on the YAC. Also, despite the fact that y886h12 contains the BDNF gene itself, only a small amount of FISH signal was apparent on the LEV deletion chromosome, most of the signal being at the insertion point on chromosome 14.

Interval XIV

The boundaries of this interval are the breakpoints of GOTY and SOA, and the only marker within it is HSPCAL3 (an HSP90α gene). The location of HSPCAL3 here is consistent with that described by James *et al.* (1994), distal of BDNF. If there really is a functional HSP90α gene here (Ozawa *et al.*, 1992), its location in the mouse genome will be of interest since, at present, BDNF is the most distal human gene in this region that is in a conserved synteny group with mouse chromosome 2 (Hanson *et al.*, 1992). The next known human genes telomeric of BDNF are LDHA/C, in 11p15.1 (interval XX) and in a conserved synteny group with mouse chromosome 7 (Sellar *et al.*, 1994b). HSPCAL3 is present on y886h12 (800 kb) and y739e5 (1300 kb) and hence within 800 kb of BDNF.

Interval XV

This interval is defined by the SOA and TRAKE breakpoints. y739e5, y691b6, y694h10, y822h6, and y959a6 all contain both of the microsatellites (D11S904 and D11S1750) found in this interval. But only D11S1750 is on y938h8 (1100 kb), a YAC that extends into interval XVI, suggesting that D11S1750 is distal of D11S904 (Fig. 4). HVBS1 (van Heyningen and Jones, 1993) is also in this interval, and FISH with an HVBS1 cosmid (FLpter value of 0.194) places it in the middle of 11p14, in subband p14.2 (Fig. 2f). D11S904 cosmids were isolated (FLpter = 0.193) and shown to be distal, but close to, HVBS1 by interphase FISH. This is consistent with the order described by Glaser *et al.* (1994), which places HVBS1 and D11S904 below and above, respectively, the breakpoint in the WAGR deletion MJ. FISH analysis puts D11S739 (Tanagami *et al.*, 1992) between HVBS1 and BDNF but distal of the GOTY deletion. However, the FLpter value of 0.187 suggests a localization distal of HVBS1 (there is also a minor signal component from this cosmid at a more centromeric location).

Interval XVI

This interval is defined by the distal deletion breakpoints in TRAKE and J1-23/BAN68. Its physical size must be <2400 kb since HVBS1 and D11S17, from the flanking intervals, have been physically mapped to a common genomic *Bss*HII fragment of this size and a 3500-kb *Mlu*I fragment (Gessler and Bruns, 1989). The

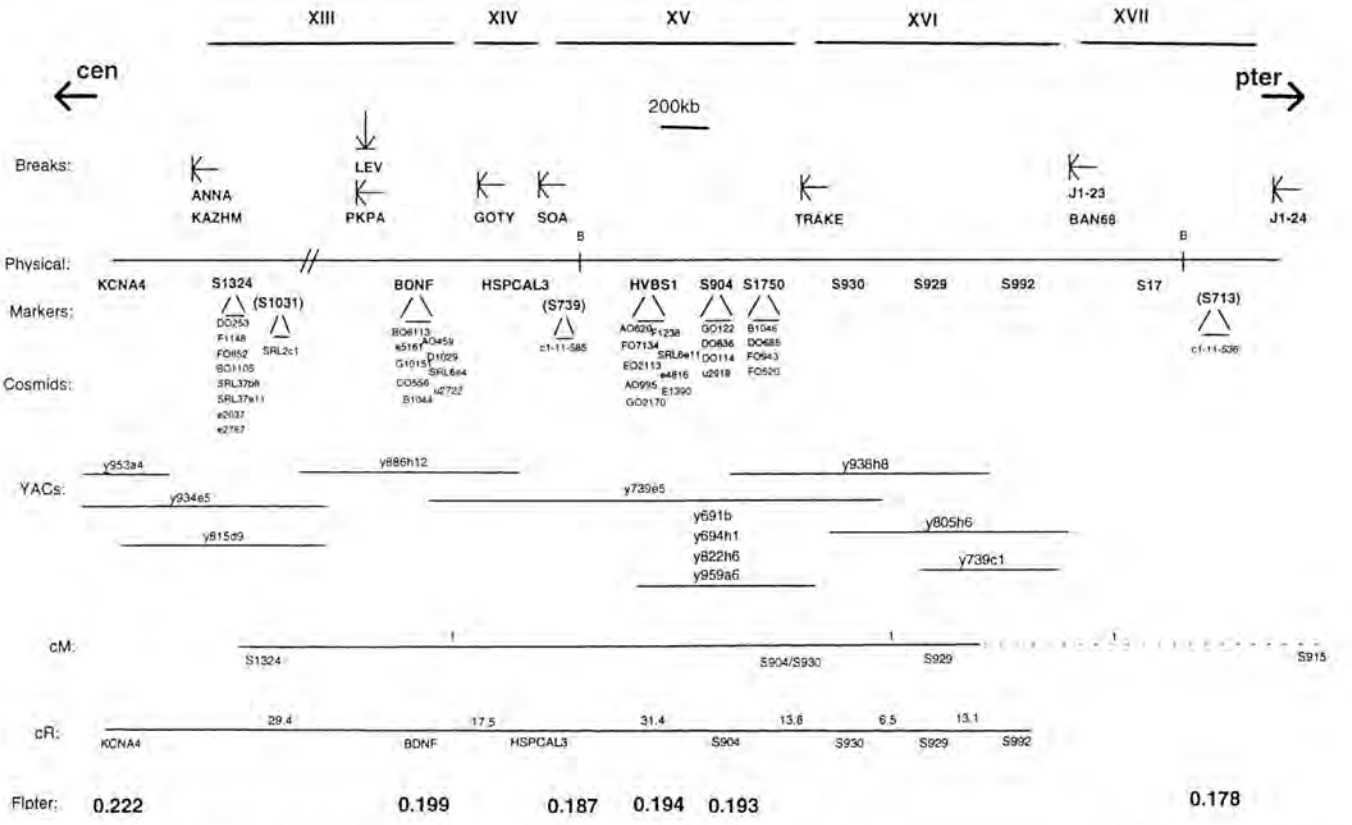


FIG. 4. Integrated map of 11p14.1–p14.2 (intervals XIII to XVII). As in Figs. 1 and 2, comparisons among the cytogenetic, physical, genetic, and radiation hybrid maps are shown. Note that here the scale bar = 200 kb. The slash lines on the physical map indicate region of unknown size that are not bridged either by the genomic PFGE map or by YACs or cosmids. In this region of the genome the physical map is not well established, and the only restriction sites indicated are the *Bss*HII sites (B) detected by Gessler and Bruns (1989).

interval contains the microsatellites D11S930 and D11S929. D11S930 is the more proximal of the two genetically (Gyapay *et al.*, 1994) and physically since it is present on y739e5 (Fig. 4). D11S992 is distal of these two microsatellites since it is absent from y938h8.

Interval XVII

This interval is bounded by the distal breakpoints of J1-23/BAN68 and J1-24 and corresponds to interval H of Tanagami *et al.* (1992). It contains only two markers, D11S17 and D11S713. D11S17 is in the distal 300 kb of the 2400-kb *Bss*HII fragment described by Gessler and Bruns (1989) that also carries HVBS1. The FLpter value of c1-11-536 (D11S713) is 0.178, close to the value for D11S865, from interval XIX, but its position on the genomic long-range restriction map is not known.

Interval XVIII

This interval between the J1-24 and RIWAR/BID7 breakpoints contains only one marker, the microsatellite D11S915. While y773d6 (850 kb) and y859a2 link D11S915 with more distal markers, there are no YACs that bridge the gap between D11S992 and D11S915 (Figs. 4 and 5).

Interval XIX

Bounded on the proximal side by the RIWAR/BID7 breaks and on the distal side by the NYMI breakpoint, this appears to be a very large interval physically since the spread of FLpter values for markers mapping here is 0.172–0.147, i.e., 2.5% of the total chromosome length, and the genetic distance encompassed is 4 cM. The most proximal group of markers in the interval is D11S865, D11S1359, and D11S1106, since they are retained on the D11S915-containing YACs y773d6 and y859a2 (Fig. 5). D11S865 (FLpter value of 0.172) is cytogenetically in 11p14.3 (Fig. 2g) and telomeric of, but close to, D11S1359 by interphase FISH. This group of markers is probably tightly linked since they cosegregate in four other YACs—y950c6, y964f4, y922b3, and y956c8 (1000 kb). These last two clones also contain the more distal marker D11S947E. If this EST represents a functional gene it will be the most centromere-proximal known gene in the 11p14.3–p15.1 region, and it will be interesting to establish whether it is in a conserved synteny group with mouse chromosome 7 (Sellar *et al.*, 1994b).

The next microsatellite in this interval is D11S928, 2 cM distal of D11S1359. There are no YACs that bridge the gap between D11S947E and D11S928 (Fig. 5), suggesting that there is quite a large gap in the physical map here. D11S928 is physically linked to the

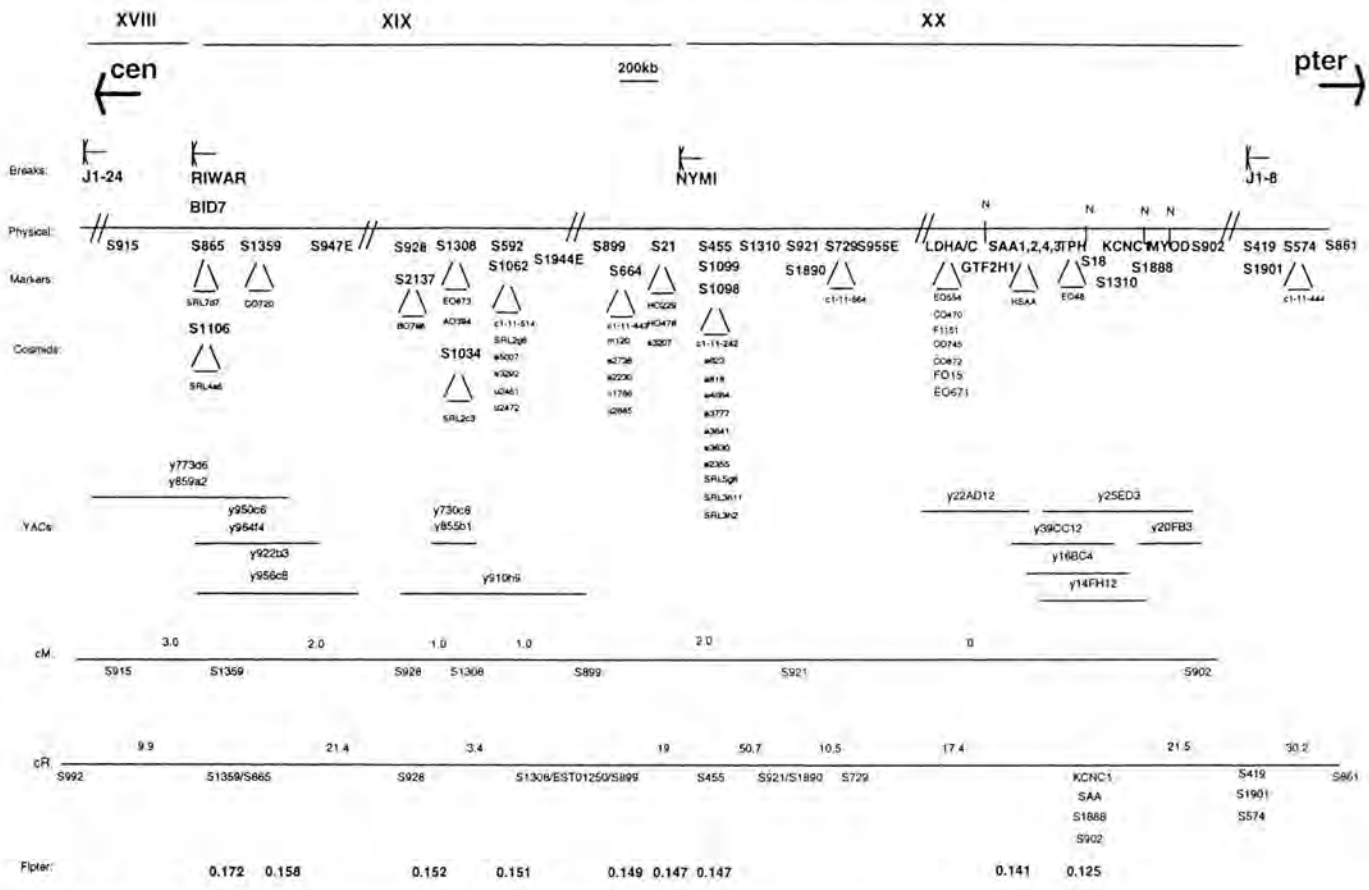


FIG. 5. Map of 11p14.3–p15.1 (intervals XVIII–XX). As in Fig. 4, only limited restriction mapping data are available in this region of the genome, and physical scale is determined mainly by the sizes of YAC clones.

next most distal genetic marker (D11S1308) by y910h9. Two other YACs (y730c8 and y855b1) closely link D11S1308 with D11S1034. y910h9 also contains the D11S592/D11S1062 loci and the EST D11S1944E. Two cosmids from this region produce FLpter values of 0.152 (ICRFc107BO798/D11S2137) and 0.151 (c1-11-514/D11S592). D11S2137 is centromeric of D11S1308 by FISH and has an apparent 11p15.1 localization on banded chromosomes (Fig. 2h). Hence the transition from 11p14.3 to p15.1 lies in the gap of interval XIX between D11S865 and D11S2137. D11S592 is telomeric of D11S2137 by interphase FISH.

In radiation hybrids (James *et al.*, 1994) D11S1944E cosegregates with D11S1308 and the more distal D11S899. However, none of the YACs analyzed here included D11S899. Other interval XIX markers not present on the YACs described here are D11S664 and D11S21. D11S21 maps toward the distal end of the interval by metaphase FISH (FLpter = 0.147) and interphase FISH versus D11S865, D11S2137, and D11S592. D11S664 produces an FLpter value of 0.149, suggesting a location just centromeric of D11S21. D11S1075, D11S1019, and D11S1043 also map within this interval by PCR analysis but could not be ordered with respect to the other markers. It is possible that they map to the gap between D11S865 and D11S928/D11S2137 where there are no YACs and few cosmids with appropriate FLpter values (0.172–0.152). Additionally, the radiation hybrid map (James *et al.*, 1994) suggests that D11S1755 and D11S1759 map between D11S1359 and D11S928.

Interval XX

This interval is defined at its centromeric end by the NYMI deletion breakpoint and distally by J1-8. D11S455 maps proximal of the SAA cluster genetically (Sellar *et al.*, 1994a) and by radiation hybrid mapping (James *et al.*, 1994); however, it produces an FLpter value (0.135) that may indicate a more distal localization, since the FLpter value for a cosmid at the SAA locus is 0.141 (Sellar *et al.*, 1994a). However, within this interval FLpter values seemed to be a poor guide to physical order. On the radiation hybrid map markers located between D11S455 and the SAA genes include D11S1310, D11S921, D11S1890, and D11S729. Both D11S921 and D11S1890 lie outside the extent of the most centromeric YAC (y22AD12) at the LDH–MYOD1 cluster (Fig. 5). However, D11S1310 is present on y14FH12 and y25ED3, suggesting that it is close to D11S18. The orientation and order of genes and markers around the SAA cluster, cen–LDHA/C–SAA1, -2, -4, -3–TPH–D11S18–KCNC1–MYOD1–tel, ascertained by YAC analysis and interphase FISH (Sellar

et al., 1994b), is at odds with that established by James *et al.* (1994), who place MYOD1 proximal to both LDHA/C and SAA. An EST D11S955E is close to, but proximal of, the LDH genes since it is absent from y22AD12.

The gene GTF2H1, encoding the 62-kDa subunit of TFIIH, has been mapped to 11p14–p15.1 by FISH (Heng *et al.*, 1994). PCR analysis on hybrid DNAs and YACs places this gene in interval XX between the LDHA/C and the SAA genes, since it is retained only on YAC y22AD12 (Fig. 5). Furthermore, restriction mapping of y22AD12 places GTF2H1 within 50 kb of LDHA.

Distal but close to SAA3 is the TPH gene, separated from the KCNC1 and MYOD1 genes by D11S18. The close physical linkage of KCNC1 and MYOD1 is consistent with their tight genetic and physical linkage (Wymore *et al.*, 1994; Sellar *et al.*, 1994b) on mouse chromosome 7. D11S1888 maps close to MYOD1 on y25ED3 and y20FB3 and is absent from J1-8, as is the microsatellite D11S902, which James *et al.* (1994) map with KCNC1, SAA, and D11S1888. However, D11S902 is more distal since it is not contained on the YACs analyzed here. On the radiation hybrid map, the next group of markers is D11S419, D11S1901, and D11S574, but these are all distal of J1-8. Between NYMI and J1-8 there are two other markers, D11S1032 and D11S1059, that are not present on any of the YACs of this interval and so have not been precisely ordered.

Recently Keats *et al.* (1994) demonstrated tight linkage of Usher syndrome type I to the region of 11p15.1 between D11S899 and the microsatellite D11S861 (Litt *et al.*, 1993), that is just distal of D11S902. In addition, linkage disequilibrium was observed in the D11S921–D11S419 region. Hence, this well mapped and gene-rich region of 11p15.1 is also the location of the gene(s) for type I Usher syndrome. Some of the genes already mapped to this critical region (Fig. 5) might be considered candidate genes for this disorder. Familial hyperinsulinism similarly shows linkage to D11S921, with linkage disequilibrium to specific D11S921/D11S491 haplotypes in some families (Glaser *et al.*, 1994).

DISCUSSION

Complete maps of regions of the genome are important for a variety of studies. As well as being essential for localizing genes, particularly those associated with inherited disorders, they allow the functional and structural organization of the genome to be studied. This map of 11p13–p15.1 illustrates some of these features. First, it highlights the unequal distribution of genes between different chromosomal regions. In the most proximal 3.5 Mb of the map (intervals I through X) that defines the distal part of 11p13, there are 4 known genes, 2 of which are involved in the WAGR syndrome, 1 EST, and many as-yet uncharacterized CpG islands. There are also 4 known genes and 1 EST (D11S947E) but very few CpG islands in the region of at least 9 Mb that extends from intervals XI into XIX and covers 11p14. Of these, only the EST is in the part of p14 considered to be the most extreme G band-like DNA (Francke, 1994). Finally, in the proximal 3–4 Mb part of 11p15.1 that is included in this map (the distal part of interval XIX through interval XX), there are 10 known genes and 2 ESTs, and limited restriction mapping in the LDH–MYOD1 region indicates the reappearance of closely spaced CpG islands (Sellar *et al.*, 1994b). This region is probably the location of the gene(s) involved in type I Usher syndrome (Keats *et al.*, 1994) and familial hyperinsulinism (Glaser *et al.*, 1994). The uneven disposition of CpG islands and genes between R and G band regions is consistent with other studies and parallels changes in replication timing and chromosome structure (Craig and Bickmore, 1994). The nature of the DNA that constitutes the small R band 11p14.2, which includes the DNA around the HVBS1 locus (Fig. 2f), is not apparent.

Second, the map spans the region where there has been a break in the conserved synteny relationship with the mouse genome—from chromosome 2 to 7. The most distally located human gene mapped here and known to be on mouse chromosome 2 is BDNF. The most centromere proximal genes on the map in a conserved synteny group with mouse chromosome 7 are LDHA/C. The intervening region corresponds to the most gene-barren region of our map. In this regard, the map location of the HSP90α gene and the genes corresponding to ESTs D11S947E, D11S1944E, and D11S955E in the mouse genome will be interesting to establish. From the map position of GTF2H1 (Fig. 5), we predict that this gene will be located on mouse chromosome 7.

The primary level of the map has utilized chromosome breakpoints, principally from individuals with full or partial WAGR syndrome. These breakpoints have been remarkably unambiguous at dividing up the region and provide absolute mapping landmarks since they have definitive ends (at the single basepair level), whereas most other map objects have length that may be imprecisely defined. We have found no evidence for complex rearrangements accompanying chromosome deletions. Because of the interest in the biology of the WT1 and PAX6 genes, there is a concentration of breakpoints flanking these genes. Aside from this, there is no evidence for clustering of breakpoints, which might indicate regions especially susceptible to breakage. Of the WAGR-associated breakpoints analyzed here and by Gessler *et al.* (1989b), none has been found with a more distal extent than that of NYMI, suggesting that haplosufficiency for a gene(s) distal of this point may not be viable. Table 2 indicates the estimated size of the deletions and the corresponding clinical phenotype of the individual from whom the cell line was derived. There is no simple relationship between the size of deletion and the degree of mental retardation reported. NAHAS, whose deletion ends just telomeric of the PAX6 gene, has quite severe mental retardation, while ALSTA, who has a deletion similar in extent, but larger, than that of NAHAS, has little or no retarda-

tion. In general, mental retardation is seen in individuals with deletions that extend more distally up the chromosome. There is either none or mild mental retardation reported in MARGA, ANNA, PKPA, and KAZHM. Telomeric of these breakpoints, GOTY, SOA, TRAKE, BAN, RIWAR, and NYMI all exhibit more severe retardation. Thus, a gene(s) predisposing to mental retardation may reside within, or telomeric to, interval XIII the location of the BDNF gene.

Our integration of microsatellite markers into the physical map has allowed us to analyze the relationship between physical and genetic distance. As well as mapping these STSs to intervals and YACs, we have used the primers to isolate cosmids by hybridization to gridded filters. In general, the cosmids isolated have mapped into the predicted physical position by FISH. However, in a few cases the cosmids mapped to a different location from the corresponding microsatellite; for example, cICRFc107B0798/D11S2137 (interval XIX) was isolated by hybridization to oligonucleotides used to amplify the D11S929 locus (interval XV). In these cases, the cosmid DNA failed to amplify with the appropriate PCR primers, and we assume that there has been fortuitous hybridization of one of the two oligonucleotides to a sequence contained in these cosmids. An added bonus of integrating the microsatellite markers into the physical map is that it provides a rapid method for analyzing the extent of new uncharacterized chromosome deletions. Markers contained within the deleted region will demark a region of apparent homozygosity (in fact hemizygosity) in the haplotype and pinpoint regions where FISH analysis will be most useful for refining the deletion endpoints.

There are a few discrepancies between the physical and the genetic maps. For example, on the genetic map D11S914 is reported as being 1 cM proximal of D11S1322 (Gyapay *et al.*, 1994). The physical map described here and the radiation map (James *et al.*, 1994) place D11S914 more distally between D11S1322 and D11S1312. There is also some variation in the relationship between genetic and physical distances across the map, from the 1 cM per Mb of DNA rule of thumb. For example, 1 cM separates D11S1324 from D11S930, markers that we estimate are >2 Mb apart (Fig. 4), and no recombination appears to separate D11S921 and D11S902, which physically (Fig. 5) seem far apart. By contrast, 3 cM separates D11S915 and D11S1359, markers that are both contained within a YAC (y773d6) of only 850 kb in size (Fig. 5), although we cannot exclude the possibility that there is an internal deletion within this YAC. Overall, the entire 11p14 region, as defined here by high-resolution FISH at metaphase (Fig. 2), spans 10 cM of recombination. These genetic distances represent the sex-averaged map. Throughout the region there appears to be more recombination on the female than on the male map. For example, between D11S929 and D11S861 there are 24.9 cM of female recombination but only 14.5 cM of male recombination (Litt *et al.*, 1993). The biological significance of these differences is not understood.

There is remarkably good agreement between the map described here and that based on radiation hybrid mapping (James *et al.*, 1994). There are a few exceptions; for example, the order of KCNA4 and D11S1324 reported by James *et al.* (1994) is inverted relative to that presented here. The more proximal location of KCNA4 is supported by both our placement of it in interval XII through breakpoint analysis and its inclusion within y953a4 (Fig. 3). D11S1324 is in the more distal interval XIII, but <1100 kb from KCNA4 (the size of y934e5). Similarly, we have mapped D11S947E distal of D11S865 and D11S1359 (Fig. 5). The largest discrepancy is in the placing of MYOD1, which James *et al.* (1994) map proximal of the LDH genes and D11S921. Both the mapping of MYOD1 on y20FB3 and y25ED3 and the order of genes on mouse chromosome 7 (Sellar *et al.*, 1994b) support a localization of MYOD1 telomeric to KCNC1 (Fig. 5). The distance separating markers on the radiation hybrid map of chromosome 11 (James *et al.*, 1994) is expressed as centiRays (cR) at an X-ray dose of 9000 rads. Over the extent of the map here, 1 cR corresponds to a mean separation of 41 kb. If this relationship is relatively constant over the entire chromosome arm, it would extrapolate to a length of 50 Mb for the short arm of chromosome 11.

Most of the YAC clones that we have examined by FISH, from the ICI, ICRF, and CEPH libraries, have been nonchimeric. However, this is not a fair representation of the proportion of chimeric clones in these libraries. We isolated and mapped markers in many more clones than is shown on this map. Any clones that contained only one marker from the map, or whose pattern of marker retention seemed aberrant, were eliminated from further study. Hence, the YACs finally analyzed on metaphase chromosomes were a selected group.

Finally, we have measured the FLpter values for cosmids from each interval where available (Table 2). The spread of these values (0.256–0.125) shows that our map covers 13% of the chromosome length. Cosmids located in 11p13 produce measurements down to values of 0.23. 11p14.1 ranges from 0.22 to 0.20, cosmids in the small subband 14.2 produce FLpter values of approximately 0.20–0.19, and 11p14.3 probably extends from values of 0.18 to 0.17. Cosmids located in 11p15.1 produce values of <0.155. There are two large gaps in the FLpter values. We have no cosmids that produce measurements between 0.187 and 0.178 (in 11p14.3) or between 0.158 and 0.152 (at the 11p14.3–p15.1 boundary). No YACs bridge these gaps either. In general, FLpter values are a guide to regional localization, but they are not dependable for ascertaining order of closely apposed markers. Interphase FISH with carefully chosen reference cosmids above and below the region of interest has been a reliable technique for this purpose.

We hope that this map, particularly the breakpoints and YACs, will continue to be used as a framework to map any new markers and genes in this part of 11p rapidly and with a high level of resolution and confi-

dence. The pinpointing of the position of the GTF2H1 gene and the region implicated in both type I Usher syndrome (Keats *et al.*, 1994) and familial hyperinsulinism (Glaser *et al.*, 1994) on this map illustrates this well. The cloned DNA resources are available to other groups who have an interest in this region of human chromosome 11.

ACKNOWLEDGMENTS

K.O. is supported by an HGMP grant no. G9029692. GCS is a Beit Memorial Research Fellow and is partly funded by a grant from the Wellcome Trust. V. v.H. is an International Scholar of the Howard Hughes Medical Institute. W.A.B. is a Lister Institute Research Fellow. This work was also aided by EC grant SCI0469. We are particularly indebted to Michael James (CEPH) for to access to information on the radiation hybrid map and Carol Jones (Denver) for providing J1 hybrids. We thank the HGMP resource centre and Denis Le Paslier of CEPH for access to YACs. In addition y886h12 was a gift from M-D Devignes (Paris) and A. Feinberg provided yD49H10. We thank the Japanese Cancer Resource Bank for gifts of cosmids. Brigitta Royer-Pokora (Heidelberg), Daniella Gerhard (St Louis) and Grady Saunders (Houston) provided other probes. Agnes Gallagher prepared the numerous oligonucleotides.

REFERENCES

Adams, M. D., Kerlavage, A. R., Fields, C., and Ventner, J. C. (1993). 3,400 new expressed sequence tags identify a diversity of transcripts in human brain. *Nature Genet.* **4:** 256–267.

Anand, R., Riley, J. H., Butler, R., Smith, J. C., and Markam, A. F. (1990). A 3.5 genome equivalent multi access YAC library: Construction, characterisation, screening and storage. *Nucleic Acids Res.* **18:** 1951–1956.

Bickmore, W. A., Porteous, D. J., Christie, S., Seawright, A., Fletcher, J. M., Maule, J. C., Couillin, P., Junien, C., Hastie, N. D., and van Heyningen, V. (1989). CpG islands surround a DNA segment located between translocation breakpoints associated with genitourinary dysplasia and aniridia. *Genomics* **5:** 685–693.

Bickmore, W. A., Longbottom, D., Oghene, K., Fletcher, J. M., and van Heyningen, V. (1993). Colocalisation of the human CD59 gene to 11p13 with the MIC11 cell surface antigen. *Genomics* **17:** 129–135.

Boehm, T., Forini, L., Kaneko, Y., Perutz, M. F., and Rabbits, T. H. (1991). The rhombotin family of cysteine-rich LIM-domain oncogenes: Distinct members are involved in T-cell translocations to human chromosomes 11p15 and 11p13. *Proc. Natl. Acad. Sci. USA* **88:** 4367–4371.

Browne, D. L., van Heyningen, V., Bickmore, W., and Litt, M. (1993). Dinucleotide repeat polymorphism at the D11S995 locus. *Hum. Mol. Genet.* **2:** 1332.

Buetow, K. H., Weber, J. L., Ludwigsen, S., Scherpbier-Heddema, T., Duyk, G. M., Sheffield, V. C., Wan, Z., and Murray, J. C. (1994). Integrated human genome-wide maps constructed using the CEPH reference panel. *Nature Genet.* **6:** 391–393.

Compton, D. A., Weil, M. M., Jones, C., Riccardi, V. M., Strong, L. C., and Saunders, G. F. (1988). Long range physical map of the Wilms' tumour–aniridia region on human chromosome 11. *Cell* **55:** 827–836.

Compton, D. A., Weil, M. M., Bonetta, L., Huang, A., Jones, C., Yeger, H., Williams, B. R. G., Strong, L. C., and Saunders, G. F. (1990). Definition of the limits of the Wilms' tumor locus on human chromosome 11. *Genomics* **6:** 309–315.

Couillin, P., Azoulay, M., Henry, I., Ravise, N., Grisard, M. C., Jeanpierre, C., Barichard, F., Metezeau, P., Candelier, J. J., Lewis, W., van Heyningen, V., and Junien, C. (1989). Characterization of a panel of somatic cell hybrids for subregional mapping along 11p and within band 11p13. *Hum. Genet.* **82:** 171–178.

Couillin, P., Le Guern, E., Vignal, A., Fizames, C., Ravise, N., Delportes, D., Reguigne, I., Rosier, M. F., Junien, C., van Heyningen, V., and Weissenbach, J. (1994). Assignment of 112 microsatellite markers to 23 chromosome 11 subregions delineated by somatic cell hybrids: Comparison with the genetic map. *Genomics* **21:** 379–387.

Craig, J. M., and Bickmore, W. A. (1994). The distribution of CpG islands on mammalian chromosomes. *Nature Genet.* **7:** 376–382.

Evans, G. A. (1993). Integrating maps of human chromosome 11. *Curr. Opin. Genet. Dev.* **3:** 418–424.

Fantes, J. A., Bickmore, W. A., Fletcher, J. M., Ballesta, F., Hanson, I. M., and van Heyningen, V. (1992). Non-radioactive *in situ* hybridization for the rapid analysis of submicroscopic deletions at the WAGR locus. *Am. J. Hum. Genet.* **51:** 1286–1294.

Fantes, J., Redeker, B., Breen, M., Boyle, S., Fletcher, J., Jones, S., Bickmore, W., Fukushima, Y., Mannens, M., Danes, S., van Heyningen, V., and Hanson, I. (1995). Aniridia-associated cytogenetic rearrangements suggest that a position effect may cause the mutant phenotype. *Hum. Mol. Genet.*, in press.

Francke, U. (1994). Digitized and differentially shaded human chromosome ideograms for genomic applications. *Cytogenet. Cell Genet.* **65:** 206–219.

Fukushima, Y., Hoovers, J., Mannens, M., Wakui, K., Ohashi, H., Ohno, T., Ueoka, Y., and Niikawa, N. (1993). Detection of a cryptic paracentric inversion within band 11p13 in familial aniridia by FISH. *Hum. Genet.* **91:** 205–209.

Gessler, M., and Bruns, G. A. P. (1989). A physical map around the WAGR complex on the short arm of chromosome 11. *Genomics* **5:** 43–55.

Gessler, M., Simola, K. O. J., and Bruns, G. A. P. (1989a). Cloning of breakpoints of a chromosome translocation identifies the AN2 locus. *Science* **244:** 1575–1578.

Gessler, M., Thomas, G. H., Couillin, P., Junien, C., McGillivray, B. C., Hayden, M., Jaschek, G., and Bruns, G. A. P. (1989b). A deletion map of the WAGR region on chromosome 11. *Am. J. Hum. Genet.* **44:** 486–495.

Gessler, M., Poustka, A., Cavenee, W., Neve, R. L., Orkin, S. H., and Bruns, G. A. P. (1990). Homozygous deletion in Wilms' tumours of a zinc-finger gene identified by chromosome jumping. *Nature* **343:** 774–778.

Gessler, M., Grupe, A., Grzeschik, K.-H., and Pongs, O. (1992). The potassium channel gene HK1 maps to human chromosome 11p14.1, close to the FSHB gene. *Hum. Genet.* **90:** 319–321.

Glaser, B., Chiu, K. C., Anker, R., Nestorowicz, A., Landau, H., Ben-Bassat, H., Shlomai, Z., Kaiser, N., Thornton, P. S., Stanley, C. A., Spielman, R. S., Gogolin-Ewens, K., Cerasi, E., Baker, L., Rice, J., Donis-Keller, H., and Permutt, M. A. (1994). Familial hyperinsulinism maps to chromosome 11p14–p15.1, 30 cM centromeric to the insulin gene. *Nature Genet.* **7:** 185–188.

Glaser, T., Housman, D., Lewis, W. H., Gerhard, D., and Jones, C. (1989). A fine structure deletion map of human chromosome 11p: Analysis of J1 series hybrids. *Somatic Cell Mol. Genet.* **15:** 477–501.

Glaser, T., Ton, C. C. T., Mueller, R., Petzl-Erler, M. L., Oliver, C., Nevin, N. C., Housman, D. E., and Maas, R. L. (1994). Absence of Pax6 gene mutations in Gillespie syndrome (partial aniridia, cerebellar ataxia, and mental retardation). *Genomics* **19:** 145–148.

Gyapay, G., Morissette, J., Vignal, A., Dib, C., Fizames, C., Millasseau, P., Marc, S., Bernardi, G., Lathrop, M., and Weissenbach, J. (1994). The 1993–1994 Généthon human genetic linkage map. *Nature Genet.* **7:** 246–250.

Hanson, I., Seawright, A., and van Heyningen, V. (1992). The human BDNF gene maps between FSHB and HVBS1 at the boundary of 11p13–p14. *Genomics* **13:** 1331–1333.

Hanson, I. M., Fletcher, J. M., Jordan, T., Brown, A., Taylor, D., Adams, R. J., Punnett, H. H., and van Heyningen, V. (1994a). Mutations at the PAX6 locus are found in heterogeneous anterior segment malformations including Peters' anomaly. *Nature Genet.* **6:** 168–173.

Heding, I. J. J., Ivens, A. C., Wilson, J., Strivens, M., Gregory, S., Hoovers, J. M. N., Mannens, M., Redeker, B., Porteous, D., van Heyningen, V., and Little, P. F. R. (1992). The generation of ordered sets of cosmid DNA clones from human chromosome region 11p. *Genomics* **86:** 6686–6690.

Heng, H. H. Q., Xiao, H., Shi, X.-M., Greenblatt, J., and Tsui, L.-C. (1994). Genes encoding general initiation factors for RNA polymerase II transcription are dispersed in the human genome. *Hum. Mol. Genet.* **3:** 61–64.

James, M. R., Richard, C. W., III, Schott, J.-J., Yousry, C., Clark, K., Bell, J., Terwilliger, J. D., Hazan, J., Dubay, C., Vignal, A., Agrapart, M., Imai, T., Nakamura, Y., Polymeropoulos, M., Weissenbach, J., Cox, D. R., and Lathrop, G. M. (1994). A radiation hybrid map of 506 STS markers spanning human chromosome 11. *Nature Genet.* **8:** 70–76.

Keats, B. J. B., Nouri, N., Pelias, M. Z., Deininger, P. L., and Litt, M. (1994). Tightly linked flanking microsatellite markers for the Usher syndrome type I locus on the short arm of chromosome 11. *Am. J. Hum. Genet.* **54:** 681–686.

Langer-Safer, P. R., Levine, M., and Ward, D. C. (1982). Immunological method for mapping genes on Drosophila polytene chromosomes. *Proc. Natl. Acad. Sci. USA* **79:** 4381–4385.

Lewis, W. H., Yeger, H., Bonetta, L., Chan, H. S. L., Kang, J., Junien, C., Cowell, J., Jones, C., and Defoe, L. A. (1988). Homozygous deletion of a DNA marker from chromosome 11p13 in sporadic Wilms' tumor. *Genomics* **3:** 25–31.

Lichter, P., Tang, C. C., Call, K., Hermanson, G., Evans, G. A., Housman, D., and Ward, D. C. (1990). High resolution mapping of human chromosome 11 by in situ hybridisation with cosmid clones. *Science* **247:** 64–69.

Litt, M., Kramer, P., Hauge, X. Y., Weber, J. L., Wang, Z., Wilkie, P. J., Holt, M. S., Mishra, S., Donis-Keller, H., Warnich, L., Retief, A. E., Jones, C., and Weissenbach, J. (1993). A microsatellite-based index map of human chromosome 11. *Hum. Mol. Genet.* **2:** 909–913.

Maisonpierre, P. C., le Beau, M. M., Espinosa, R., III, Ip, N. Y., Belluscio, L., de la Monte, S. M., Squinto, S., Furth, M. E., and Yancopoulos, G. D. (1991). Human and rat brain-derived neurotrophic factor and neurotrophin-3: Gene structure, distributions and chromosomal localisations. *Genomics* **10:** 558–568.

Miwa, T., Sudo, K., Nakamura, Y., and Imai, T. (1993). Fifty sequence-tagged sites on human chromosome 11. *Genomics* **17:** 211–214.

Ozawa, K., Murakami, Y., Eki, T., Soeda, E., and Yokoyama, K. (1992). Mapping of the gene family for human heat-shock protein 90α to chromosomes 1, 4, 11, and 14. *Genomics* **12:** 214–220.

Philipson, L. H., Eddy, R. L., Shows, T. B., and Bell, G. I. (1993). Assignment of human KCNA4 (Kv1.4, PCN2) to chromosome 11q13.4–q14.1. *Genomics* **15:** 463–464.

Redeker, E., Hoovers, J. M. N., Alders, M., van Moorsel, C. J. A., Ivens, A. C., Gregory, S., Kalikin, L., Bliek, J., de Galan, L., van den Bogaard, R., Visser, J., van der Voort, R., Feinberg, A. P., Little, P. F. R., Westerveld, A., and Mannens, M. (1994). An integrated physical map of 210 markers assigned to the short arm of human chromosome 11. *Genomics* **21:** 538–550.

Rose, E. A., Glaser, T., Jones, C., Smith, C. L., Lewis, W. H., Call, K. M., Minden, M., Champagne, E., Bonetta, L., Yeger, H., and Houseman, D. E. (1990). Complete physical map of the WAGR region of 11p13 localizes a candidate Wilms' tumor gene. *Cell* **60:** 495–508.

Royer-Pokora, B., Ragg, S., Heckl-Ostreicher, B., Held, M., Loos, U., Call, K., Glaser, T., Housman, D., Saunders, G., Zabel, B., Williams, B., and Poustka, A. (1991). Direct pulsed field gel electrophoresis of Wilms' tumours shows that DNA deletions in 11p13 are rare. *Genes Chromo. Cancer* **3:** 89–100.

Seawright, A., Fletcher, J. M., Fantes, J. A., Morrison, H., Porteous, D. J., Steven, S. L., Hastie, N. D., and van Heyningen, V. (1988). Analysis of WAGR deletions and related translocations with gene-specific DNA probes, using FACS-selected cell hybrids. *Somatic Cell Mol. Genet.* **14:** 21–30.

Sellar, G. C., Jordan, S. A., Bickmore, W. A., Fantes, J. A., van Heyningen, V., and Whitehead, A. S. (1994a). The human serum amyloid A protein (SAA) superfamily gene cluster: Mapping to chromosome 11p15.1 by physical and genetic linkage analysis. *Genomics* **19:** 221–227.

Sellar, G. C., Oghene, K., Boyle, S., Bickmore, W. A., and Whitehead, A. S. (1994b). Organization of the region encompassing the human serum amyloid A (SAA) gene family on chromosome 11p15.1. *Genomics* **23:** 492–495.

Smith, M. W., Clark, S. P., Hutchinson, J. S., Wei, Y. H., Churukian, A. C., Daniels, L. B., Diggle, K. L., Gen, M. W., Romo, A. J., Lin, Y., Selleri, L., McElligott, D. L., and Evans, G. A. (1993). A sequence-tagged site map of human chromosome 11. *Genomics* **17:** 699–725.

Tanagami, A., Tokino, T., Takiguchi, S., Mori, M., Glaser, T., Park, J. W., Jones, C., and Nakamura, Y. (1992). Mapping of 262 DNA markers into 24 intervals on human chromosome 11. *Am. J. Hum. Genet.* **50:** 56–64.

Ton, C. C., Hirvonen, H., Miwa, H., Weil, M. M., Monaghan, P., Jordan, T., van Heyningen, V., Hastie, N. D., Meijers-Heijboer, H., Drechsler, M., Royer-Pokora, B., Collins, F., Swaroop, A., Strong, L. C., and Saunders, G. F. (1991). Positional cloning and characterization of a paired box- and homeobox-containing gene from the aniridia region. *Cell* **67:** 1059–1074.

Trask, B. J., Massa, H. F., Kenwrick, S., and Gitschier, J. (1991). Mapping of human chromosome Xq28 by 2-color fluorescence in situ hybridisation of DNA sequences to interphase cell nuclei. *Am. J. Hum. Genet.* **48:** 1–15.

van Heyningen, V., Bickmore, W. A., Seawright, A., Fletcher, J. M., Maule, J., Fekete, G., Gessler, M., Gruns, G. A. P., Huerre-Jeanpierre, C., Junien, C., Williams, B. R. G., and Hastie, N. D. (1990). Role for the Wilms' tumor gene in genital development? *Proc. Natl. Acad. Sci. USA* **87:** 5383–5386.

van Heyningen, V., and Jones, C. (1993). Report on the committee on the genetic constitution of chromosome 11. *Cytogenet. Cell Genet. Genome Priority Rep.* **1:** 365–401.

Watkins, P. C., Eddy, R., Beck, A. K., Vellucci, V., Leverone, B., Tanzi, R. E., Gusella, J. F., and Shows, T. B. (1987). DNA sequence and regional assignment of the human follicle stimulating hormone β-subunit gene to the short arm of human chromosome 11. *DNA* **6:** 205–212.

Wymore, R. S., Korenberg, J. R., Kinoshita, K. D., Aiyar, J., Coyne, C., Chen, X.-N., Hustad, C. M., Copeland, N. G., Gutman, G. A., Jenkins, N. A., and Chandy, K. G. (1994). Genomic organization, nucleotide sequence, biophysical properties, and localization of the voltage-gated K^+ channel gene KCNA4/Kv1.4 to mouse chromosome 2/human 11p14 and mapping of KCNC1/Kv3.1 to mouse 7/human 11p14.3–p15.2 and KCNA1 to human 12p13. *Genomics* **20:** 191–202.

Zehetner, G., and Lehrach, H. (1994). The reference library system—Sharing biological material and experimental data. *Nature* **367:** 489–491.

Expression of the Wilms' Tumor Gene *WT1* in Human Malignant Mesothelioma Cell Lines and Relationship to Platelet-Derived Growth Factor A and Insulin-Like Growth Factor 2 Expression

Anthonie W. Langerak, Kathleen A. Williamson, Kiyoshi Miyagawa, Anne Hagemeijer, Marjan A. Versnel, and Nicholas D. Hastie

Departments of Immunology (A.W.L., M.A.V.) and Cell Biology and Genetics (A.H.), Erasmus University, Rotterdam, The Netherlands; MRC Human Genetics Unit, Western General Hospital, Edinburgh, United Kingdom (K.A.W., K.M., N.D.H.)

Mutations in the *WT1* tumor suppressor gene are known to contribute to the development of Wilms' tumor (WT) and associated gonadal abnormalities. *WT1* is expressed principally in the fetal kidney, developing gonads, and spleen and also in the mesothelium, which lines the coelomic cavities. These tissues develop from mesenchymal components that have subsequently become epithelialized, and it has therefore been proposed that *WT1* may play a role in this transition of cell types. To test the possible involvement of this gene in malignant mesothelioma, we have first studied its expression in a panel of human normal and malignant mesothelial cell lines. *WT1* mRNA expression levels varied greatly between the cell lines and no specific chromosomal aberration on 11p, which could be related to the variation in *WT1* expression in these cell lines, was observed. Furthermore, no gross deletions, rearrangements, or functionally inactivating point mutations in the *WT1* coding region were identified. All four *WT1* splice variants were observed at similar levels in these cell lines. The *WT1* gene encodes a zinc-finger transcription factor and the four protein isoforms are each believed to act as transcriptional repressors of certain growth factor genes. Lack of *WT1* expression is thus predicted to result in growth stimulation of tumor cells. Binding of one particular WT1 isoform construct to the insulin-like growth factor 2 (*IGF2*) and platelet-derived growth factor A (*PDGFA*) gene promoters has been demonstrated to result in repression of these genes in transient transfection studies. Analysis of *IGF2* and *PDGFA* mRNA expression levels compared with *WT1* mRNA expression levels failed to demonstrate an inverse correlation in the mesothelial cell lines, which endogenously express these genes. Finally, the putative role of WT1 in the transition of cell types was investigated. No obvious correlation between *WT1* expression levels and cell morphology of the malignant mesothelial cell lines was evident from this study. Moreover, no change in *WT1* expression was observed in normal mesothelial cells which were, by alteration of culture conditions, manipulated to switch from the mesenchymal to epithelial morphology. *Genes Chromosom Cancer 12:87–96 (1995).*

INTRODUCTION

Human malignant mesothelioma is a mesodermally derived tumor, which is most often found in the pleura. Its incidence is strongly associated with exposure to asbestos fibers (Wagner et al., 1960). Malignant mesothelioma is thought to develop from mesothelial cells, which form a specialized epithelium lining the coelomic cavities. Malignant mesothelioma was mentioned as one of the so-called second risk tumors in a few patients who had recovered from the pediatric kidney malignancy Wilms' tumor (WT) (Austin et al., 1986). This suggests the possible involvement of a common underlying genetic event in both malignancies.

The *WT1* locus on 11p13 has been identified as one of the chromosomal loci contributing to WT development (Call et al., 1990; Gessler et al., 1990). *WT1* encodes a protein with DNA-binding capacity, elicited by four zinc-fingers at the C-terminal part, which bind to DNA sequences with the core consensus element 5'-GCGGGGGCG-3' (Rauscher et al., 1990). The WT1 protein additionally possesses transcriptional regulatory activity exerted through the N-terminal glutamine- and proline-rich regions of the protein (Madden et al., 1991). Alternative splicing of the *WT1* gene at two independent splice sites has been shown to result in the formation of four *WT1* splice variants. In tumor tissue and fetal kidney there appears to be little variation in the ratios of these four isoforms (Haber et al., 1991; Brenner et al., 1992).

In transient transfection studies insulin-like growth factor 2 (*IGF2*), platelet-derived growth factor A (*PDGFA*), and *IGF1R* have recently been identified as potential target genes for transcriptional repression by WT1 (Drummond et al., 1992; Gashler et al., 1992; Wang et al., 1992; Werner et

Received May 30, 1994; accepted July 28, 1994.

Address reprint requests to Dr. Anthonie W. Langerak, Department of Immunology, Erasmus University, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands.

al., 1993). These and other results have led to the hypothesis that the WT1 protein functions as a tumor suppressor gene product and that reduction or absence of *WT1* expression that is seen in a proportion of WTs results in an increased expression of certain growth factor genes. However, transactivation by WT1 was also seen, depending on the presence of wild-type TP53 protein, the number of WT1 binding sites in the promoter of the target gene, or the presence of specific missense mutations within the transregulatory domain (Maheswaran et al., 1993; Park et al., 1993a; Wang et al., 1993).

Despite these results on repression or activation of certain target genes by WT1 in transient assays, not much is known about the physiological role of WT1. It has been suggested that WT1 may play a role in mediating the shift from a mesenchymal to an epithelial phenotype, as it is expressed in the nephrogenic epithelia, in epithelial cells of the gonads, and in the mesothelium (Pritchard-Jones et al., 1990; Pelletier et al., 1991; Armstrong et al., 1992). These are all cells of mesodermal origin that have undergone the transition to the epithelial morphology. However, other cell types undergoing this transition do not express the *WT1* gene, whereas in embryonic mice distinct regions in the spinal cord and brain that are of ectodermal origin display *WT1* expression, arguing that there should be other tissue-specific roles for WT1 in development (Armstrong et al., 1992). Recently, a crucial role for *WT1* in early urogenital development was established in a model system by gene targeting in murine embryonic stem cells (Kreidberg et al., 1993). In these *WT1* knockout mice a failure in kidney development was observed. This was suggested to be caused by inhibition of inductive events leading to the formation of the metanephric kidney. In addition, abnormal development of the gonads, the heart, and the mesothelium was seen as well. Abnormalities in the phenotypes of these tissues support the idea of a role for WT1 in the mesenchymal to epithelial cell transition.

As *WT1* expression was observed in the human and mouse developing and mature mesothelium (Pritchard-Jones et al., 1990; Armstrong et al., 1992; Park et al., 1993b), we were interested to determine whether *WT1* was also expressed in its malignant counterpart as this might indicate a possible involvement of *WT1* in the pathogenesis of malignant mesothelioma. In this respect, it is worth noting that cytogenetic analysis of 40 confirmed mesothelioma patients revealed karyotypic abnormalities in several chromosomes, but only in a few cases were rearrangements in 11p seen (Hagemeijer et al., 1990). We have studied expression of the *WT1* gene in a panel of human normal and malignant mesothelial cell lines. Expression levels were related to the morphology (epithelial or mesenchymal) of the mesothelioma cells in vitro in order to clarify putative WT1 involvement in cell type transition. We also investigated whether gross alterations or point mutations could be detected at the *WT1* locus, whether the four different alternatively spliced mRNAs were present in the various normal and malignant mesothelial cell lines, and whether the *WT1* mRNA expression level in these cell lines could be correlated to their *PDGFA* and *IGF2* mRNA levels.

MATERIALS AND METHODS

Cell Lines, Growth Conditions, Characterization, and Cytogenetics

Experiments were performed using the human pleural malignant mesothelioma cell lines Mero-14, -25, -41, -48b, -48c, -72, -82, -83, -84, -95, -96, and -123 (Versnel et al., 1988, 1989) and the normal mesothelial cell lines NM-1, -4, -5, -9, and -12 (Versnel et al., 1991, 1993; Langerak et al., in preparation). All mesothelioma cell lines were derived from mesothelioma patients whose diagnosis was based on routine cytology, which was histologically or ultrastructurally confirmed. Cell lines were routinely cultured as described earlier (Versnel et al., 1988, 1989). Cytogenetic analysis was performed as described earlier (Versnel et al., 1988).

Northern and Southern Blot Analyses and Probes

Northern blotting and Southern blotting were performed as described in Langerak et al. (1992). For hybridization of Northern and Southern blots a 1.8 kb *Eco*RI *WT1* fragment derived from the WT33 cDNA was used (Call et al., 1990). Northern blots were rehybridized with the 1.3 kb *Eco*RI *PDGFA* fragment (Betsholtz et al., 1986), the 1.4 kb *Xba*I-*Eco*RI *IGF2* fragment from pIGF-II (Jansen et al., 1985), and the 0.7 kb *Eco*RI-*Pst*I glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) fragment (Benham et al., 1984). Rehybridization of Southern blots was performed with a 1.3 kb *Pst*I *IGF2* fragment from pKT218 (Jansen et al., 1985).

Reverse Transcription (RT) Polymerase Chain Reaction (PCR) Analysis

RT of RNA, isolated from the normal and malignant mesothelial cell lines, and subsequent PCR

analysis were performed as described previously (Langerak et al., 1992). For amplification of the alternative splice variants the sense primers B297 (5′-TTG GTC GAC ATG ACC TGG AAT CAG/C ATG-3′; located in *WT1* exon 4) or B439 (5′-CTT GTA CGG TCG GCA TCT-3′; located in *WT1* exon 7) were used in combination with antisense primer B298 (5′-TGC AAG CTT CAG CTG AAG GGT/C TTC/T TC-3′; located in *WT1* exon 10) (Little et al., 1992). Thirty-five cycles of 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C were performed. PCR products were analyzed on 10% polyacrylamide gels using *Pst*I digested lambda DNA as a marker.

Point Mutation Analysis

Chemical cleavage mismatch analysis using hydroxylamine and osmium tetroxide (HOT analysis) was performed as described (Cotton et al., 1988; Prosser et al., 1990). Templates were generated by RT-nested PCR (Hanson et al., 1993), a strategy required due to low yields of available RNA. The *WT1* region encoding the transregulatory domain was amplified with outer primers D609 sense (5′-CAA ACA GGA GCC GAG CTG G-3′) and D610 antisense (5′-GCA CAT CCT GAA TGC CTC TG-3′) followed by inner primers 1 and 3 (Brown et al., 1992). The DNA-binding domain was similarly amplified with primers C582 sense (5′-AAA TGG ACA GAA GGG CAG AGC-3′) and C583 antisense (5′-TTG GAA GTT GGA TGA AGA AGA TC-3′) followed by primers 2 and 4 (Brown et al., 1992). PCR conditions for D609/D610 are step 1, 1 min at 94°C; step 2, 30 cycles of 30 sec at 94°C, 1 min at 54°C, and 1–2 min at 72°C; step 3, 10 min at 72°C. Touchdown PCR conditions for C582/C583 are step 1, 1 min at 94°C; step 2, 30 cycles of 30 sec at 92°C, 1 min at 62–57°C and 2 min at 72°C; step 3, 10 min at 72°C. Samples generating cleaved fragments after HOT analysis were subjected to a second round of RT-nested PCR. Then the products were purified by β-Agarase I (New England Biolabs, Beverly, MA) and subsequently sequenced directly as described (Winship, 1989).

RESULTS

WT1 mRNA Expression in Mesothelial Cell Lines

Expression of the *WT1* gene was studied on Northern blots, containing total RNA from normal and malignant mesothelial cell lines of human origin. *WT1* mRNA was found to be consistently expressed in the normal mesothelial cell lines, although some variation in the levels was observed (Fig. 1). The *WT1* expression level was highly variable in the investigated panel of malignant mesothelioma cell lines, ranging from very high (Mero-25) to nearly or totally undetectable (Mero-41, -72, -82, -83, -95) (Fig. 1). *WT1* mRNA expression levels were normalized over *GAPDH* mRNA levels after quantification by densitometric analysis (see Table 1).

Based on their histology, malignant mesotheliomas can be classified as epithelial, mesenchymal, or biphasic. The morphology of the cultured mesothelioma cells in monolayer can differ from the primary tumor-tissue morphology (see Table 1). In an attempt to clarify the possible role of the *WT1* gene product in the transition of certain cell types from a mesenchymal toward an epithelial morphology, *WT1* mRNA expression levels in the cultured mesothelioma cells were therefore related to the epithelial or fibrous/mesenchymal morphology of these cell lines. Although the highest *WT1* mRNA level was observed in Mero-25, which has the clearest epithelial phenotype, *WT1* expression was not completely confined to cell lines showing an epithelial or biphasic morphology. *WT1* mRNA was undetectable in several cell lines with a fibrous morphology like Mero-41 and -72, but other fibrous cell lines did express *WT1* (Mero-83, -96, and -123). In cell lines that were predominantly either fibrous or epithelial, expression was observed occasionally (Mero-48b, -48c, and -84), and in others not at all (Mero-82 and -95).

The putative involvement of *WT1* in the mesenchymal to epithelial shift was further studied using a second approach. It has been described that cultured normal mesothelial cells can adopt a mesenchymal (fibrous) or epithelial cell shape depending on the presence or absence of epidermal growth factor (EGF) in the culture medium, respectively (Connell and Rheinwald, 1983). Therefore, the *WT1* mRNA level was determined in two normal mesothelial cell lines under these conditions. The two cell lines showed a more epithelial morphology upon removal of EGF [and hydrocortisone (HC)] for 3 days from the standard culture medium. However, analysis of RNA isolated from the mesothelial cells cultured for 72 hr in the absence of EGF and HC did not result in a significantly altered level of *WT1* mRNA (data not shown).

Alternative Splicing Pattern of *WT1*

The *WT1* gene is capable of producing four different mRNAs by alternative splicing, which can lead to the insertion of an extra 51 bp (exon 5) upstream of the zinc-fingers and/or 9 bp (giving rise

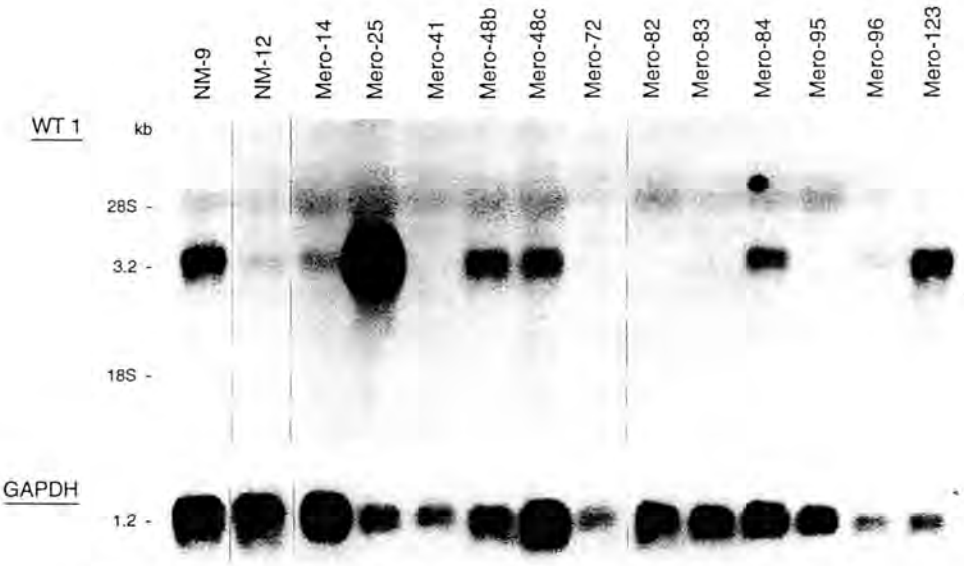


Figure 1. Northern blot analysis with 25 μg total RNA from normal (NM) and malignant (Mero) mesothelial cell lines. RNA was hybridized to *WT1* and *GAPDH* probes.

TABLE 1. Summary of Cytogenetics, Tumor Cell Line Morphology, and *WT1* Expression in Normal (NM) and Malignant (Mero) Mesothelial Cell Lines

Cell line	Tumor cell line morphology	Modal chromosome No.	No. of copies of chromosome (arm)			*WT1* mRNA level[a]	*WT1* gene structure (Southern blot)	*WT1* gene (mutations)	*WT1* mRNA (alternative splicing)
			Normal No. 11	Rearranged No. 11p	Total No. 11p				
NM-1	—	46	2	—	2	0.81	ND[b]	ND	4 isoforms
NM-4	—	46	2	—	2	0.28	ND	ND	ND
NM-5	—	46	2	—	2	0.12	ND	ND	ND
NM-9	—	46	2	—	2	0.26	Normal	ND	ND
NM-12	—	46	2	—	2	0.08	ND	ND	ND
Mero-14	Fibrous	75	3 (4)	—	3 (4)	0.04	Normal	Not found	4 isoforms
Mero-25	Epithelial	67	3 (4)	1 × inv (11)(p11q14)	4 (5)	9.59	Normal	Not found	4 isoforms
Mero-41	Fibrous	72	4	—	4	0	Normal	ND	NA[c]
Mero-48a	Epithelial (fibrous)[d]	71–75	3	1 × mar (t(9p;11p))	4	ND	Normal	Not found	ND
Mero-48b	Fibrous (epithelial)	71–75	3	1 × mar (t(9p;11p))	4	0.43	Normal	Neutral transition	ND
Mero-48c	Fibrous (epithelial)	71–75	3	1 × mar (t(9p;11p))	4	0.12	Normal	Neutral transition	4 isoforms
Mero-72	Fibrous	42	2	—	2	0	Normal	ND	NA
Mero-82	Fibrous (epithelial)	49	2	—	2	0	Normal	ND	NA
Mero-83	Fibrous	75–85	2	2 × add 11p15	4	0.04	Normal	Not found	4 isoforms
Mero-84	Fibrous (epithelial)	38	0	t(6p + ;11p−), +der(11)t(11;22)	2	0.14	Normal	Not found	4 isoforms
Mero-95	Biphasic	54–58	3	—	3	0	Normal	ND	NA
Mero-96	Fibrous	72–78	2 (3)	2 × 6q−(6p::11p)	4 (5)	0.26	Normal	Not found	4 isoforms
Mero-123	Fibrous	55	3	—	3	0.91	ND	Neutral transition	4 isoforms

[a]Shown over *GAPDH* expression levels as determined by densitometry.
[b]ND, not determined.
[c]NA, not applicable.
[d]In parentheses: minority of the cells.

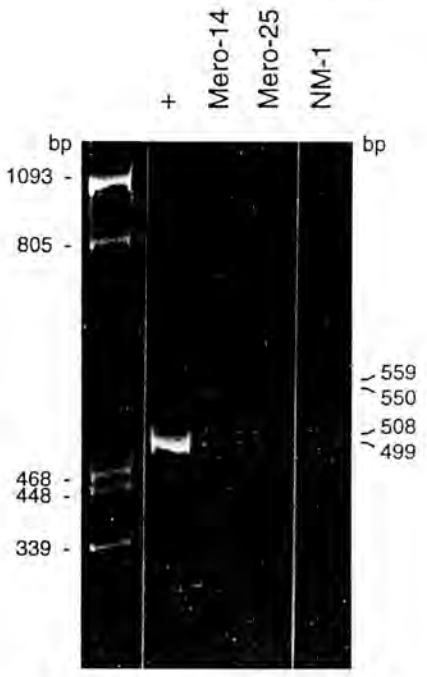


Figure 2. RT PCR analysis with RNA from malignant mesothelioma cell lines Mero-14 and -25 and normal mesothelial cell line NM-1. WT33 cDNA was used as a positive control (+). In the left lane *Pst*I digested lambda DNA was loaded as a size marker.

to the KTS amino acid sequence) between zinc-fingers 3 and 4 (Haber et al., 1991; Brenner et al., 1992). In this study the occurrence of these distinct *WT1* mRNA forms was examined in clonal cell lines. For this purpose cDNA derived from total RNA of several of the *WT1* expressing malignant mesothelioma cell lines (see Table 1) was used in a PCR reaction with primers B297 and B298. Four distinct DNA fragments of 499 bp (−51 bp, −KTS), 508 bp (−51 bp, +KTS), 550 bp (+51 bp, −KTS), and 559 bp (+51 bp, +KTS), resulting from two independent alternative splicing events, were observed (see Table 1). All four splice variants were detected in normal mesothelial cell line NM-1 as well. In Figure 2 these results are shown for the cell lines NM-1 and Mero-14 and -25. Using primers B439 and B298 in all cell lines studied, two distinct fragments of 320 and 329 bp were seen, which result from the 9 bp alternative splicing event (data not shown). In our *WT1* mRNA expressing mesothelial cell lines, little variation was observed in the ratios of the four isoforms.

Cytogenetic and Genomic Data Concerning *WT1*

The variation in *WT1* mRNA expression that we observed in the malignant mesothelioma cell lines could be due to differences in the whole or partial copy number of chromosome arm 11p. Furthermore, rearrangements of the *WT1* gene or gene amplification may also be involved. To see if any of these possibilities may explain the variation in *WT1* mRNA level in the mesothelioma cell lines, cytogenetic analysis and Southern blotting were employed.

Cytogenetic data from the mesothelioma cell lines were obtained by studying metaphase cells. Analysis of chromosome arm 11p in the mesothelioma cell lines did not point toward any specific chromosomal aberration that could be correlated to their *WT1* mRNA expression level (see Table 1). The aneuploidy of chromosome arm 11p and the *WT1* mRNA level in the various cell lines did not correlate either (Table 1).

To see if gene rearrangements had occurred in those cell lines showing very low or undetectable amounts of *WT1* mRNA, the *WT1* gene was studied by Southern blot analysis. No differences were found in the *Eco*RI and *Hin*dIII digestion pattern of DNA from any of the malignant mesothelioma cell lines studied compared to the pattern of normal mesothelial cell line NM-9 (Fig. 3; also data not shown). This indicated that gross rearrangements in the *WT1* gene had not occurred in the malignant mesothelial cell lines and thus were not likely to be the cause of undetectable *WT1* mRNA expression in Mero-41, -72, -82, and -95.

By Southern blot analysis we did detect small differences in the intensity of the bands for the various cell lines, which could in principle be caused by variation in the number of *WT1* gene copies. However, rehybridization of the filter containing *Hin*dIII digested DNA with a probe for the *IGF2* gene, which is also located on the short arm of chromosome 11 at 11p15, showed similar differences in intensity (data not shown). This meant that the *WT1* gene was not differentially amplified in any of the cell lines. Variation in the number of copies of 11p may exist but in general the number of chromosome arm 11p largely balanced the total chromosome number, which suggests that the small differences in intensity are most probably due to small variations in loading of the gels.

WT1 Point Mutation Analysis

The HOT technique of chemical cleavage mismatch analysis was used to scan virtually the entire coding sequence of *WT1* for point mutations. Only the first 256 bp in exon 1 were omitted from this analysis, as the high GC content of this region renders it refractory to PCR amplification. The malig-

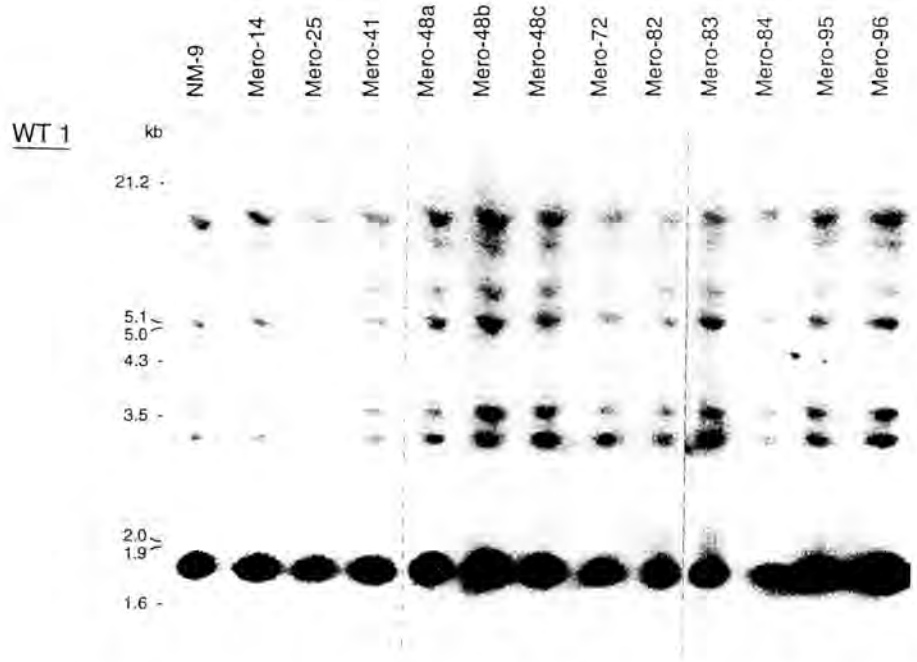


Figure 3. Southern blot analysis of the *WT1* gene. *Eco*RI-digested genomic DNA from a normal (NM-9) and several malignant (Mero) mesothelial cell lines was hybridized to a ^{32}P-labeled *WT1* probe.

nant mesothelioma cell lines Mero-14, -48b, -48c, -83, -84, and -123 were scanned for point mutations in exons 1–6, encoding the transregulatory domain, and Mero-14, -25, -48b, -48c, -83, -84, -96, and -123 were scanned for point mutations in exons 6–10, encoding the DNA-binding domain. The only base change detected was a novel C to T transition at nucleotide 768 (numbered according to Gessler et al., 1990). This exon 1 transition is a third base change and does not cause amino acid substitution (AAC/T encodes an Asn residue). It is present in the homo/hemizygous state in Mero-48b, -48c, and -123 (data not shown). No functionally inactivating nonsense or missense mutations were detected (see Table 1).

WT1 mRNA Expression in Relation to *PDGFA* and *IGF2* mRNA Expression

In transient transfection assays WT1 was reported to repress the expression of *PDGFA* and *IGF2* promoter constructs. In order to see if the described variation in *WT1* mRNA expression in our mesothelial cell lines could be related to different levels of *PDGFA* and *IGF2* mRNA, blots were rehybridized with probes for these two genes and analyzed by densitometry. The results from a representative experiment are presented as arbitrary units over *GAPDH* signals in Table 2.

TABLE 2. mRNA Expression Levels of *WT1*, *PDGFA*, and *IGF2* (6.0 and 4.8 kb) in Normal (NM) and Malignant (Mero) Mesothelial Cell Lines, Expressed as Relative Densitometric Units Over *GAPDH* Expression Levels

			IGF2	
Cell line[a]	*WT1*	*PDGFA*[b]	6.0 kb	4.8 kb
NM-1	0.81	0.65	0	0.53
NM-4	0.28	0.78	0.19	0.66
NM-9	0.26	ND[c]	0	0.23
NM-5	0.12	0.62	0.12	0.48
NM-12	0.08	ND	0.23	0.48
Mero-25	9.59	1.42	0	0.73
Mero-123	0.91	2.22	0	0.68
Mero-48b	0.43	1.41	0.28	1.02
Mero-96	0.26	3.74	1.72	6.55
Mero-84	0.14	1.55	0.02	0.23
Mero-48c	0.12	0.50	0.29	0.43
Mero-14	0.04	0.04	0	0.16
Mero-83	0.04	1.18	0.01	0.18
Mero-82	0	1.02	0	0.21
Mero-72	0	2.59	2.37	2.62
Mero-95	0	2.62	0.07	0.44
Mero-41	0	7.54	0.24	1.27

[a]Cell lines are arranged in descending order of *WT1* expression levels.
[b]Totaled levels of 2.8, 2.3, and 1.9 kb *PDGFA* transcripts.
[c]ND, not determined.

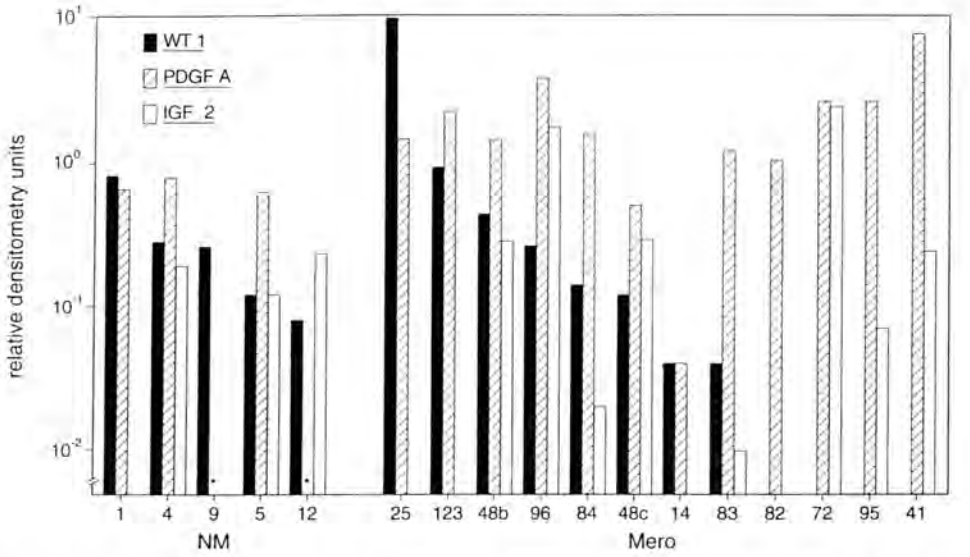


Figure 4. Relative densitometric units of the mRNA expression levels of *WT1*, *PDGFA* (2.8, 2.3, and 1.9 kb totaled) and *IGF2* (6.0 kb) in normal (NM) and malignant (Mero) mesothelial cell lines. The data are presented on a log scale. See Table 2 for the exact densitometric units. Cell lines are arranged in descending order of *WT1* expression levels. Expression levels that were not determined are indicated by an asterisk.

The densitometric data of the three *PDGFA* transcripts (2.8, 2.3, and 1.9 kb) were totaled, as expression of these three messengers is under control of a single promoter region that contains consensus WT1 binding sequences. Our mesothelioma cell lines demonstrated variation in *PDGFA* mRNA expression, but in general the expression level was higher than in normal mesothelial cell lines. In a few cell lines lacking *WT1* mRNA, a relatively high *PDGFA* expression was seen (Mero-41, -72, and -95), but other cell lines which did show *WT1* mRNA expression, i.e., Mero-96 and -123, displayed a similar *PDGFA* mRNA level. Cell line Mero-25, in which a very high *WT1* mRNA level was seen, showed an intermediate level of *PDGFA* expression. This intermediate *PDGFA* level, however, was also seen in cell lines with a lower *WT1* mRNA content than Mero-25. So, as is also illustrated in Figure 4, no clear correlation was observed between *WT1* and *PDGFA* expression in our mesothelial cell lines.

The 6.0 kb *IGF2* transcript is the product of the fetal P3 promoter, which contains WT1 consensus binding sequences, whereas the 4.8 kb transcript is expressed from a different fetal promoter (P4), also containing consensus WT1 binding sequences. Relatively high *WT1* mRNA levels were found in cell lines which do not express the 6.0 kb *IGF2* transcript (NM-1 and Mero-25 and -123), but other cell lines which demonstrated very low or no 6.0 kb *IGF2* mRNA expression, like NM-9 and Mero-14, -82, -83, -84, and -95, showed a low or intermediate *WT1* mRNA level. In the panel of normal and mesothelial cell lines no clear relationship, reciprocal or otherwise, was observed between the 6.0 kb *IGF2* and *WT1* mRNA level (see also Fig. 4). The same holds true for the expression level of the 4.8 kb *IGF2* transcript.

DISCUSSION

The WT gene *WT1* on 11p13 acquired its name, as it was originally mapped by deletion analysis of individuals with the WAGR (WT, aniridia, genitourinary abnormalities, and mental retardation) syndrome (Riccardi et al., 1978; van Heyningen et al., 1985). The demonstration of constitutional and somatic intragenic deletions in the *WT1* gene in a proportion of WT patients has confirmed that it is a WT predisposition gene (Haber et al., 1990; Cowell et al., 1991; Huff et al., 1991; Brown et al., 1992; Tadokoro et al., 1992). In expression studies in human and mouse embryos the *WT1* gene was reported to be involved in normal genitourinary development (Pritchard-Jones et al., 1990; Armstrong et al., 1992). Because of this limited spatial expression, *WT1* was suggested to be important in tissues which are of mesodermal origin and which undergo a mesenchymal to epithelial transition, although expression was also observed in the spinal cord and developing brain. In situ hybridization studies showed high *WT1* expression in the developing Sertoli cells of the testis and granulosa cells of the ovary (Pelletier et al., 1991; Armstrong et al., 1992).

As the studies by Pritchard-Jones et al. (1990) and Armstrong et al. (1992) had revealed the expression of *WT1* in the mesothelium, a specialized epithelium lining the coelomic cavities, an obvious question was if expression of the *WT1* gene could be detected in mesothelioma as well. This prompted us to study *WT1* expression in a panel of normal and malignant human mesothelial cell lines. Northern blot analysis revealed the consistent presence of *WT1* mRNA in cultured normal mesothelial cells, whereas in cultured malignant mesothelioma cells a variation in the expression level, ranging from very high to undetectable, was seen. We also found that the apparent lack of *WT1* mRNA expression in several of these cell lines probably was not due to deletions or rearrangements in the *WT1* gene. Furthermore, differences in the *WT1* mRNA expression level between different cell lines could not be accounted for by gene amplification or a specific chromosomal aberration on 11p. Differences in transcription initiation or RNA degradation thus most probably account for the variation in *WT1* mRNA expression between the malignant mesothelioma cell lines.

When the *WT1*-expressing malignant mesothelioma cell lines were analyzed for more subtle alterations within the coding sequence, no nonsense or missense mutations were found. Three lines contained an identical C to T transition in the sequence encoding the transregulatory domain. However, this mutation is predicted to be silent at the protein level and therefore most likely pathologically insignificant. Recently a homozygous *WT1* missense mutation that alters a Ser residue in the transregulatory domain has been reported for a single case of human peritoneal mesothelioma (Park et al., 1993b). This case is unusual in that the mesothelioma was not asbestos-related and was not actually a malignant tumor but rather a developmental abnormality. In addition, Park et al. (1993b) found no *WT1* mutations in 32 specimens of asbestos-related mesothelioma. For our samples it is possible that there are undetected *WT1* mutations in the 5′-most coding region of exon 1 or in the untranslated or intronic sequences of the gene. The cell lines that fail to show *WT1* mRNA expression may additionally have mutations in the promoter/control regions of the gene. Differences in the occurrence of the four alternative splicing products, which may result in altered specificity for DNA binding sites (Bickmore et al., 1992), were not observed in our panel of *WT1* expressing mesothelial cell lines. All four variants were identified earlier in WT tissue and in fetal kidney (Haber et al., 1991; Brenner et al., 1992). In these tissues the transcripts with the 9 bp alternative splice were suggested to be slightly predominant, whereas in our mesothelial cell lines we did not observe this, but a more quantitative assay has to be performed to unravel this putative discrepancy.

Taken together, the results obtained in our panel of malignant mesothelioma cell lines thus suggest that the *WT1* gene may play a role as a tumor suppressor gene in a minority of human mesotheliomas. To test this in vivo, we started to study primary tumor material from mesothelioma patients. *WT1* mRNA expression could be observed in cells from pleural effusions of four malignant mesothelioma patients (data not shown). These pleural fluids, however, contain tumor cells in combination with several other cell types, which means that this expression cannot be simply attributed to tumor cells, even though pleural fluids with a high percentage of mesothelioma cells were analyzed. Immunofluorescence staining with WT1 antibodies and/or RNA in situ hybridization would be more informative in this respect.

As the ovarian surface epithelium is considered to be a specialized mesothelium (Papadaki and Beilby, 1971) and several ovarian carcinoma cell lines demonstrated a comparable *PDGFA* and *PDGFB* mRNA expression to malignant mesothelioma cell lines (Versnel et al., 1994), we also analyzed several ovarian carcinoma cell lines for *WT1* mRNA expression. Comparable to the expression in malignant mesothelioma cell lines, in three of six serous ovarian carcinoma cell lines studied, *WT1* expression was observed on Northern blots, while in the other three no *WT1* transcripts were detected (data not shown). Furthermore, we recently observed a very high *WT1* mRNA level in cell line COV-434, which is derived from a granulosa tumor of the ovary and thus is not of mesothelial origin (data not shown). This expression is in agreement with the observed *WT1* expression in ovarian granulosa cells (Armstrong et al., 1992).

Malignant mesotheliomas are classified as epithelial, fibrous/mesenchymal, or biphasic. We therefore tried to correlate the morphology of the various malignant mesothelioma cell lines with their *WT1* mRNA expression level. Although the highest expression was found in cell line Mero-25, which has the most obvious epithelial morphology, no clear correlation could be observed between *WT1* mRNA expression and morphology. Moreover, normal mesothelial cells which can switch in morphology depending on the addition or removal of EGF from the culture medium, showed similar

WT1 expression levels independent of the phenotype of the cells. So in these mesothelial cell lines no evidence could be found for a WT1 role in the mesenchymal to epithelial transition of cells. However, this may be different in vivo. Therefore, mesothelioma tissue from patients with a biphasic (i.e., with mesenchymal and epithelial elements) malignant mesothelioma should be studied with WT1 antibodies or by RNA in situ hybridization to investigate the putative correlation between *WT1* expression and morphology in vivo.

It has been shown in transient transfection assays that WT1 represses *PDGFA* (Gashler et al., 1992; Wang et al., 1992) and *IGF2* mRNA expression (Drummond et al., 1992). Our panel of normal and malignant mesothelial cell lines showing variation in *WT1* mRNA expression was analyzed for *PDGFA* and *IGF2* mRNA levels as well. No clear relationship, reciprocal or otherwise, between *WT1* and *PDGFA* or *IGF2* expression was found in our cell lines. The fact that no clear correlation could be found in cells endogenously expressing these genes is in contrast with the repression seen in the forementioned studies. However, these data were obtained in cells upon transfection of *WT1* expression constructs together with *PDGFA* and *IGF2* reporter constructs, whereas we looked at endogenous expression levels in a panel of cell lines. It may also be that in our mesothelial cell lines other factors are also involved in regulating *PDGFA* and *IGF2* mRNA expression, thereby masking regulation by WT1. Alternatively, mutations in the WT1 binding sequences in the promoters of these genes may prohibit WT1 regulation. It remains to be determined, whether WT1 can regulate expression of these genes in a physiological context. Evidence for this may come from stable transfection of the *WT1* gene in Mero cell lines lacking *WT1* or knocking out the endogenous *WT1* gene expression in *WT1* expressing cell lines.

In summary, we have shown that *WT1* mRNA is consistently expressed in normal mesothelial cell lines and that there is no expression in a minority of malignant mesothelioma cell lines. No indications were found for chromosomal aberrations, deletions, rearrangements, functionally inactivating missense or nonsense mutations, or an aberrant alternative splicing pattern in these cell lines. The *WT1* expression level does not seem to correlate with the mesenchymal or epithelial morphology of the various cell lines in vitro. No inverse correlation between *WT1* and *PDGFA* or *IGF2* mRNA expression was seen in our panel of mesothelial cell lines, which endogenously express these genes.

ACKNOWLEDGMENTS

We thank Prof. Dr. R. Benner for continuous support and Mrs. E. Postma for technical assistance. Dr. M. Jansen kindly provided the *IGF2* probe. Mr. T.M. van Os is acknowledged for photographic assistance and Mrs. A.C. de Vries for secretarial assistance. This study was financially supported by a grant from the Dutch Cancer Society.

REFERENCES

Armstrong JF, Pritchard-Jones K, Bickmore WA, Hastie ND, Bard JBL (1992) The expression of the Wilms' tumour gene, *WT1*, in the developing mammalian embryo. Mech Dev 40:85–97.

Austin MB, Fechner RE, Roggli VL (1986) Pleural malignant mesothelioma following Wilms' tumor. Am J Clin Pathol 86:227–230.

Benham FJ, Hodgkinson S, Davies KE (1984) A glyceraldehyde-3-phosphate dehydrogenase pseudogene on the short arm of the human X-chromosome defines a multigene family. EMBO J 3:2635–2640.

Betsholtz C, Johnsson A, Heldin C-H, Westermark B, Lind P, Urdea MS, Eddy R, Shows TB, Philpott K, Mellor AL, Knott TJ, Scott J (1986) cDNA sequence and chromosomal localization of human platelet-derived growth factor A-chain and its expression in tumor cell lines. Nature 320:695–699.

Bickmore WA, Oghene K, Little MH, Seawright A, van Heyningen V, Hastie ND (1992) Modulation of DNA binding specificity by alternative splicing of the Wilms' tumour *wt1* gene transcript. Science 257:235–237.

Brenner B, Wildhardt G, Schneider S, Royer-Pokora B (1992) RNA polymerase chain reaction detects different levels of four alternatively spliced *WT1* transcripts in Wilms' tumors. Oncogene 7:1431–1433.

Brown KW, Watson JE, Poirier V, Mott MG, Berry PJ, Maitland NJ (1992) Inactivation of the remaining allele of the *WT1* gene in a Wilms' tumour from a WAGR patient. Oncogene 7:763–768.

Call KM, Glaser T, Ito CY, Buckler AJ, Pelletier J, Haber DA, Rose EA, Kral A, Yeger H, Lewis W, Jones C, Housman DE (1990) Isolation and characterization of a zinc-finger polypeptide gene at the human chromosome Wilms' tumor locus. Cell 60:509–520.

Connell ND, Rheinwald JG (1983) Regulation of the cytoskeleton in mesothelial cells: Reversible loss of keratin and increase in vimentin during rapid growth in culture. Cell 34:245–253.

Cotton RG, Rodrigues NR, Campbell RD (1988) Reactivity of cytosine and thymine in single-base-pair mismatches with hydroxylamine and osmium tetroxide and its application to the study of mutations. Proc Natl Acad Sci USA 85:4397–4401.

Cowell JK, Wadey RB, Haber DA, Call KM, Housman DE, Pritchard J (1991) Structural rearrangements of the *WT1* gene in Wilms' tumour cells. Oncogene 6:595–599.

Drummond IA, Madden SL, Rohwer-Nutter P, Bell GI, Sukhatme VP, Rauscher FJ III (1992) Repression of the insulin-like growth factor II gene by the Wilms' tumor suppressor WT1. Science 257:674–678.

Gashler AL, Bonthron DT, Madden SL, Rauscher FJ III, Collins T, Sukhatme VP (1992) Human platelet-derived growth factor A-chain is transcriptionally repressed by the Wilms' tumor suppressor WT1. Proc Natl Acad Sci USA 89:10984–10988.

Gessler M, Poustka A, Cavenee W, Neve RL, Orkin SH, Bruns GAP (1990) Homozygous deletion in Wilms' tumours of a zinc-finger gene identified by chromosome jumping. Nature 343:774–778.

Haber DA, Buckler AJ, Glaser T, Call KM, Pelletier J, Sohn RL, Douglass EC, Housman DE (1990) An internal deletion within an 11p13 zinc-finger gene contributes to the development of Wilms' tumor. Cell 61:1257–1269.

Haber DA, Sohn RL, Buckler AJ, Pelletier J, Call KM, Housman DE (1991) Alternative splicing and genomic structure of the Wilms' tumor gene *WT1*. Proc Natl Acad Sci USA 88:9618–9622.

Hagemeijer A, Versnel MA, van Drunen E, Moret M, Bouts MJ, van der Kwast ThH, Hoogsteden HC (1990) Cytogenetic analysis of malignant mesothelioma. Cancer Genet Cytogenet 47:1–28.

Hanson IM, Seawright A, Hardman K, Hodgson S, Zaletayev D,

Fekete G, van Heyningen V (1993) *PAX6* mutations in aniridia. Hum Mol Genet 2:915–920.

Huff V, Miwa H, Haber DA, Call KM, Housman DE, Strong L, Saunders GF (1991) Evidence for *WT1* as a Wilms' tumor (WT) gene: Intragenic germinal deletion in bilateral WT. Am J Hum Genet 48:997–1003.

Jansen M, van Schaik FMA, van Tol H, van den Brande JL, Sussenbach JS (1985) Nucleotide sequences of cDNAs encoding precursors of human insulin-like growth factor-II (IGF-II) and an IGF-II variant. FEBS Lett 179:243–246.

Kreidberg JA, Sariola H, Loring JM, Maeda M, Pelletier J, Housman D, Jaenisch R (1993) WT-1 is required for early kidney development. Cell 74:679–691.

Langerak AW, Dirks RPH, Versnel MA (1992) Splicing of the platelet-derived growth factor A-chain mRNA in human malignant mesothelioma cell lines and regulation of its expression. Eur J Biochem 208:589–596.

Little MH, Prosser J, Condie A, Smith PJ, van Heyningen V, Hastie ND (1992) Zinc finger point mutations within the *WT1* gene in Wilms' tumor patients. Proc Natl Acad Sci USA 89:4791–4795.

Madden SL, Cook DM, Morris JF, Gashler A, Sukhatme VP, Rauscher FJ III (1991) Transcriptional repression mediated by the WT1 Wilms' tumor gene product. Science 253:1550–1553.

Maheswaran S, Park S, Bernard A, Morris JF, Rauscher FJ III, Hill DE, Haber DA (1993) Physical and functional interactions between WT1 and p53 proteins. Proc Natl Acad Sci USA 90:5100–5104.

Papadaki L, Beilby JOW (1971) The fine structure of the surface epithelium of the human ovary. J Cell Sci 8:445–465.

Park S, Tomlinson G, Nisen P, Haber DA (1993a) Altered transactivational properties of a mutated *WT1* gene product in a WAGR-associated Wilms' tumor. Cancer Res 53:4757–4760.

Park S, Schalling M, Bernard A, Maheswaran S, Shipley GC, Roberts D, Fletcher J, Shipman R, Rheinwald J, Demetri G, Griffin J, Minden M, Housman DE, Haber DA (1993b) The Wilms' tumor gene *WT1* is expressed in murine mesoderm-derived tissues and mutated in a human mesothelioma. Nat Genet 4:415–420.

Pelletier J, Schalling M, Buckler AJ, Rogers A, Haber DA, Housman DE (1991) Expression of the Wilms' tumour gene *WT1* in the murine urogenital system. Genes Dev 5:1345–1356.

Pritchard-Jones K, Fleming S, Davidson D, Bickmore W, Porteous D, Gosden C, Bard J, Buckler A, Pelletier J, Housman D, van Heyningen V, Hastie ND (1990) The candidate Wilms' tumour gene is involved in genitourinary development. Nature 346:194–197.

Prosser J, Thompson AM, Cranston G, Evans HJ (1990) Evidence that p53 behaves as a tumour suppressor gene in sporadic breast tumours. Oncogene 5:1573–1579.

Rauscher FJ III, Morris JF, Tournay OE, Cook DM, Curran T (1990) Binding of the Wilms' tumor locus zinc-finger protein to the EGR-1 consensus sequence. Science 250:1259–1262.

Riccardi VM, Sujanski E, Smith AC, Francke U (1978) Chromosomal imbalance in the aniridia-Wilms' tumor association: 11p interstitial deletion. Pediatrics 61:604–610.

Tadokoro K, Fujii H, Ohshima A, Kakizawa Y, Shimizu K, Sakai A, Sumiyoshi K, Inoue T, Hayashi Y, Yamada M (1992) Intragenic homozygous deletion of the *WT1* gene in Wilms' tumor. Oncogene 7:1215–1221.

van Heyningen V, Boyd PA, Seawright A, Fletcher JM, Fantes JA, Buckton KE, Spowart G, Porteous DJ, Hill RE, Newton MS, Hastie ND (1985) Molecular analysis of chromosome 11 deletions in aniridia-Wilms' tumour syndrome. Proc Natl Acad Sci USA 82:8592–8596.

Versnel MA, Hagemeijer A, Bouts MJ, van der Kwast ThH, Hoogsteden HC (1988) Expression of c-*sis* (PDGF B-chain) and PDGF A-chain genes in ten human malignant mesothelioma cell lines derived from primary and metastatic tumours. Oncogene 2:601–605.

Versnel MA, Bouts MJ, Hoogsteden HC, van der Kwast ThH, Delahaye M, Hagemeijer A (1989) Establishment of human malignant mesothelioma cell lines. Int J Cancer 44:256–260.

Versnel MA, Claesson-Welsh L, Hammacher A, Bouts MJ, van der Kwast ThH, Eriksson A, Willemsen R, Welma SM, Hoogsteden HC, Hagemeijer A, Heldin C-H (1991) Human malignant mesothelioma cell lines express PDGF β-receptors, whereas cultured normal mesothelial cells express predominantly PDGF α-receptors. Oncogene 6:2005–2011.

Versnel MA, Langerak AW, van der Kwast ThH, Hoogsteden HC, Hagemeijer A (1993) Expression of PDGF chains and PDGF receptors in normal and malignant mesothelial cell lines. Eur Respir Rev 3:186–188.

Versnel MA, Haarbrink M, Langerak AW, de Laat PAJM, Hagemeijer A, van den Berg L, Schrier PI (1994) Human ovarian tumours from epithelial origin express PDGF in vitro and in vivo. Cancer Genet Cytogenet 73:60–64.

Wagner JC, Sleggs CA, Marchand P (1960) Diffuse pleural mesothelioma and asbestos exposure in the North-Western Cape Province. Br J Ind Med 17:260–270.

Wang ZY, Madden SL, Deuel TF, Rauscher FJ III (1992) The Wilms' tumor gene product *WT1* represses transcription of the platelet-derived growth factor A-chain gene. J Biol Chem 267:21999–22002.

Wang ZY, Qiu QQ, Deuel TF (1993) The Wilms' tumor gene product *WT1* activates or suppresses transcription through separate functional domains. J Biol Chem 268:9172–9175.

Werner H, Re GG, Drummond IA, Sukhatme VP, Rauscher FJ III, Sens DA, Garvin AJ, LeRoith D, Roberts C (1993) Increased expression of the insulin-like growth factor I receptor gene, *IGF1R*, in Wilms' tumor is correlated with modulation of *IGF1R* promoter activity by the *WT1* Wilms' tumor gene product. Proc Natl Acad Sci USA 90:5828–5832.

Winship PR (1989) An improved method for directly sequencing PCR amplified material using dimethyl sulphoxide. Nucleic Acids Res 17:1266.

Human Molecular Genetics, Vol. 2, No. 3 259–264

Evidence that WT1 mutations in Denys–Drash syndrome patients may act in a dominant-negative fashion

Melissa H.Little+, Kathleen A.Williamson, Marcel Mannens[1], Anna Kelsey[2], Christine Gosden, Nicholas D.Hastie and Veronica van Heyningen*

MRC Human Genetics Unit, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, UK, [1]Institute of Human Genetics (AMC), University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands and [2]Department of Pathology, Royal Manchester Children's Hospital, Pendlebury, Manchester M27 1HA, UK

Received November 16, 1992; Revised and Accepted January 5, 1993

ABSTRACT

The triad of nephropathy, partial gonadal dysgenesis and Wilms' tumour (WT) is known as Denys–Drash syndrome (DDS). The WT predisposition gene WT1, which plays a vital role in both genital and renal development, is known to be mutated in DDS patients. The WT1 mutations in these patients are constitutional point mutations clustered in the zinc finger (ZF) encoding exons, particularly the exons encoding ZF2 and ZF3. The predicted functional alteration in WT1 is thought to underlie DDS aetiology either by abolishing binding of the WT1 ZF domain to its normal target DNA binding site(s), perhaps blocking the binding of the wild type WT1 present (dominant negative mutation), and/or by conferring the ability to recognise novel but inappropriate DNA binding sites (dominant mutation). We report here on the analysis of WT1 in a further five cases of DDS. In each case a constitutional point mutation was detected in either ZF2 or ZF3. Three of these mutations are novel, with two affecting the conserved histidine and cysteine residues crucial for ZF tertiary structure. The protein product of the third is predicted to lack ZF2, 3 and 4 as a result of a chain termination mutation, and is presumably incapable of binding DNA. However since the DDS phenotype is only elicited by mutations which lead to loss or alteration of ZF function (presumably DNA binding) while the N-terminal upstream portion of the gene remains intact, we suggest that a dominant negative mechanism is at work here.

INTRODUCTION

Denys–Drash syndrome (DDS) comprises the triad of partial gonadal dysgenesis, congenital or infantile nephropathy and Wilms' tumour (WT) (1,2). Patients usually present within the first three years of life (3) with renal failure or nephrotic syndrome, often accompanied by hypertension. The unifying feature of DDS is the nephropathy. This always results from glomerular abnormalities, usually focal or diffuse mesangial sclerosis, with such abnormalities histologically visible in the podocyte layer of glomerular cells (4). When possible, renal function is restored by renal transplantation at an early age, however patients with a slower onset of renal failure or those possessing original renal tissue show a high incidence of WT. Even after nephrectomy patients are still at risk for gonadoblastoma.

An association between these anomalies was first made in an individual with XX/XY mosaicism (1), and subsequently in two XY individuals with ambiguous external genitalia (2). XY patients can also present with apparently normal female genitalia, but ambiguous or dysplastic internal genitalia, sometimes including persistent Mullerian duct derivatives (uterus, fallopian tubes). XX patients are usually phenotypically normal females; however, in common with the XY patients, the internal genitalia are often found to be dysplastic. Indeed, it is now appreciated that XX individuals with normal external genitalia presenting with this form of nephropathy are at risk for WT and should be considered DDS patients (5). In general, the term DDS has been expanded to include patients with this form of nephropathy together with either gonadal dysgenesis, WT or both (3).

Genital anomalies are also present in association with WT in >50% of boys with WAGR syndrome (WT, aniridia, genitourinary abnormalities and mental retardation) (6, 7). These patients carry a constitutional heterozygous deletion of the short arm of chromosome 11, involving band 11p13. The WT predisposition gene WT1 was isolated from this region by positional cloning (8,9). WT1 is thought to act as a transcriptional regulator, a function consistent with the presence of four zinc finger (ZF) motifs at the C-terminus and an N-terminal proline-glutamine-rich transregulatory domain (8, 9). Intragenic microdeletions (10, 11, 12, 13) and point mutations (14) within WT1 have been found in both sporadic and hereditary WT. The predicted pleiotropic effect of constitutional WT1 mutation on genital development (15, 16) is now strongly supported by observations of WT1 mutations in two WT patients with cryptorchidism and hypospadias (12). It has recently been shown that DDS patients carry constitutional point mutations within the ZF region of one copy of the WT1 gene (17, 18, 19), with 20 such cases reported to date. The two central ZFs, ZF2 and ZF3, are the most commonly affected. Point mutations were reported in patients with WT and/or gonadoblastoma, and also in patients

* To whom correspondence should be addressed

+ Present address: Centre for Molecular Biology and Biotechnology, University of Queensland, St Lucia, 4072 Brisbane, Australia

with nephropathy and gonadal dysgenesis but no tumour. In every case where tumour material was examined, generation of homozygosity for the constitutional mutation was observed. Thus it seems that a constitutional missense mutation within the ZF region of one copy of WT1 is sufficient to produce gonadal dysgenesis, glomerulopathy and predisposition to WT or gonadoblastoma, whereas the total deletion of one copy of WT1, as is the case in WAGR, (11p del) (16) or a more N-terminal mutation (11,12) often produces much milder genital anomalies (usually cryptorchidism and/or hypospadias) and no overt kidney disease. It was therefore proposed that the WT1 mutations present in DDS patients produce a mutant protein with altered function which overrides the normal activity of the remaining wild type WT1 allele. With rare exception (see later) all of the reported mutations in DDS affect amino acids predicted to play a critical role in ZF DNA-binding (20).

In this study we have investigated a further five DDS patients for WT1 mutations. While all patients showed the characteristic renal pathology, the severity of the genital anomalies and presence of a tumour varied. All patients were found to have constitutional WT1 point mutations. However, the observation of a chain termination mutation in ZF2 in one case, as well as a case reported by Baird et al (19), suggests that the DDS phenotype can arise from loss as well as alteration of specific WT1 DNA binding, suggesting a dominant negative rather than a dominant mode of action.

RESULTS

Patients

The clinical features of the 5 patients investigated in this study are summarised in Table 1A. The severity of the DDS phenotype is variable within this series. Patient 4 exhibited a number of other anomalies not generally associated with DDS but not uncommon in other WT patients. Horseshoe kidney has previously been reported in association with sporadic WT (21) and in Beckwith–Wiedemann syndrome (BWS) patients (22). Craniostenosis and prominent metopic suture have also been observed in BWS patients (22). The respiratory distress and hypertension seen in patients 3 and 5 has been observed in other DDS patients (2, 3). While two of the patients did not present with WT, this may have been due to the early advent of death

Table 1A. Summary of the clinical features of the patients described in this paper.

PATIENT	1	2	3	4	5
GENOTYPE	46, XY	46, XX	NA	46, XY	46, XY
GENITALIA	NORMAL FEMALE	NORMAL FEMALE	NORMAL FEMALE	MICROPENIS, CRYPTORCHIDISM	HYPOSPADIAS
WILMS TUMOR	NIL	BILATERAL	UNILATERAL	BILATERAL	NIL
'DRASH' NEPHROPATHY	YES	YES	YES	YES	YES
OTHER FEATURES			CEREBRAL ATROPHY, PYSCHOMOTOR DELAY, CHRONIC BRONCHITIS, LOW IgG	GROSS MOTOR DELAY, CRANIOSTENOSIS, PROMINENT METOPIC SUTURE, HORSESHOE KIDNEY	HYPERTENSION EXTREME OEDEMA, RESPIRATORY INFECTIONS AND PNEUMONIA

Table 1B. Summary of the genetic features of the patients described in this paper.

PATIENT	1*	2	3	4	5
LOCATION	ZF3	ZF3	ZF2	ZF2	ZF2
POINT MUTATION	C to T	G to A	T to G	C to T	C to G
AMINO ACID CHANGE	394R to W	396D to N	360C to G	362R to STOP	373H to Q
CONSTITUTIONAL MUTATION	HETEROZYGOUS	HETEROZYGOUS	HETEROZYGOUS	HETEROZYGOUS	HETEROZYGOUS
TUMOUR MUTATION	N/A	HOMOZYGOUS	HETEROZYGOUS	HOMOZYGOUS	N/A

Shaded boxes represent previously undescribed mutations or phenomena. *The mutation in patient 1 was independently detected by Baird et al (19).

or transplantation. Tumour material was analysed from all three patients who developed WT.

Diagnostic chorionic villus sampling (CVS) was requested, primarily because of advanced maternal age, in a subsequent pregnancy from the parents of patient 1. At the time this individual went for CVS the pregnancy was found to be non viable, and an evacuation of the products of conception was performed. These were used for karyotyping and WT1 mutation analysis.

WT1 mutation analysis

Chemical mismatch cleavage (Fig. 1) or direct sequence analysis (Figs. 2 and 3) of PCR products from these DDS patients identified the presence of WT1 point mutations in ZF2 or ZF3 in all individuals (Table 1B). These mutations were constitutionally heterozygous (that in patient 4 was also verified by Mark Patterson, Cambridge, personal communication). Homozygosity for the mutant WT1 allele was seen in the tumours from patients 2 and 4. The tumour from patient 3 retained both alleles (Fig. 3B). The mutations seen in patients 1 and 2 (Figs. 1 and 2) have previously been reported in other DDS patients (17, 18, 19) however the other three patients show novel DDS point mutations. Analysis of constitutional DNA from the only available parents, those of patients 1, 3, and 5, revealed no WT1 mutations (Figs. 2B, 3A and B), establishing that the mutations in these DDS patients arose *de novo*.

Predicted consequence of detected mutations

The close similarity in the amino acid sequence of EGR1 and WT1 led to the suggestion, and in vitro demonstration (23, 24), that they bind similar DNA targets. NMR and X-ray crystallography analysis of EGR1 DNA-binding (20) has revealed the key amino acids which confer binding specificity. The ZF3 mutations of ^{394}arginine (R) to tryptophan (W) in patient 1, and of ^{396}aspartic acid (D) to asparagine (N) in patient 2 (Fig. 2A) both affect DNA-binding by disrupting hydrogen bonding with a critical guanine residue in the target site. Such mutations may be expected, in principle, to act in a dominant or dominant negative fashion.

The mutations found in patients 3 and 5 affect either a conserved cysteine (C) or histidine (H) residue (Fig. 3A) both of which are zinc chelating amino acids crucial to the tertiary structure of this class of zinc finger proteins ($C_2.H_2$ zinc finger motif). Therefore, as the mutant ZF2 in these patients will not fold into a finger structure, DNA-binding will be compromised and may be prevented. Even if such conformational changes do not de-stabilise the surrounding ZFs, the specificity and affinity of WT1-DNA binding is likely to be affected.

The point mutation in patient 4 converts ZF2 ^{362}R into a termination codon (Fig. 3A) which will completely eliminate binding of mutant WT1 protein to DNA by truncating the last three ZFs. This patient provides strong evidence that the DDS mutations do not act in a dominant fashion as this effectively fingerless form of WT1 is extremely unlikely to have a novel DNA binding specificity.

Screening for common DDS mutations using restriction enzymes

As the number of DDS patients analysed for WT1 mutations increases, certain mutations appear to predominate. The most common WT1 mutation, representing 12 of the 20 mutations now detected (17, 18, 19), is a C to T transition in ZF3 resulting in an R to W amino acid change. A number of other mutations have also been reported more than once. We therefore set out to establish rapid restriction-based assays for these prevalent DDS WT1 mutations, eliminating the need for more labour intensive methods of mutational analysis in such cases.

The two most common DDS mutations, ^{394}R to W (60%) and ^{396}D to N (15%), abolish an RsrII site in ZF3 (Fig. 2A), preventing the digestion of a 349bp PCR fragment (primers 798/801) into 167bp and 182bp fragments (Fig. 2B). As the presence of a mutation in this case prevents digestion, patient samples should be control digested with a known cutting enzyme.

Figure 1. Chemical mismatch cleavage patterns of ZF3 PCR product for patients 1 and 2. Modification was performed with both hydroxylamine (Ha) and osmium tetroxide (OsO_4). Patient 1: lane 1, negative control; lane 2, constitutional DNA. Patient 2: lane 1, negative control; lane 2, left tumour DNA; lane 3, right tumour DNA; lane 4, constitutional DNA; lane 5, parental constitutional DNA. The nature of the mutation in patient 1 (C to T) results in cleavage of Ha modified fragments, whereas the nature of the mutation in patient 2 (G to A) results in cleavage of both Ha and OsO_4 modified fragments.

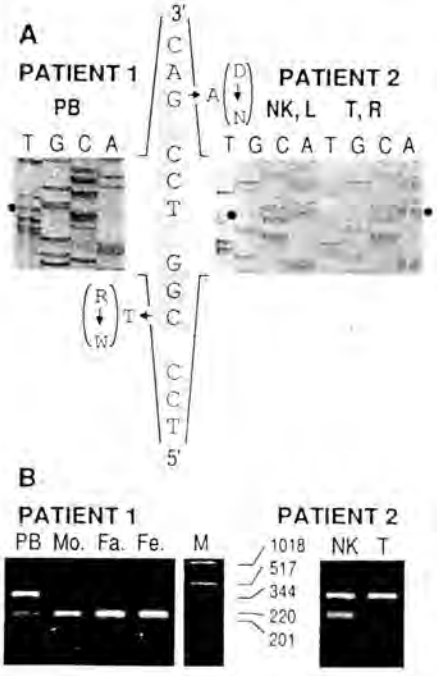


Figure 2.A. Sequencing of ZF3 for patients 1 and 2, showing point mutations. PB = peripheral blood; NK,L = normal kidney, left; T,R = tumour, right. The recognition sequence of RsrII is shaded. **B.** RsrII restriction digest patterns for patients 1 and 2. Mo. = mother; Fa. = father; Fe. = foetus; M = molecular weight markers.

The third reported DDS mutation in this portion of ZF3, ^{396}D to glycine (G), creates a HaeIII site resulting in a pattern of 4 bands (21bp, 60bp, 81bp and 182bp) rather than 3 bands (21bp, 60bp and 263bp).

The PCR product for ZF2 (primers C323/796) contains a single HphI site, which cleaves the 352bp fragment into a 70bp and a 282bp fragment. Chemical mismatch cleavage analysis of patient 3 revealed a ^{360}C to G mutation in ZF2. This T to G mutation creates a new HphI site (Fig. 3A) within the 282bp HphI fragment resulting in 32bp and 250bp bands (Fig. 3B). This provides another restriction-based screen for a DDS mutation, but as this mutation has only been described once it may be of less use than the other two digests.

Prenatal screening

From what is now known of the nature of WT1 mutations in DDS patients, the likelihood of siblings presenting with DDS is highly unlikely, as it would require either germline mosaicism for such a mutation in a parent or two distinct *de novo* DDS WT1 mutations occurring in the same family. There has however been one report of nephrotic syndrome and WT in siblings (25). During this study, we were asked to examine tissue from the nonviable foetal sibling of patient 1 to ascertain whether it carried a WT1 mutation. As the likelihood of two *de novo* DDS mutations was minute, we first identified the point mutation present in the affected child, patient 1, and then used PCR and restriction digest screening to look for the ^{394}R to W mutation in the foetal sample. The foetal sample was normal at this base (Fig. 2B) and for all other mutations detectable by restriction digest screening. Cytogenetic analysis of foetal tissue revealed that the foetus was 47,XY,+21, which may have been a contributing factor to the cause of death *in utero*.

DISCUSSION

Mutations within the 11p13 Wilms' tumour gene have now been described in WT patients both with and without any associated congenital anomalies. However, the proportion of WT patients with identified WT1 mutations is still small, except for the subgroup of WT patients displaying the Denys-Drash syndrome of nephropathy and gonadal dysgenesis. Almost all DDS patients analysed have a single constitutional WT1 point mutation and these predominantly result in an amino acid change within a ZF. As these mutations appeared to act in a dominant fashion, it was possible that the mutant WT1 protein bound to and regulated a novel and inappropriate DNA target (dominant) or that it was able to interfere with or inhibit the normal activity of the wild type protein (dominant-negative).

It may be envisaged that in DNA-binding proteins, the substitution of critical amino acid residues in the DNA-binding domain may alter the specificity of the DNA-binding. This has been described for the steroid hormone receptors which belong to a family of ZF genes where four C residues complex one zinc molecule. Alteration of a single amino acid in the ZF region of the glucocorticoid receptor alters the DNA binding specificity such that the receptor can now bind both the glucocorticoid and oestrogen responsive elements (26). The first DDS point mutations described (17) are predicted to disrupt WT1 binding in a similar manner (20). However, a case was recently reported with a mutation of a ZF1 zinc chelating C (18) . In this study we have found two further cases of DDS with mutation in zinc chelating C and H residues in ZF2 (patients 3 and 5, Table 1B). Such mutations are unlikely to confer a unique new binding specificity. It is probable that they will result in a de-stabilisation of ZF tertiary structure and compromise DNA binding.

The concept that loss, as opposed to alteration, of DNA-binding can lead to the severe DDS phenotype is clearly shown by the chain termination mutation in ZF2 of patient 4. An even more clear-cut loss-of-binding is seen in the exon 6 chain termination mutation which results in complete absence of all four zinc fingers in DDS patient GOS389, described by Baird et al (19). Such truncation mutations indicate that DDS mutations may act to effectively destroy WT1-DNA binding without altering the rest of the protein. As only one WT1 allele is mutated, this appears to confer onto the mutant protein a dominant effect over the wild type molecule. There are many examples of DNA-binding transcription factors whose activity is modulated or facilitated by protein-protein interactions, such as homo-multimerisation. The steroid hormone receptors contain a dimerisation domain which consists of a series of discontinuous hydrophobic heptad repeats, not dissimilar to a leucine (L)-zipper motif (27). Although there is no evidence to date that WT1 binds as a dimer, exon 4 of the WT1 gene does encode a pseudo-L-zipper motif similar to that in the steroid hormone receptors. This motif consists of three continuous hydrophobic heptad repeats together with three other possible discontinuous heptads. These heptads are perfectly conserved in the mouse WT1 gene. It has been shown in some L-zipper proteins that the introduction of mutants containing the dimerisation component but no functional domain cause the accumulation of inactive mutant-wild type protein oligomers (28). Given that DDS can arise from truncation mutations, we hypothesise that the DDS phenotype may result from the formation of inactive mutant-wild type WT1 protein dimers. While this has yet to be formally tested, we have isolated clones to which WT1 specifically binds and these contain both continuous and inverted repeat sequences, suggesting a double

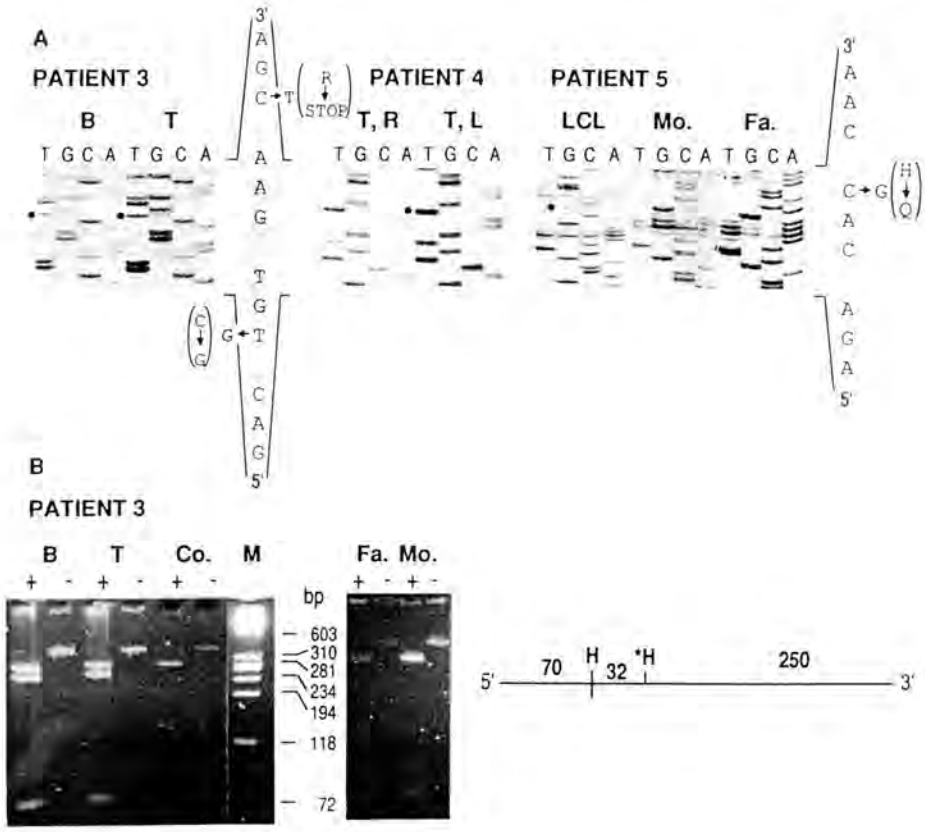


Figure 3.A. Sequencing of ZF2 for patients 3, 4 and 5, showing point mutations. B = blood; T = tumour (R, right or L, left); LCL = lymphoblastoid cell line; Mo. = mother; Fa. = father. The recognition sequence of HphI is shaded. **B.** HphI restriction digest patterns for patient 3. Co. = control; M = molecular weight markers; Fa. = father; Mo. = mother; + = HphI; − = no HphI. The HphI site created by the T to G point mutation (*H) cuts the 282bp fragment into a 250bp and a 32bp fragment, the latter migrating too fast to be detected on the gel.

binding site (24). In addition, studies currently in progress characterising WT1 binding to a candidate target promoter have revealed a double WT1 footprint, suggesting that two adjacent molecules of WT1 protein bind to the promoter (Andrew Ward, Oxford, personal communication). Whether WT1 dimerises remains to be tested formally. If it does, then the mutant form of the protein in DDS patients is likely to be acting in a dominant-negative fashion by complexing with the wild type protein, and preventing DNA-binding of this complex.

All previous studies have demonstrated LOCH wherever tumour tissue was available from DDS patients (17, 18, 19) or from other constitutional WT1 mutation patients (11,12). This suggests that while DDS mutations appear to have a dominant effect over normal glomerular and genital development, tumorigenesis requires mutation of both WT1 alleles. However, we have previously described two non-DDS WT patients in which only one WT1 allele was found to be mutated in the tumours (14, 29) and proposed that while mutation of both WT1 alleles is one pathway to tumour formation, this is sufficient but not essential. It now appears that DDS is no exception to this observation as we describe in this study a tumour from patient 3 which retains both constitutional WT1 alleles. In this patient, it is possible that there has been a second mutation in either WT1 exons 1, 2, 3 or 5 (not yet analysed) or alternatively that a second mutation has occurred at another WT locus other than WT1. 11p15 specific allele loss has been reported in WT and this may represent the second mutation in this tumour. This has not been previously observed in a DDS tumour.

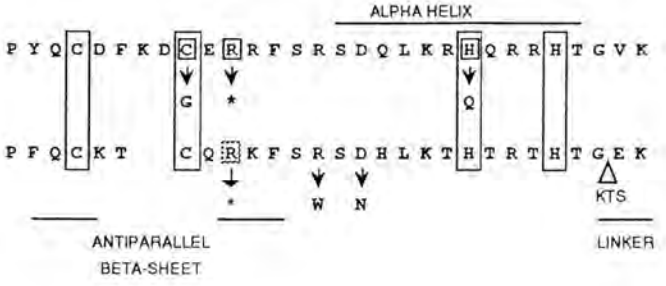


Figure 4. Diagram of ZF2 and 3 indicating the conserved cysteine and histidine residues and the mutations found in this series of DDS patients. Also indicated is a ZF3 R to STOP mutation which we have previously reported in a bilateral WT patient with no evidence for genital anomalies.

In summary, we have identified and characterised WT1 mutations in five more DDS patients, further supporting a role for WT1 in both renal and genital development. Our observation of a ZF2 truncation mutation indicates that DDS WT1 mutations can most likely lead to abolition of DNA-binding. Consequently we have hypothesised that WT1 binds as a dimer and that it is the formation of wild type/mutant heterodimers which interferes with normal WT1 activity. We feel that this is evidence that DDS mutants act in a dominant-negative fashion resulting from loss or reduction in normal WT1 DNA-binding.

MATERIALS AND METHODS

DNA extraction and PCR

DNA was prepared from lymphoblastoid cell lines (LCL) or peripheral blood samples from 5 DDS patients as previously described (29). Tumour sample DNA was extracted from 3 of these patients. When available, DNA was also prepared from parental peripheral blood or LCLs. The oligonucleotide primers used for amplification of WT1 exons 4, 6, 7 (ZF1), 8 (ZF2), and 9 (ZF3) lay in the preceding and following intronic sequences. For exon 10 (ZF4), only the ZF region of the exon was examined. Many of these oligonucleotides have been previously described (14). The primers used were as follows.

Exon 4:	C486 (5′ AAA CAG TTG TGT ATT ATT TTG TGG 3′) and C152 (5′ ACT TTC TTC ATA AGT TCT AAG CAC 3′); 225bp
Exon 6:	C178 (5′ AAG CTT CAC TGA CCC TTT TTC CCT TC 3′) and C177 (5′ GAA TTC CAA AGA GTC CAT CAG TAAGG 3′);230bp
Exon 7 (ZF1):	C155 (5′ GAC CTA CGT GAA TGA TCA CAT G 3′) and 945 (14); 348bp
Exon 8 (ZF2):	C323 (5′ CCT TTA ATG AGA TCC CCT TTT CC 3′) and 796 (14); 352bp
Exon 9 (ZF3):	798 and 801 (14); 349bp
Exon 10 (ZF4):	802 and 447 (14); 354bp or C911 (5′ ACT TCA CTC GGG CCT TGA TAG 3′) and C912 (5′ GTG GAG A-GT CAG ACT TGA AAG 3′); 275bp

PCR reactions were 50μl total volume containing 200ng of genomic DNA, 200μM nucleotides, 1μM of each primer, 1×Promega PCR buffer and 1U of Promega Taq Polymerase. Reactions were denatured at 92C for 45s, annealed at the appropriate temperature for 1 min and elongated at 72C for 1–1.5 min for 30 cycles (Hybaid Thermal Cycler; tube control). All PCR fragments were purified from low-melting temperature agarose (SeaPlaque) using Mermaid (Bio101).

Chemical mismatch cleavage analysis and direct sequencing

Chemical mismatch cleavage analysis was performed as previously described (14,30) using both hydroxylamine and osmium tetroxide. One lane of a PCR fragment sequencing reaction was run as a size marker. When a mismatch was detected, independently amplified fragments were directly sequenced as previously described (14,31) using either the same primers as were used for the PCR reaction or internal primers. This enabled the characterisation of any specific base changes and determined whether the sample was homo- or heterozygous for that mutation.

Restriction analysis

After amplification excess paraffin oil was removed and the PCR reactions were chloroform extracted, precipitated with ethanol, washed two times in 70% ethanol, vacuum dried and resuspended in PCR-quality H_2O. Restriction digests were performed in 1×restriction digestion buffer of appropriate salt composition and concentration for the restriction enzyme (Boehringer Mannheim or New England Biolabs) used. Digests were performed overnight and the results run out on either a 2% agarose gel (RsrII digests) or a 6% Nusieve gel (HphI digests) adjacent to a ϕX174 HaeIII size marker.

Cytogenetics

Phytohaemagglutinin-stimulated peripheral blood lymphocyte cultures from patient 1 were set up to provide mitotic chromosomes for cytogenetic analysis (32). Foetal tissues from the missed abortion in the mother of patient 1 were also cultured for karyotypic analysis (33).

ACKNOWLEDGEMENTS

We thank Dr Kathryn Pritchard-Jones, Royal Marsden Hospital, Sutton, Surrey, for her co-operation and helpful discussion. Special thanks to Agnes Gallagher for oligonucleotide synthesis and Douglas Stuart for photography. Dr Paul Hoban at the Paterson Institute for Cancer Research, Manchester, The Royal Manchester Children's Hospital and Booth Hall Children's Hospital, Manchester, the Universitatsklinikum Essen, Germany and Dr R.S. Trompeter at the Hospitals for Sick Children, Great Ormond Street, London provided invaluable clinical material. ML was funded by the Royal Society as an Endeavour Postdoctoral Fellow.

REFERENCES

1. Denys, P., Malvaux, P., van den Berghe, H., Tanghe, W. and Prosemans, W. (1967) *Arch. Franc. Ped.*, 24, 729–739.
2. Drash, A., Sherman, F., Hartmann, W.H. and Blizzard, R.M. (1970) *J. Pediatr.*, 76, 585–593.
3. Eddy, A.A. and Mauer, S.M. (1985) *J. Pediatr.*, 106, 584–587.
4. Habib, R., Loirat, C., Gubler, M.C., Niaudet, P., Bensman, A., Levy, M. and Broyer, M. (1985) *Clin. Nephrol.*, 24, 267–278.
5. Friedman, A.L. and Findlay, J.L. (1987) *Am. J. Med. Genet. (Suppl.)*, 3, 293–296.
6. Turleau, C., deGrouchy, J., Tournade, M.F., Gagnadoux, M-F. and Junien,C. (1984) *Clin. Genetics.*, **26**, 356–362.
7. Breslow, N., Beckwith, J.B., Ciol, M. and Sharples, K. (1988) *Cancer Res.*, **48**, 1653–657.
8. Call, K.M., Glaser, T., Ito, C.Y., Buckler, A.J., Pelletier, J., Haber, D.A., Rose, E.A., Kral, A., Yeger, H., Lewis, W.H., Jones, C. and Housman, D.E. (1990) *Cell*, **60**, 509–520.
9. Gessler, M., Poutska, A., Cavenee, W., Neve, R.L., Orkin, S.H. and Bruns, G.A.P. (1990) *Nature*, **343**, 774–778.
10. Haber, D.A., Buckler, A.J., Glaser, T., Call, K.M., Pelletier, J., Sohn, R.L., Douglass, E.C. and Housman, D.E. (1990) *Cell*, 61, 1257–1269.
11. Huff, V., Miwa, H., Haber, D.A., Call, K.M., Housman, D., Strong, L.C. and Saunders, G.F. (1991) *Am. J. Hum. Genet.*, 48, 997–1003.
12. Pelletier, J., Bruening, W., Li, F.P., Haber, D.A., Glaser, T. and Housman, D.E. (1991a) *Nature*, 353, 431–434.
13. Brown, K.W., Watson, J.E., Poirier, V., Mott, M.G., Berry, P.J. and Maitland, N.J. (1992) *Oncogene*, 7, 763–768.
14. Little, M.H., Prosser, J., Condie, A., Smith, P.J., van Heyningen, V. and Hastie, N.D. (1992a) *Proc. Natl. Acad. Sci. USA.*, **89**, 4791–4795.
15. Pritchard-Jones, K., Fleming, S., Davidson, D., Bickmore, W., Porteous, D., Gosden, C., Bard, J., Buckler, A., Pelletier, J., Housman, D.E., van Heyningen, V. and Hastie, N.D. (1990) *Nature*, 346, 194–197.
16. van Heyningen, V., Bickmore, W.A., Seawright, A., Fletcher, J., Maule, J., Fekete, G., Gessler, M., Bruns, G.A.P., Huerre-Jeanpierre, C., Junien, C., Williams, B.R.G. and Hastie, N.D. (1990) *Proc. Natl. Acad. Sci. USA.*, **87**, 5383–5386.
17. Pelletier, J., Bruening, W., Keshtan, C.E., Mauer, S.M., Manivel, J.C., Striegel, J.L., Houghton, D.C., Junien, C., Habib, R., Fouser, L., Fine, R.N., Silverman, B.L., Haber, D.A. and Housman, D.E. (1991b) *Cell*, **67**, 437–447.
18. Bruening, W., Bardessy, N., Silverman, B.L., Cohn, R.A., Machin, G.A., Aronson, A.J., Housman, D. and Pelletier, J. (1992) *Nature Genetics*, 1, 144–148.
19. Baird, P.N., Santos, A., Groves, N., Jadresic, L. and Cowell, J.K. (1992) *Human Molecular Genetics*, 1, 301–305.
20. Pavletich, N.P. and Pabo, C.O. (1991) *Science*, 252, 809–817.
21. Miller, R.W., Fraumeni, J.F. and Manning, M.D. (1964) *N. Engl. J. Med.*, **270**, 922–927.
22. Sotelo-Avila, C., Gonzalez-Crussi, F. and Fowler, J.W. (1980) *J. Pediatr.*, **96**, 47– 50.
23. Rauscher III, F.J., Morris, J.F., Tournay, O.E., Cook, D.M. and Curran, T. (1990) *Science*, **250**, 1259–1262.
24. Bickmore, W.A., Oghene, K., Little, M.H., Seawright, A., van Heyningen, V. and Hastie, N.D. (1992) *Science*, **257**, 235–237.
25. Zunin, C. and Soave, F. (1964) *Ann. Pediatr.*, **203**, 29–38.
26. Umesono, K. and Evans, R.M. (1989) *Cell*, **57**, 1139–1146.
27. Forman, B.M. and Samuels, H.H. (1990) *Mol. Endocrinol.*, **4**, 1293–1301.
28. Smeal, T., Angel, P., Meek, J. and Karin, M. (1989) *Genes Dev.*, **3**, 2091–2100.
29. Little, M.H., Byrne, J., Clarke, J., Dunn, R. and Smith, P.J. (1992b) *Eur. J. Cancer.*, **28A (11)**, 1879–1883.
30. Cotton, R.G.H., Rodrigues, N.R. and Campbell, R.D. (1988) *Proc. Natl. Acad. Sci. USA*, 85, 4397–4401.
31. Winship, P.R. (1989) *Nucleic Acids Research*, 17, 1266.
32. Gosden, C.M., Davidson, C. and Robertson, M. (1992) In Rooney, D. and Czeplekowski, B. (eds.) Practical Series 'Human Cytogenetics'. OUP, Oxford, *in press.*
33. Gosden, C.M. (1992) In Brock, D.J.H., Rodeck, C.H. and Ferguson-Smith, M.A. (eds.) Prenatal Diagnosis and Screening. Churchill Livingston, Edinburgh, *in press.*

Do children with diffuse mesangial sclerosis in association with mutations of the Wilms' tumour suppressor gene (WT1) require bilateral nephrectomy?

Key words: Diffuse mesangial sclerosis – Wilms' tumour suppressor gene – Nephrectomy

Sirs,

Denys-Drash syndrome (DDS), characterised by the association of an early-onset nephropathy [commonly diffuse mesangial sclerosis (DMS)], pseudohermaphroditism in males and a high risk for developing Wilms' tumour (WT) was first described in 1967 [1]. More recent publications have proposed extending the concept of the syndrome to those with only two features of the disease, including the nephropathy [2]. The development of WT in DDS has been linked to germline mutations of the WT1 gene (chromosomal position 11p13), which encodes a zinc finger protein thought to have transcriptional regulatory function. These mutations almost always occur de novo and the majority are clustered within exon 9 of the WT1 gene. It has been suggested that as DMS is intimately associated with DDS, all phenotypic females presenting with DMS should have chromosome studies performed to detect unexpected XY karyotypes and all children undergo ultrasonography to exclude WT [3].

We present a 17-month-old boy with DDS in whom intensive investigation and intervention led to the prevention of WT. He presented with hypertensive encephalopathy and oedema, and was found to have renal failure (sodium 136 mmol/l, potassium 7.6 mmol/l, urea 25.7 mmol/l, creatinine 311 μmol/l) with nephrotic syndrome [urine protein/creatinine ratio 3,875 mg/mmol (normal 0–25), serum albumin 15 g/l]. Physical examination revealed hypospadias with bilaterally descended testes and scars from previous bilateral hernia repairs. His initial treatment consisted of positive pressure ventilation, intravenous labetalol and peritoneal dialysis (PD). Immunological causes for renal failure were excluded and his renal tract was structurally normal on ultrasound.

Renal biopsy showed one of nine glomeruli to be globally sclerosed; the remainder all showed diffuse mesangial hypercellularity with expansion of mesangial matrix and sclerosis. There was focal tubular atrophy with interstitial fibrosis and the vessels showed marked medial hypertrophy. The findings were consistent with a diagnosis of DMS. His karyotype was 46 XY. DNA analysis [4] revealed a mutation in exon 9 (encoding zinc finger 3) of one copy of the WT1 gene, a CGG codon (encoding arginine) having mutated to TGG (encoding tryptophan). Neither parent carried this mutation.

A diagnosis of DDS was made. He was discharged on PD 36 days after admission, clinically well with his hypertension controlled with captopril. Despite improvement in the intrinsic renal function, we elected to proceed to bilateral nephrectomy because of the considerable risk for the development of WT. As part of the pre-operative work-up, an abdominal computed tomographic scan was performed which was entirely normal. At surgery, a small nodular area, 1.4 cm in diameter, was noted close to the left renal hilum. Histological examination revealed a cyst lined with cuboidal ciliated epithelium and surrounded by groups of immature tubules, primitive ducts with a collar of fibromuscular tissue, mesenchyme, bundles of smooth muscle and islands of fat. Blastemal cells were not prominent, but the appearances were compatible with a cystic intralobar nephrogenic rest, blending imperceptibly into the renal parenchyma. There was no evidence of malignancy. The remaining renal tissue showed most of the glomeruli to be globally sclerosed with severe tubular atrophy and a heavy interstitial inflammatory infiltrate, consistent with end-stage kidney in DMS. There were no peri-operative complications, and he is currently well on PD.

Whilst the natural history of nephrogenic rests in those without WT1 mutations is somewhat variable, a small proportion develop into WT. In those with WT1 mutations in association with DDS, WT occurs in over 90% [5]. Two of the three children with DDS on our end-stage renal failure (ESRF) programme presented with bilateral WT. By electing for bilateral nephrectomy in this child, we have removed the risk of WT and its treatment-associated morbidity, including prolongation of time to consideration for transplantation. It can be aruged that early bilateral nephrectomy is preferable to serial radiological examinations in this population. This is not a problem in those with nephropathy, as the time-course from presentation to ESRF is short, although the role of bilateral nephrectomy in those who have not yet developed nephropathy is more debatable.

We recommend that all children with early-onset proteinuria, especially where DMS is present on biopsy, require DNA analysis to exclude mutations of the WT1 gene and that bilateral nephrectomy is considered early in those children with DDS, WT1 mutation and overt nephropathy. We would be most interested to hear the opinions from other centres.

N. J. A. Webb[1], M. A. Lewis[1], K. Williamson[4], V. van Heyningen[4], J. Bruce[2], M. Lendon[3], and R. J. Postlethwaite[1]
Departments of [1] Nephrology, [2] Surgery, and [3] Pathology
Royal Manchester Children's Hospital
Pendlebury
Manchester, UK [4] MRC Human Genetics Unit
Western General Hospital
Edinburgh, UK

References

1. Denys P, Malvaux P, Berghe H van den, Tanghe W, Proesmans W (1967) Association d'un syndrome anatomo-pathologique de pseudohermaphroditisme masculin, d'une tumeur de Wilms, d'une nephropathie parenchymateuse et d'un mosaicisme XX/XY. Arch Fr Pediatr 24: 729–739
2. Habib R, Loirat C, Gubler MC, Niaudet P, Bensman A, Levy M, Broyer M (1985) The nephropathy associated with male pseudohermaphroditism and Wilms tumour (Drash syndrome): a distinctive glomerular lesion – report of ten cases. Clin Nephrol 24: 269–278

3. Habib R, Gubler M, Antignac C, Gagnadoux M (1993) Diffuse mesangial sclerosis: a congenital nephropathy with nephrotic syndrome. Adv Nephrol 22: 43–57
4. Little MH, Williamson KA, Mannens M, Kelsey A, Gosden C, Hastie ND, Heyningen V van (1993) Evidence that WT1 mutations in Denys Drash syndrome patients may act in a dominant negative fashion. Hum Mol Genet 2: 259–264
5. Coppes MJ, Haber DAH, Grundy PE (1994) Genetic events in the development of Wilms tumour. N Engl J Med 331: 586–590

Renal anomalies in Down syndrome

Key words: Renal anomalies – Down syndrome

Sirs,

We read with interest the comments by Ehrich [1] and by Robson and Leung [2] in this journal about renal replacement therapy and renal abnormalities, respectively, in children with Down syndrome. Since there are only a few reports, we reviewed the outpatient charts of all children with Down syndrome who were seen in the Pediatric Cardiology Clinic at James H. Quillen College of Medicine and East Tennessee State University, Johnson City, Tennessee. This report describes our findings and a review of the literature.

Fifty-four children (34 males, 20 females) aged 2 months to 24 years (median 4.5 years) with Down syndrome were seen in our clinic over the last 8 years. Clinical history and laboratory data were reviewed for any possible renal disorder. Follow-up data were obtained from the primary physician either by questionnaires or by phone, or by both. Of the 54 patients, 40 had congenital heart disease. All had normal urinalysis. Serum creatinine and blood urea nitrogen were measured in 28 children and were normal. Thirteen children had flat plate of the abdomen immediately after cardiac catheterization to visualize the kidney, ureter, and bladder; these were normal in all.

Six children had symptoms related to their urinary tract; 5 of these, all females, had recurrent urinary tract infections. Further investigations with one or more of the following tests: voiding cystourethrography, abdominal ultrasound, intravenous pyelography, and blood urea nitrogen and serum creatinine were performed in these 3 patients, but all were within normal limits. One male child had urethral stricture that required repeated dilatations following surgical correction. This child had a normal functioning kidney as indicated by a normal blood urea nitrogen and serum creatinine. No further details are available about this child. Of the 6 patients, 2 developed acute renal failure following angiotensin converting enzyme (ACE) inhibitor therapy for cardiac failure and died. One of them had normal kidneys by gross and microscopic examination at autopsy; the other patient did not have an autopsy.

From our limited retrospective clinical data, the incidence of renal anomalies in children with Down syndrome does not appear to be higher than in the normal population. It should be noted that our series did not include fetuses, still-borns, or infants with Down syndrome who may have died in the nursery with renal disease. Hitherto, three autopsy studies have reported renal anomalies in children with Down syndrome. In 1960, Berg et al. [3] reported 4 patients with renal agenesis or hypoplasia and 1 with horseshoe kidney of 141 autopsies (2.8%) of patients with Down syndrome. In 1973, Egli and Stalder [4] reported renal anomalies in 7 of 103 autopsies (6.7%) of children with Down syndrome (cysts 2, hydronephrosis 1, hydroureter 3, ureteral stenosis 2, megacystitis 3). In 1991, Ariel et al. [5] reported renal anomalies, such as renal hypoplasia, cysts, and obstructive uropathy (4% in infants and children, 15% in still-borns and fetuses), in 124 autopsy cases of Down syndrome.

Besides these and other reports [6, 7] of congenital renal anomalies, there are few isolated reports of acquired renal disorders in children with Down syndrome, such as chronic glomerulonephritis [2], mesangiocapillary glomerulonephritis [8], and immunolactoid glomerulopathy [9]. Nonstructural renal disorders, such as hyperuricemia and a decrease in the clearance of uric acid and creatinine, are also reported in children with Down syndrome [10].

Although 6 patients in our series had signs and symptoms of renal disease, none had renal pathology except for 1 child who had urethral stricture. Two children (a 5-year-old male and a 5-month-old female) with heart failure and Down syndrome developed renal failure after ACE inhibitor therapy and died before renal replacement therapy. An association between ACE inhibitor therapy and acute renal dysfunction has been noted in patients with bilateral renal diseases such as polycystic kidney [11], bilateral renal artery stenosis, and scleroderma [12]. Neither of our 2 patients had any preexisting renal disease. Both had normal urinalysis and normal blood levels of urea and creatinine prior to ACE inhibitor therapy. One also had a normal gross and microscopic examination of both kidneys at autopsy. Nevertheless, several minor renal abnormalities, such as immature glomeruli [7], renal hypoplasia or relatively small kidney [13], maturation delay of nephrogenic zone of cortex, and persistent fetal lobulation, are reported in children with Down syndrome [13, 14]. These abnormalities may predispose to a high risk for renal failure when exposed to ACE inhibitors.

From our data and those of others [3–5], the incidence of clinically significant renal anomalies does not appear to be higher in children with Down syndrome than in the general population. We, however, recommend that caution should be exercised when using ACE inhibitors in children with Down syndrome and congestive heart failure. A larger clinical study of a similar cohort may further delineate this issue.

Int. J. Exp. Path. (1994), **75**, 147–155

Current Status Review

Towards an understanding of Wilms' tumour

KATHLEEN A. WILLIAMSON AND VERONICA VAN HEYNINGEN
MRC Human Genetics Unit, Western General Hospital, Edinburgh, Scotland

Wilms' tumour: histopathology and statistics

Wilms' tumour (WT) or nephroblastoma is a paediatric renal malignancy first described by Max Wilms in 1899. It affects approximately 1 in 10 000 children, making it the most common solid tumour of childhood. Diagnosis is normally made between 2 and 4 years of age (Breslow *et al.* 1988), with the overall survival rate at present exceeding 80% (Mehta *et al.* 1991).

The histology of WT most clearly demonstrates the embryonal nature of this malignancy, with the constituent elements of the tumour recapitulating the early stages of nephrogenesis. Post pronephric and mesonephric renal development begins with the condensation of metanephric mesenchyme, by a reciprocal inductive interaction with the ureteric bud, to form the metanephric blastema (Figure 1). This in turn becomes epithelialized and progresses through the multiple stages of nephrogenesis to form the permanent mammalian kidney, the metanephros. WT is thought to arise from aberrant development of the metanephric blastema, with the cells being held at the early, embryonal stage. The transient immature epithelial structures characteristic of this stage, such as renal vesicles, comma, S-shaped and glomeruloid bodies, persist in the tumour, whereas the development of mature renal structures, such as fully formed glomeruli and proximal and distal tubules, is blocked. In addition to the blastemal and epithelial elements WT also contain stroma which, together with the epithelium, is thought to derive from the blastema (Pritchard-Jones & Fleming 1991). The majority of WT contain approximately equal proportions of these three elements and are therefore said to display triphasic or classic histology. Cell types not normally found in the kidney are present in a minority of WT. Such heterotopic elements include mesodermally derived striated and smooth muscle cells, chondrocytes, adipocytes, and also neuroblasts and ganglion cells (Sotelo-Avila 1990). This histological picture suggests than an early pluripotential stem cell, involved in kidney development and perhaps in other mesodermal pathways too, is immortalized in WT. In normal development such cells are committed to their correct fates but in WT development aberrant differentiation into progeny cell types is found in the disorganized tumour tissue.

Abnormally persistent islands of nephrogenic cells, present beyond the cessation of nephrogenesis but with little potential for metastasis, are called nephrogenic rests (NR), of which there are two main categories: intralobar nephrogenic rests (ILNR) and perilobar nephrogenic rests (PLNR). Each has a distinct histology and is not categorized solely on lobar position (Beckwith *et al.* 1990). In association with other blastemal derivatives NRs form benign nephroblastomatous lesions, which are thought to be precursors of WT, intermediate between normal nephrogenesis and renal malignancy. Infant autopsies suggest that these lesions (PLNR type) are present in approximately 1% of the general population (Beckwith *et al.* 1990). Statistically, only about 1 in 100 such individuals goes on to develop WT; therefore, presumably, in the majority of cases nephroblastomatosis does not progress to the tumorigenic state.

Almost 8% of WT cases present in association with other developmental abnormalities, in particular sporadic aniridia (iris hypoplasia, 0.8%), genitourinary (GU) abnormalities (3%) (most commonly hypospadias and cryptorchidism (75% of GU abnormalities)) and hemihypertrophy (3%) (Breslow *et al.* 1988). These abnormalities are also often part of syndromes such as the WAGR syndrome (*W*T, *a*niridia, *G*U abnormalities and mental *r*etardation), Denys–Drash syndrome, DDS (nephropathy, partial gonadal dysgenesis and WT) and Beck-

Correspondence: Kathleen A. Williamson, MRC Human Genetics Unit, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, Scotland.

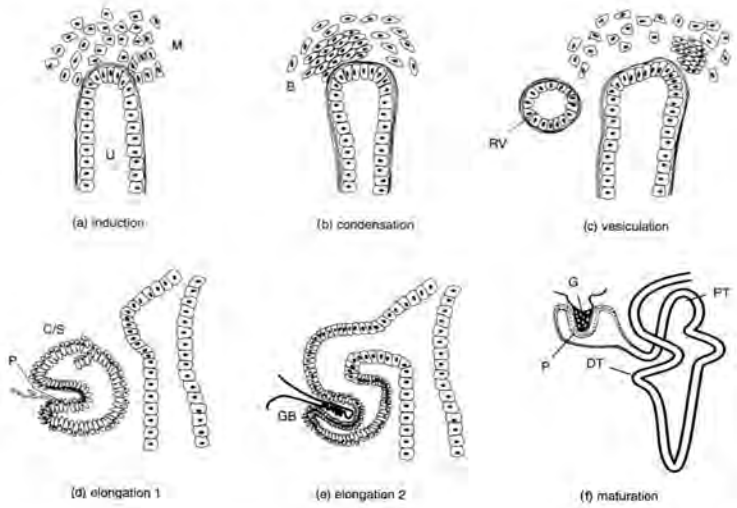


Figure 1. Development of the metanephros. a, Induction: U-ureteric bud, M-metanephric mesenchyme; b, condensation: B-metanephric blastema; c, vesiculation: RV-renal vesicle; d, elongation 1: C/S-comma-/S-shaped body, P-podocytes; e, elongation 2: GB-glomeruloid body; f, maturation: G-glomerulus, PT-proximal tubule, DT-distal tubule.

with–Wiedemann syndrome, BWS (foetal overgrowth, hemihypertrophy and embryonal tumours). The proposal that there may be pathogenetic heterogeneity for WT with respect to the above abnormalities is based on the finding that WT presenting with aniridia and DDS are associated with ILNRs whereas those presenting with hemihypertrophy and BWS are associated with PLNRs (Beckwith *et al.* 1990). Unlike ILNR-WT, PLNR-WT are predominantly blastemal/epithelial, contain structures from the later stages of nephrogenesis, and rarely develop heterotopic elements, that is, the blastema in the latter appears to be more committed than in the former.

A further variability observed with WT relates to the age of onset. The majority of WT patients present as sporadic unilateral cases, with a median age at diagnosis of 3 years (Breslow *et al.* 1988). Bilateral and familial patients, together often referred to as hereditary WT (Knudson 1985), constitute only 7% and 1% of cases, respectively. Although less frequent than unilateral WT, the bilateral cases have a lower median age at diagnosis of 2 years. In both cases females tend to present at a slightly later age. WT associated with aniridia and/or GU abnormalities also show a younger age distribution than those associated with hemihypertrophy, highlighting a further difference between ILNR-WT and PLNR-WT (Beckwith *et al.* 1990), and most probably reflecting the earlier onset of the blastemal defect in ILNR-WT.

The earlier presentation of bilateral tumours is consistent with the two-hit model for tumorigenesis proposed by Knudson in 1971. This model has been applied to WT (Knudson & Strong 1972) and developed further to suggest that both functional copies of a tumour suppressor gene, or anti-oncogene, have to be mutated to permit tumour formation (Knudson 1985). Bilateral cases have one germline (constitutional) mutation which represents the first hit, and then require only one subsequent somatic hit at the same locus to disrupt the remaining copy of the gene. Unilateral cases are however expected to develop sporadically from two somatic hits at the same locus and in the same cell. The number of target cells for the second mutational event are obviously far fewer in comparison with germline cases and hence unilateral tumours tend to develop later in childhood.

As described, WT age of onset patterns are consistent with Knudson's model for tumour suppressor genes. However, the lower frequency of bilateral tumours compared with unilateral tumours and the paucity of familial cases suggest that constitutional mutations leading to WT development are genetically deleterious (van Heyningen *et al.* 1990).

Evidence for genetic heterogeneity in WT

The fact that WT can present in association with other developmental abnormalities was critical in defining a chromosomal location for a WT gene. Sporadic aniridia patients, a high proportion of whom develop WT, often have cytogenetically detectable deletions involving the short arm of chromosome 11, at band p13 (Francke *et al.* 1979). Many of these deletion patients have the more complex WAGR phenotype (also including GU abnormalities and mental retardation), which is representative of a contiguous gene syndrome. A key patient with a large 11p13 deletion and partial WAGR, WT but no aniridia, defined aniridia as a separate locus (Davis *et al.* 1988; Gessler *et al.* 1989; van Heyningen *et al.* 1990) and helped to refine the position of a putative WT gene at this region.

The majority of WT patients do not, however, have

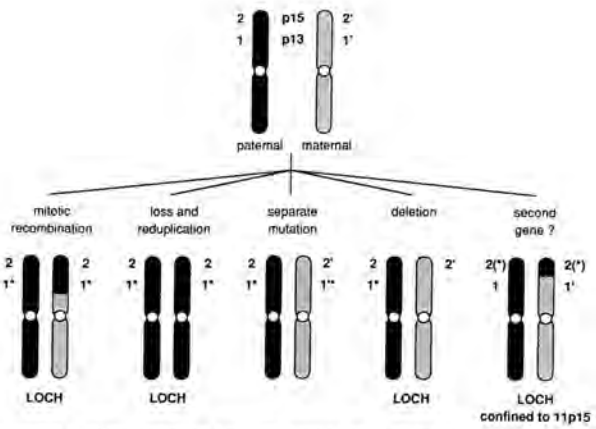


Figure 2. Mechanisms for functional loss of both copies of a tumour suppressor gene in WT development. 1-locus at 11p13, 2-locus at 11p15, the maternal allele is primed. *Hit; LOCH, loss of constitutional heterozygosity.

cytogenetically detectable alterations at 11p13. Knudson's two-hit model predicts functional loss of both copies of a tumour suppressor gene in WT development (Knudson 1985). The second hit can be achieved in several different ways (Figure 2). Most easily, homozygosity for the first hit can be achieved by mitotic recombination or loss and reduplication. Alternatively, the second allele can be disrupted by a separate mutation or deletion of part or whole of the relevant chromosomal region. Except when there is an independent second mutation, these events will result in allele loss or loss of constitutional heterozygosity (LOCH) at the target gene and usually at neighbouring loci also. Thirty to forty per cent of WT show LOCH and, surprisingly, it is almost exclusively the maternal allele that is lost (Schroeder *et al.* 1987; Williams *et al.* 1989; Pal *et al.* 1990). A further surprise is that in many cases the allele loss is not observed at 11p13 but is confined to the more telomeric position 11p15 (Mannens *et al.* 1988; Reeve *et al.* 1989; Henry *et al.* 1989; Koufos *et al.* 1989; Wadey *et al.* 1990). This suggests the possibility that there is a second WT gene, with duplication (if the remaining homologue has not been hit and is then duplicated) or loss of both copies of this 11p15 gene contributing to tumour development. Alternatively, 11p13 and 11p15 loci could act cumulatively, with a single hit at each locus initiating WT development. Further evidence for the existence of an 11p15 WT gene comes from chromosome translocations and paternal duplications of this region which have been observed in, and implicated in the aetiology of, BWS (Henry *et al.* 1991). As previously mentioned, BWS patients are at a high risk for developing embryonal tumours, the most frequent of which is WT.

Familial WT does not appear to involve genes on chromosome 11. Cytogenetic and linkage studies have produced conflicting reports regarding linkage of a third WT gene to chromosome 16q, where LOCH in WT has been detected (Maw *et al.* 1992; Huff *et al.* 1992; Coppes *et al.* 1992a). Alterations of chromosome 1, including trisomy 1q, have also been reported for WT (Douglass *et al.* 1985; McDowell *et al.* 1989; Kaneko *et al.* 1983; Golden *et al.* 1992); however, further analysis is required as the location of the familial locus/loci remains undetermined at this date.

The apparent involvement of multiple loci and the potential for synergistic interactions between them may be one of the reasons, together with genetic background, for the incomplete penetrance and variable expression observed in WT. Individuals carrying the same chromosomal alterations, whether at the relatively crude cytogenetic level or more highly resolving molecular level (see later), can display differing degrees of severity regarding WT development and associated abnormalities. It is also found that males are more frequently affected than females with GU abnormalities, with the development of hypospadias and/or cryptorchidism being particularly variable.

Isolation of WT1: a WT predisposition gene

Molecular analysis of overlapping 11p13 WAGR deletions, such as the previously mentioned partial WAGR case (Davis *et al.* 1988), defined the chromosomal location of a putative WT gene. The position and extent of the critical deleted region was confirmed by the discovery of a small homozygous 11p13 deletion in a sporadic WT (Lewis *et al.* 1988). This deletion covers approximately 345 kb of DNA (Rose *et al.* 1990), a region that would encompass at least part if not all of a putative WT gene and which was small enough in which to search for candidate gene sequences. This positional cloning approach led to the identification in 1990 of a gene from this region (Call *et al.* 1990), the WT1 gene, which has subsequently been confirmed both at the biological and the genetic level as a WT predisposition gene.

Biological analysis of WT1: expression studies

Candidate genes should display expression patterns consistent with their proposed function and associated phenotype. Expression of WT1 is detected in metanephric blastema, renal vesicles and, most strongly, in the podocyte cells of glomeruloid bodies (see Figure 1) (Pritchard-Jones *et al.* 1990), in keeping with a role in renal development. As mentioned above, these structures are present only during active nephrogenesis and

WT1 renal expression is accordingly restricted to the foetal kidney (Pritchard-Jones *et al.* 1990). In WT the persistence of these structures correlates with continued WT1 expression, at levels comparable to those in foetal kidney (Call *et al.* 1990; Pritchard-Jones *et al.* 1990). It would therefore appear that in normal development WT1 expression is required during the initial stages of nephrogenesis, but expression must then be turned off to allow nephron maturation. The continued expression of WT1 in tumours suggests that mutations affecting this gene may contribute towards alteration of the balance between proliferation and differentiation. These expression studies also show that it is the epithelial and not the differentiated mesenchymal derivatives of blastema that express WT1 (Pritchard-Jones *et al.* 1990; Pritchard-Jones & Fleming 1991). This explains why stromal predominant WT have far lower levels of WT1 mRNA compared with epithelial predominant and triphasic WT (Pritchard-Jones *et al.* 1990; Miwa *et al.* 1992a; Gerald *et al.* 1992; Yeger *et al.* 1992).

WT1 expression is also detected in some non-renal tissues. Human studies have shown that the developing genital ridge and the Sertoli and granulosa cells of the mature gonad express WT1, as do mesothelial cells (Pritchard-Jones *et al.* 1990), spleen and some haematopoietic cells (Call *et al.* 1990; Miwa *et al.* 1992b). A functional role for WT1 in these cell types is more clearly demonstrated by newly published results from WT1 knock-out mice (which completely lack functional WT1) (Kreidberg *et al.* 1993). These non-viable mice display renal and gonadal agenesis and developmental defects of the mesothelium. The gonadal and mesothelial cells share a common differentiation pathway with the renal system in that all three tissues initially develop from mesenchyme that subsequently becomes epithelialized. The only other cells that show this developmentally regulated conversion are the endothelial cells lining blood vessels and the trophectoderm in pre implantation embryos (Ekblom 1989). The transition of mesenchyme to epithelium appears therefore to be a rare event and it is possible that WT1 is either directly or indirectly involved in this switch in cell types.

The gonadal expression of WT1 is however most interesting in relation to the GU abnormalities so frequently seen with WT (Breslow *et al.* 1988). WT patients have an approximately threefold increased risk for developing such abnormalities, and in WAGR and DDS XY individuals the risk frequency is > 80%. It was initially proposed that a gene contributing to gonadal development was distinct from but closely linked to a WT gene and hence alteration of one would similarly disrupt the other. With the renal and gonadal systems originating

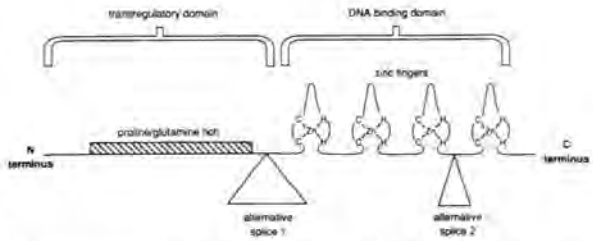


Figure 3. Schematic diagram of the WT1 protein, indicating the position of functional domains and alternative splices.

from the same mesodermal components early in development (for a general review of this topic see Browder *et al.* 1991), the finding that WT1 expression was common to both supported the proposal that this single gene may be acting pleiotropically, playing a crucial role early in the development of both systems (van Heyningen *et al.* 1990; Pritchard-Jones *et al.* 1990). Constitutional alterations of WT1 that contribute to aberrant renal development could therefore also contribute to aberrant gonadal development, explaining the high incidence of GU abnormalities in WT patients and also perhaps suggesting a reduced reproductive capacity, helping to explain the low frequency of familial WT.

Genetic analysis of WT1: function studies

The WT1 gene maps close to the aniridia gene (AN2, PAX6) and comprises ten exons spanning approximately 50 kb of DNA (Call *et al.* 1990; Haber *et al.* 1991; Gessler *et al.* 1992). The 3.2 kb mRNA encodes a 49–52 kDa protein that localizes to the nucleus (Morris *et al.* 1991; Telerman *et al.* 1992). WT1 protein contains several classic motifs (Figure 3) (Call *et al.* 1990); exons 1–6 encode a domain rich in proline (P) and glutamine (G) residues which appears to act as a transregulatory domain (these domains influence the transcription of target genes, probably by attracting and binding other factors required by the transcription complex) and exons 7–10 each encode a C_2H_2-type zinc finger (ZF) motif, conferring sequence-specific DNA binding properties (Rauscher *et al.* 1990; Bickmore *et al.* 1992). In addition exons 2–4 have the potential to encode a protein dimerization domain similar to the leucine zipper motif (Madden *et al.* 1993). The expression pattern, cellular localization and motifs of WT1 suggest that it functions as a tissue-specific transcription factor.

Two independent alternative splices within the coding region of WT1 give rise to four proposed protein isoforms (see Figure 3) (Haber *et al.* 1991). Alternative splice I comprises exon 5, which encodes 17 amino acids. Inclusion of this splice introduces six additional serine (S) or threonine (T) residues into the transregulatory

domain. The phosphorylation of S and T residues is a common regulatory mechanism used by many proteins, including transcription factors; however, WT1 does not appear to exhibit significant levels of phosphorylation (Morris *et al.* 1991) and splice I may therefore be important for other aspects of WT1 activity. No functional differences between the + and − 17 amino acid isoforms have been described. The second alternative splice utilizes two splice donor sites at the end of exon 9, which encodes the third ZF. If the downstream splice donor site is used an extra three amino acids (lysine, threonine and serine KTS) are added, increasing the linker region between ZF3 and ZF4. The four different splice forms have been designated A (−17aa−KTS) B (+17aa−KTS) C (−17aa+KTS) and D (+17aa+KTS) and are present in normal kidney at a ratio of approximately 1A:2.5B:4C:8D (Haber *et al.* 1991). In all but a single WT (see later) and in expressing murine tissues this ratio is generally conserved (Haber *et al.* 1991; Brenner *et al.* 1992), suggesting that perturbation of it may be deleterious to development.

WT1 ZF2,3 and 4 display >60% homology to the three ZFs of EGR1, a member of the early growth response gene family (Sukhatme *et al.* 1988). EGR1 is a transcription factor that activates the expression of target genes (Lemaire *et al.* 1990) via the consensus DNA binding sequences 5'GCG GGG GCG 3' (Christy & Nathans 1989). X-ray crystallography studies have identified conserved residues within the EGR1 ZF motifs that are critical for binding this target sequence (Pavletich & Pabo 1991). In-vitro studies have shown that −KTS WT1 (the ZF isoform most similar to the EGR1 ZFs) can also bind this sequence (Rauscher *et al.* 1990) and a distinct set of sequences to which +KTS WT1 binds (Bickmore *et al.* 1992). The fact that + and − KTS WT1 can have different DNA binding sites, and can therefore potentially regulate different genes, strengthens the proposal that the maintenance of the isoform ratio is crucial for normal development.

In-vivo studies to find the true target genes for WT1 are lacking but a number of putative targets have been studied using transient transfection assays where WT1 can be overexpressed. The insulin-like growth factor II (IGF-II), insulin-like growth factor I receptor (IGF-IR) and platelet derived growth factor A chain (PDGF-A) genes are often overexpressed in WT (interestingly, IGF-IR and PDGF-A have also been implicated in the malignant transformation of mesothelial cells (Lee *et al.* 1993)). The overexpression of IGF-II may simply be a reflection of the embryonal nature of WT or result from other mechanisms thought to participate in WT development (see later); however, IGF-II, and IGF-IR and PDGF-A, do have the ability to aid tumorigenesis through autocrine growth stimulation. The increased expression levels may therefore represent a direct deregulation from WT1 and all three genes do contain EGR1 consensus binding sequences within their promoters. When these promoters or the EGR1 promoter are each linked to reporter genes and coexpressed with WT1 ZF constructs, expression from the reporter gene is repressed (Drummond *et al.* 1992; Werner *et al.* 1993; Gashler *et al.* 1992; Wang *et al.* 1992; Madden *et al.* 1991). Similarly, finger swap experiments where the transregulatory domain of WT1 is joined to the ZF domain of EGR1 converts the latter from transcriptional activator to repressor (Madden *et al.* 1991). One model proposed from these studies suggests that EGR1 activates expression of growth promoting genes, perhaps allowing proliferation of the metanephric blastema, and then WT1 is required to repress their expression and so allow differentiation to proceed, that is EGR1 and WT1 may act antagonistically. This model cannot however explain the action of the +KTS WT1 isoforms, which do not bind the EGR1 consensus sequence (Bickmore *et al.* 1992).

Genetic analysis of WT1: mutation studies

The most fundamental way to confirm that a candidate gene is involved in a particular phenotype is to search for mutations in that gene in affected individuals. As previously mentioned, the majority of WT patients do not have cytogenetically detectable alterations at 11p13; however, by using more sensitive molecular detection methods WT1 mutations have been found in 10–15% of tumours tested. These submicroscopic mutations include intragenic deletions, insertions, duplications and single base changes and they have been detected in sporadic unilateral, bilateral, familial WT and DDS patients, and as second hits in WAGR tumours.

With one exception (Little *et al.* 1992a), the reported WT1 mutations found in sporadic unilateral WT all result in transcripts that can no longer code for at least one of the ZF motifs, causing disruption of the DNA binding domain (Haber *et al.* 1990; Cowell *et al.* 1991; Tadokoro *et al.* 1992; Coppes *et al.* 1993). These mutations are predicted to produce WT1 protein that has lost all DNA binding capacity. Binding experiments using one of these WT1 mutants, which lacks ZF3, has demonstrated almost complete loss of binding to the EGR1 consensus sequence (Rauscher *et al.* 1990). A similar result is also suggested by the WT1 mutation where the coding potential for all of the ZF motifs is destroyed (Tadokoro *et al.* 1992). This would produce a fingerless protein which seems unlikely to have retained any capacity to bind DNA directly.

Mutations predicted to produce fingerless WT1 protein are also detected in bilateral (again there is one exception (Little *et al.* 1992a)) (Huff *et al.* 1991; Pelletier *et al.* 1991a) and WAGR tumours (Brown *et al.* 1992; Baird *et al.* 1992a; Gessler *et al.* 1993) and in one of the two familial cases known to involve inheritance of WT1 mutations (see later) (Pelletier *et al.* 1991a). The last category is predicted to be extremely rare as the vast majority of heritable tumours involving WT1 derive from de-novo germline mutation, which, as stated earlier, is perhaps because individuals with constitutional WT1 mutations have a reduced reproductive capacity.

In WAGR individuals the above type of mutation is of course the second hit, required only for tumorigenesis. The hypospadias and cryptorchidism that develops in WAGR individuals appears to result from WT1 protein haplo-insufficiency (having only 50% of wild type levels, as one allele is constitutionally deleted). GU abnormalities developing in bilateral WT individuals may also result from a 50% constitutional loss of functional WT1 protein, suggesting that these mutations are functionally analogous to the WAGR situation. The possible presence of an expressed N-terminal region of WT1 does not appear to induce a novel phenotype; however, the effect of these mutations has not been analysed at the protein level.

In all but four tumours, whenever LOCH has been analysed homozygosity for WT1 mutations has been observed. These four exceptions (Haber *et al.* 1990; Little *et al.* 1992a; Little *et al.* 1993) introduce the possibility that the loss of only one copy of WT1 can, under some circumstances, lead to tumour formation. This could occur either through involvement of a second locus, possibly at 11p15, or through the action of a dominant-negative mutation in WT1. This type of mutation causes interference of the wild type function by the mutant protein, perhaps through heterodimer formation (Herskowitz 1987). There is evidence that the WT1 mutant lacking ZF3 functions as a dominant-negative (Haber *et al.* 1992).

More clear-cut evidence for dominant-negative mutation is presented in DDS cases. The majority (29 out of 31) of these patients have constitutional WT1 point mutations, most of which are missense mutations within ZF2 or ZF3 (Pelletier *et al.* 1991b; Bruening *et al.* 1992; Baird *et al.* 1992b; Coppes *et al.* 1992b; Little *et al.* 1993). Sixty-one per cent of DDS mutations show alteration at one of two specific, conserved residues in ZF3, which correspond to critical binding residues identified in the EGR1 X-ray crystallography studies (Pavletich & Pabo 1991). In comparison to the WAGR phenotype, DDS is more severe, comprising nephropathy as well as partial or complete gonadal dysgenesis in virtually 100% of XY patients. This suggests that DDS constitutionally heterozygous WT1 mutations act in a dominant or dominant-negative manner. Dominant mutations imply a gain of function, such as novel and inappropriate target binding. The finding of three DDS-associated nonsense mutations (Baird *et al.* 1992b; Ogawa *et al.* 1993a; Little *et al.* 1993) which truncate the ZF domain suggests that the mechanism is more likely to be dominant-negative (Little *et al.* 1993). This is not the sole mechanism implicated in this phenotype, however, as the only WT1 mutation described which perturbs the isoform ratio was also found in a DDS individual (Bruening *et al.* 1992).

The above studies provide us with a general pattern of the types of WT1 mutation associated with particular subtypes of WT. No strict correlations can be inferred, however, again because of the incomplete penetrance and variable expressivity of the WT mutant phenotype. On this theme, there are currently four examples of individuals displaying very different phenotypes but having identical WT1 mutations. Two of these examples involve inherited WT1 mutations, where the carrier parent does not have the GU abnormalities (Pelletier *et al.* 1991a) or DDS phenotype (Coppes *et al.* 1992b) that develops in the child. Also, an XY DDS individual has the same constitutional WT1 nonsense mutation (Little *et al.* 1993) as a female who has only sporadic unilateral WT (Coppes *et al.* 1993). Finally, an identical 11p13 deletion has been found in two related children, a boy having the extremely severe phenotype of WAGR together with DDS and a girl having only aniridia (Henry *et al.* 1993). The last two examples again show that XY individuals are more sensitive to WT1 mutations than XX individuals.

Evidence for an imprinting effect in WT

Genomic imprinting refers to the monoallelic expression of genes in a parental-specific pattern. Murine studies have identified four genes controlled in this way, including the Igf2 and Igf2 receptor (Igf2r) genes (DeChiara *et al.* 1991; Barlow *et al.* 1991) which are expressed from only the paternally and only the maternally inherited alleles, respectively. Recent experiments have shown that human IGF-II is imprinted in the same direction as its mouse homologue (Ohlsson *et al.* 1993; Giannoukakis *et al.* 1993). IGF-II maps to 11p15, where, as previously mentioned, LOCH is observed in some WT. The preferential loss of the maternal 11p in WT together with duplication of the corresponding paternal region has the potential therefore to produce a double dose of any exclusively paternally expressed gene located here, such as IGF-II (see Figure 2). Such paternal disomy for

11p15 is observed in BWS (Henry *et al.* 1991), and overexpression of a growth factor is concordant with the overgrowth or hemihypertrophy characteristic of this syndrome. Many WT overexpress IGF-II (Reeve *et al.* 1985; Scott *et al.* 1985) even with approximately 60% not displaying 11p LOCH. Statistically, it is likely that 3 out of 4 of these WT will still have a double dose of IGF-II as imprinting is relaxed at the normally silent maternal allele, resulting in biallelic IGF-II expression (Rainier *et al.* 1993; Ogawa *et al.* 1993b).

Loss, as opposed to relaxation of imprinting, of maternal 11p15 in WT may additionally result in loss of an exclusively maternally expressed tumour suppressor gene (a possible candidate gene is H19 (Zhang & Tycko 1992)). WT1, which remains the only WT predisposition gene currently cloned, is not imprinted in foetal kidney or WT (Little *et al.* 1992b; Zhang & Tycko 1992).

Summary

Many areas of research are contributing to our understanding of WT and the role of WT1 in development of the renal and genitourinary systems. Characterization of putative target genes and the control of their expression continues. The importance of isoform ratios and imprinting effects are also under active investigation, often using animal models. The accumulating mutation data, together with evolutionary studies, illuminate WT1 structure–function relationships, highlighting the regions critical in normal development and tumorigenesis. And last, but by no means least, the hunt for the WT2 and WT3 genes continues.

References

Baird P.N., Groves N., Haber D.A., Housman D.E. & Cowell J.K. (1992a) Identification of mutations in the WT1 gene in tumours from patients with the WAGR syndrome. *Oncogene* **7,** 2141–2149.

Baird P.N., Santos A., Groves N., Jadresic L. & Cowell J.K. (1992b) Constitutional mutations in the WT1 gene in patients with Denys-Drash syndrome. *Hum. Mol. Genet.* **1,** 301–305.

Barlow D.P., Stoger R., Herrmann B.G., Saito K. & Schweifer N. (1991) The mouse insulin-like growth factor type-2 receptor is imprinted and closely linked to the Tme locus. *Nature* **349,** 84–87.

Beckwith J.B., Kiviat N.B. & Bonadio J.F. (1990) Nephrogenic rests, nephroblastomatosis, and the pathogenesis of Wilms' tumor. *Pediatr. Pathol.* **10,** 1–36.

Bickmore W.A., Oghene K., Little M.H., Seawright A., van Heyningen V. & Hastie N.D. (1992) Modulation of DNA binding specificity by alternative splicing of the Wilms' tumor wt1 gene transcript. *Science* **257,** 235–237.

Brenner B., Wildhardt G., Schneider S. & Royer-Pokora B. (1992) RNA polymerase chain reaction detects different levels of four alternatively spliced WT1 transcripts in Wilms' tumors. *Oncogene* **7,** 1431–1433.

Breslow N., Beckwith B.J., Ciol, M. & Sharples K. (1988) Age distribution of Wilms' tumor: report from the National Wilms' Tumor Study. *Cancer Res.* **48,** 1653–1657.

Browder L.W., Erickson C.A. & Jeffery W.R. (1991) *Developmental Biology*. 3rd ed. Saunders College, Philadelphia.

Brown K.W., Watson J.E., Poirier V., Mott M.G., Berry P.J. & Maitland N.J. (1992) Inactivation of the remaining allele of the WT1 gene in a Wilms' tumour from a WAGR patient. *Oncogene* **7,** 763–768.

Bruening W., Bardeesy N., Silverman B.L., Cohn R.A., Machin G.A., Aronson A.J., Housman D. & Pelletier J (1992) Germline intronic and exonic mutations in the Wilms's tumour gene (WT1) affecting urogenital development. *Nature Genet.* **1,** 144–148.

Call K.M., Glaser T., Ito C.Y., Buckler A.J., Pelletier J., Haber D.A., Rose E.A., Kral A., Yeger H., Lewis W.H., Jones C. & Housman D.E. (1990) Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. *Cell* **60,** 509–520.

Christy B. & Nathans D. (1989) DNA binding site of the growth factor-inducible protein Zif268. *Proc. Natl Acad. Sci. USA* **86,** 8737–8741.

Coppes M.J., Bonetta L., Huang A., Hoban P., Chilton-MacNeill S., Campbell C.E., Weksberg R., Yeger H., Reeve A.E. & Williams B.R.G. (1992a) Loss of heterozygosity mapping in Wilms' tumor indicated the involvement of three distinct regions and a limited role for nondisjunction or mitotic recombination. *Genes, Chromosomes Cancer* **5,** 326–334.

Coppes M.J., Liefers G.J., Higuchi M., Zinn A.B., Balfe J.W. & Williams B.R.G. (1992b) Inherited WT1 mutation in Denys–Drash syndrome. *Cancer Res.* **52,** 6125–6128.

Coppes M.J., Liefers G.J., Paul P., Yeger H. & Williams B.R.G. (1993) Homozygous somatic WT1 point mutations in sporadic unilateral Wilms' tumor. *Proc. Natl Acad. Sci. USA* **90,** 1416–1419.

Cowell J.K., Wadey R.B., Haber D.A., Call K.M., Housman D.E. & Pritchard J. (1991) Structural rearrangements of the WT1 gene in Wilms' tumour cells. *Oncogene* **6,** 595–599.

Davis L.M., Stallard R., Thomas G.H., Couillin P., Junien C., Nowak N.J. & Shows T.B. (1988) Two anonymous DNA segments distinguish the Wilms' tumor and aniridia loci. *Science* **241,** 840–842.

DeChiara T.M., Robertson E.J. & Efstratiadis A. (1991) Parental imprinting of the mouse insulin-like growth factor II gene. *Cell* **64,** 849–859.

Douglass E.C., Wilimas J.A., Green A.A. & Look A.T. (1985) Abnormalities of chromosomes 1 and 11 in Wilms' tumor. *Cancer Genet. Cytogenet.* **14,** 331–338.

Drummond I.A., Madden S.L., Rohwer-Nutter P., Bell G.I., Sukhatme V.P. & Rauscher III, F.J. (1992) Repression of the insulin-like growth factor II gene by the Wilms' tumor suppressor WT1. *Science* **257,** 674–678.

Ekblom P. (1989) Developmentally regulated conversion of mesenchyme to epithelium. *FASEB J.* **3,** 2141–2150.

Francke U., Holmes L.B., Atkins L. & Riccardi V.M. (1979) Aniridia-Wilms' tumor association: evidence for specific deletion of 11p13. *Cytogenet. Cell Genet.* **24,** 185–192.

Gashler A.L., Bonthron D.T., Madden S.L., Rauscher III F.J., Collins T. & Sukhatme V.P. (1992) Human platelet-derived growth factor A chain is transcriptionally repressed by the Wilms' tumor suppressor WT1. *Proc. Natl Acad. Sci. USA* **89,** 10984–10988.

Gerald W.L., Gramling T.S., Sens D.A. & Garvin A.J. (1992) Expression of the 11p13 Wilms' tumor gene, WT1, correlates with histologic category of Wilms' tumor. *Am. J. Pathol.* **140,** 1031–1037.

Gessler M., Konig A. & Bruns G.A.P. (1992) The genomic organisation and expression of the WT1 gene. *Genomics* **12,** 807–813.

Gessler M., Thomas G.H., Couillin P., Junien C., McGillivray B.C., Hayden M., Jaschek G. & Bruns G.A.P. (1989) A deletion map of the WAGR region on chromosome 11. *Am. J. Hum. Genet.* **44,** 486–495.

Gessler M., Konig A., Moore J., Qualman S., Arden K., Cavenee W. & Bruns G. (1993) Homozygous inactivation of WT1 in a Wilms' tumor associated with the WAGR syndrome. *Genes, Chromosomes Cancer* **7,** 131–136.

Giannoukakis N., Deal C., Paquette J., Goodyer C.G. & Polychronakos C. (1993) Parental genomic imprinting of the human IGF2 gene. *Nature Genet.* **4,** 98–101.

Golden W.L., Sudduth K.W. & Lovell M.A. (1992) Clonal evolution of a Wilms' tumor. *Cancer Genet. Cytogenet.* **60,** 102–104.

Haber D.A., Buckler A.J., Glaser T., Call K.M., Pelletier J., Sohn R.L., Douglass E.C. & Housman D.E. (1990) An internal deletion within an 11p13 zinc finger gene contributes to the development of Wilms' tumor. *Cell* **61,** 1257–1269.

Haber D.A., Sohn R.L., Buckler A.J., Pelletier J., Call K.M. & Housman D.E. (1991) Alternative splicing and genomic structure of the Wilms tumor gene WT1. *Proc. Natl Acad. Sci USA* **88,** 9618–9622.

Haber D.A., Timmers H Th. M., Pelletier J., Sharp P.A. & Housman D.E. (1992) A dominant mutation in the Wilms' tumor gene WT1 cooperates with the viral oncogene E1A in transformation of primary kidney cells. *Proc. Natl Acad. Sci. USA* **89,** 6010–6014.

Henry I., Bonaiti-Pellie C., Chehensse V., Beldjord C., Schwartz C., Utermann G. & Junien C. (1991) Uniparental paternal disomy in a genetic cancer-predisposing syndrome. *Nature* **351,** 665–667.

Henry I., Grandjouan S., Couillin P., Barichard F., Huerre-Jeanpierre C., Glaser T., Philip T., Lenoir G., Chaussain J.L. & Junien C. (1989) Tumor-specific loss of 11p15.5 alleles in del11p13 Wilms' tumor and in familial adrenocortical carcinoma. *Proc. Natl Acad. Sci. USA* **86,** 3247–3251.

Henry I., Hoovers J., Barichard F., Bertheas M.F., Puech A., Prieur F., Gessler M., Bruns G., Mannens M. & Junien C. (1993) Pericentric intrachromosomal insertion responsible for recurrence of del(11)(p13p14) in a family. *Genes, Chromosomes Cancer* **7,** 57–62.

Herskowitz I. (1987) Functional inactivation of genes by dominant negative mutations. *Nature* **329,** 219–222.

Huff V., Miwa H., Haber D.A., Call K.M., Housman D., Strong L.C. & Saunders G.F. (1991) Evidence for WT1 as a Wilms Tumor (WT) Gene: Intragenic germinal deletion in Bilateral WT. *Am. J. Hum Genet.* **48,** 997–1003.

Huff, V., Reeve A.E., Leppert M., Strong L.C., Douglass E.C., Geiser C.F., Li F.P., Meadows A., Callen D.F., Lenoir G. & Saunders G.F. (1992) Nonlinkage of 16q markers to familial predisposition to Wilms' tumor. *Cancer Res.* **52,** 6117–6120.

Kaneko Y., Kondo K., Rowley J.D., Moohr J.W. & Maurer H.S. (1983) Further chromosome studies on Wilms' tumor cells of patients without aniridia. *Cancer Genet. Cytogenet.* **10,** 191–197.

Knudson Jr A.G. (1971) Mutation and cancer: statistical study of retinoblastoma. *Proc. Natl Acad. Sci. USA* **68,** 820–823.

Knudson Jr A.G. (1985) Hereditary cancer, oncogenes, and antioncogenes. *Cancer Res.* **45,** 1437–1443.

Knudson Jr A.G. & Strong L.C. (1972) Mutation and cancer: a model for Wilms' tumor of the kidney. *J. Natl Cancer Inst.* **48,** 313–324.

Koufos A., Grundy P., Morgan K., Aleck K.A. Hadro T., Lampkin B.C. Kalbakji A. & Cavenee W.K. (1989) Familial Wiedemann-Beckwith syndrome and a second Wilms' tumor locus both map to 11p15.5. *Am. J. Hum. Genet.* **44,** 711–719.

Kreidberg J.A., Sariola H., Loring J.M., Maeda M., Pelletier J., Housman D. & Jaenisch R. (1993) WT1 is required for early kidney development. *Cell* **74,** 679–691.

Lee T.C., Zhang Y., Aston C., Hintz R., Jagirdar J., Perle M.A., Burt M. & Rom W.N. (1993) Normal human mesothelial cells and mesothelioma cell lines express insulin-like growth factor I and associated molecules. *Cancer Res.* **53,** 2858–2864.

Lemaire P., Vesque C., Schmitt J., Stunnenberg H., Frank R. & Charnay P. (1990) The serum-inducible mouse gene Krox-24 encodes a sequence-specific transcriptional activator. *Mol. Cell. Biol.* **10,** 3456–3467.

Lewis W.H., Yeger H., Bonetta L., Chan H.S.L., Kang J., Junien C., Cowell J., Jones C. & Dafoe L.A. (1988) Homozygous deletion of a DNA marker from chromosome 11p13 in sporadic Wilms' tumor. *Genomics* **3,** 25–31.

Little M.H., Dunn R., Byrne J.A., Seawright A., Smith P.J., Pritchard-Jones K., van Heyningen V. & Hastie N.D. (1992b) Equivalent expression of paternally and maternally inherited WT1 alleles in normal fetal tissue and Wilms' tumours. *Oncogene* **7,** 635–641.

Little M.H., Prosser J., Condie A., Smith P.J., van Heyningen V. & Hastie N.D. (1992a) Zinc finger point mutations within the WT1 gene in Wilms' tumor patients. *Proc. Natl Acad. Sci. USA* **89,** 4791–4795.

Little M.H., Williamson K.A., Mannens M., Kelsey A., Gosden C., Hastie N.D. & van Heyningen V. (1993) Evidence that WT1 mutations in Denys-Drash syndrome patients may act in a dominant-negative fashion. *Hum. Mol. Genet.* **2,** 259–264.

Madden S.L., Cook D.M., Morris J.F., Gashler A., Sukhatme V.P. & Rauscher III, F.J. (1991) Transcriptional repression mediated by the WT1 Wilms' tumor gene product. *Science* **253,** 1550–1553.

Madden S.L., Cook D.M. & Rauscher III, F.J. (1993) A structure-function analysis of transcriptional repression mediated by the WT1, Wilms' tumor suppressor protein. *Oncogene* **8,** 1713–1720.

Mannens M., Slater R.M., Heyting C., Bliek J., de Kraker J., Coad N., de Pagter-Holthuizen P. & Pearson P.L. (1988) Molecular nature of genetic changes resulting in loss of heterozygosity of chromosome 11 in Wilms' tumours. *Hum. Genet.* **81,** 41–48.

Maw M.A., Grundy P.E., Millow L.J., Eccles M.R., Dunn R.S., Smith P.J., Feinberg A.P., Law D.J., Paterson M.C., Telzerow P.E., Callen D.F., Thompson A.D., Richards R.I. & Reeve A.E. (1992) A third Wilms' tumor locus on chromosome 16q. *Cancer Res.* **52,** 3094–3098.

McDowell H., Howard P., Martin J., Hart C. & Crampton J. (1989) Chromosome 1 studies in Wilms' tumor. *Cancer Genet. Cytogenet.* **43,** 203–209.

Mehta M.P., Bastin K.T. & Wiersma S.R. (1991) Treatment of Wilms' tumour. *Drugs* **42,** 766–780.

Miwa H., Beran M. & Saunders G.F. (1992b) Expression of the Wilms' tumor gene (WT1) in human leukemias. *Leukemia* **6,** 405–409.

Miwa H., Tomlinson G.E., Timmons C.F., Huff V., Cohn S.L., Strong L.C. & Saunders G.F. (1992a) RNA expression of the WT1 gene in Wilms' tumors in relation to histology. *J. Natl Cancer Inst.* **84,** 181–187.

MORRIS J.F., MADDEN S.L., TOURNAY O.E., COOK D.M., SUKHATME V.P. & RAUSCHER III F.J. (1991) Characterisation of the zinc finger protein encoded by the WT1 Wilms' tumor locus. *Oncogene* **6,** 2339–2348.

OGAWA O., ECCLES M.R., YUN K., MUELLER R.F., HOLDAWAY M.D.D. & REEVE A.E. (1993a) A novel insertional mutation at the third zinc finger coding region of the WT1 gene in Denys–Drash syndrome. *Hum. Mol. Genet.* **2,** 203–204.

OGAWA O., ECCLES M.R., SZETO J., MCNOE L.A., YUN K., MAW M.A., SMITH P.J. & REEVE A.E. (1993b) Relaxation of insulin-like growth factor II gene imprinting implicated in Wilms' tumour. *Nature* **362,** 749–751.

OHLSSON R., NYSTROM A., PFEIFER-OHLSSON S., TOHONEN V., HEDBORG F., SCHOFIELD P., FLAM F. & EKSTROM T.J. (1993) IGF2 is parenterally imprinted during human embryogenesis and in the Beckwith-Wiedemann syndrome. *Nature Genet.* **4,** 94–97.

PAL N., WADEY R.B., BUCKLE B., YEOMANS E., PRITCHARD J. & COWELL J.K. (1990) Preferential loss of maternal alleles in sporadic Wilms' tumour. *Oncogene* **5,** 1665–1668.

PAVLETICH N.P. & PABO C.O. (1991) Zinc finger-DNA recognition: crystal structure of a Zif268-DNA complex at 2.1A. *Science* **252,** 809–817.

PELLETIER J., BRUENING W., KASHTAN C.E., MAUER S.M., MANIVEL J.C., STRIEGEL J.E., HOUGHTON D.C., JUNIEN C., HABIB R., FOUSER L., FINE R.N., SILVERMAN B.L., HABER D.A. & HOUSMAN D. (1991b) Germline mutations in the Wilms' tumor suppressor gene are associated with abnormal urogenital development in Denys–Drash syndrome. *Cell* **67,** 437–447.

PELLETIER J., BRUENING W., LI F.P., HABER D.A., GLASER T. & HOUSMAN D.E. (1991a) WT1 mutations contribute to abnormal genital system development and hereditary Wilms' tumour. *Nature* **353,** 431–434.

PRITCHARD-JONES K. & FLEMING S. (1991) Cell types expressing the Wilms' tumour gene (WT1) in Wilms' tumours: implications for tumour histogenesis. *Oncogene* **6,** 2211–2220.

PRITCHARD-JONES K., FLEMING S., DAVIDSON D., BICKMORE W., PORTEOUS D., GOSDEN C., BARD J., BUCKLER A., PELLETIER J., HOUSMAN D., VAN HEYNINGEN V. & HASTIE N. (1990) The candidate Wilms' tumour gene is involved in genitourinary development. *Nature* **346,** 194–197.

RAINIER S., JOHNSON L.A., DOBRY C.J., PING A.J., GRUNDY P.E. & FEINBERG A.P. (1993) Relaxation of imprinted genes in human cancer. *Nature* **362,** 747–749.

RAUSCHER III, F.J., MORRIS J.F., TOURNAY O.E., COOK D.M. & CURRAN T. (1990) Binding of the Wilms' tumor locus zinc finger protein to the EGR-1 consensus sequence. *Science* **250,** 1259–1262.

REEVE A.E., ECCLES M.R., WILKINS R.J., BELL G.I. & MILLOW L.J. (1985) Expression of insulin-like growth factor-II transcripts in Wilms' tumour. *Nature* **317,** 258–260.

REEVE A.E., SIH S.A., RAIZIS A.M. & FERNBERG A.P. (1989) Loss of allelic heterozygosity at a second locus on chromosome 11 in sporadic Wilms' tumor cells. *Mol. Cell. Biol.* **9,** 1799–1803.

ROSE E.A., GLASER T., JONES C., SMITH C.L., LEWIS W.H., CALL K.M., MINDEN M., CHAMPAGNE E., BONETTA L., YEGER H. & HOUSMAN D.E. (1990) Complete physical map of the WAGR region of 11p13 localizes a candidate Wilms' tumor gene. *Cell* **60,** 495–508.

SCHROEDER W.T., CHAO LIAN-YU, DAO D.D., STRONG L.C., PATHAK S., RICCARDI V., LEWIS W.H. & SAUNDERS G.F. (1987) Nonrandom loss of maternal chromosome 11 alleles in Wilms' tumors. *Am. J. Hum. Genet.* **40,** 413–420.

SCOTT J., COWELL J., ROBERTSON M.E., PRIESTLEY L.M., WADEY R., HOPKINS B., PRITCHARD J., BELL G.I., RALL L.B., GRAHAM C.F. & KNOTT T.J. (1985) Insulin-like growth factor-II gene expression in Wilms' tumour and embryonic tissues. *Nature* **317,** 260–262.

SOTELO-AVILA C. (1990) Nephroblastoma and other pediatric renal cancers. In *Tumors and Tumor-Like Conditions of the Kidneys and Ureters.* 1st edn. Ed. J.N. Eble (Contemporary Issues In Surgical Pathology, 16). Churchill Livingstone, New York, pp. 71–121.

SUKHATME V.P., CAO XINMIN, CHANG L.C., TSAI-MORRIS CHON-HWA, STAMENKOVICH D., FERREIRA P.C.P., COHEN D.R., EDWARDS S.A., SHOWS T.B., CURRAN T., LE BEAU M.M. & ADAMSON E.D. (1988) A zinc finger-encoding gene coregulated with c-fos during growth and differentiation, and after cellular depolarization. *Cell* **53,** 37–43.

TADOKORO K., FUJII H., OHSHIMA A., KAKIZAWA Y., SHIMIZU K., SAKAI A., SUMIYOSHI K., INOUE T., HAYASHI Y. & YAMADA M. (1992) Intragenic homozygous deletion of the WT1 gene in Wilms' tumor. *Oncogene* **7,** 1215–1221.

TELERMAN A., DODEMONT H., DEGRAEF C., GALAND P., BAUWENS S., VAN OOSTVELDT P. & AMSON R.B. (1992) Identification of the cellular protein encoded by the human Wilms' tumor (WT1) gene. *Oncogene* **7,** 2545–2548.

VAN HEYNINGEN V., BICKMORE W.A., SEAWRIGHT A., FLETCHER J.M., MAULE J., FEKETE G., GESSLER M., BRUNS G.A.P., HUERRE-JEANPIERRE C., JUNIENS C., WILLIAMS B.R.G. & HASTIE N.D. (1990) Role for the Wilms' tumor gene in genital development? *Proc. Natl Acad. Sci. USA* **87,** 5383–5386.

WADEY R.B., PAL N., BUCKLE B., YEOMANS E., PRITCHARD J. & COWELL J.K. (1990) Loss of heterozygosity in Wilms' tumour involves two distinct regions of chromosome 11. *Oncogene* **5,** 901–907.

WANG Z.Y., MADDEN S.L., DEUEL T.F. & RAUSCHER III F.J. (1992) The Wilms' tumor gene product, WT1, represses transcription of the platelet-derived growth factor A-chain gene. *J. Biol. Chem.* **267,** 21999–22002.

WERNER H., RE G.G., DRUMMOND I.A., SUKHATME V.P., RAUSCHER III F.J., SENS D.A., GARVIN A.J., LEROITH D. & ROBERTS JR C.T. (1993) Increased expression of the insulin-like growth factor I receptor gene, IGF1R, in Wilms tumor is correlated with modulation of IGF1R promoter activity by the WT1 Wilms tumor gene product. *Proc. Natl Acad. Sci. USA* **90,** 5828–5832.

WILLIAMS J.C., BROWN K.W., MOTT M.G. & MAITLAND N.J. (1989) Maternal allele loss in Wilms' tumour. *Lancet* 283–284.

YEGER H., CULLINANE C., FLENNIKEN A., CHILTON-MACNEILL S., CAMPBELL C., HUANG A., BONETTA L., COPPES M.J., THORNER P. & WILLIAMS B.R.G. (1992) Coordinate expression of Wilms' tumor genes correlates with Wilms' tumor phenotypes. *Cell Growth Differentiation* **3,** 855–864.

ZHANG Y. & TYCKO B. (1992) Monoallelic expression of the human H19 gene. *Nature Genet.* **1,** 40–44.