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Molecular Genetic Analysis of Wilms' Tumor

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ABSTRACT

Wilms' tumor, a childhood malignancy of the kidney, is one of the most common pediatric tumors. The disease occurs in both sporadic and hereditary forms and is associated with a number of congenital disorders. Wilms' tumor appears to be genetically heterogeneous although only a single Wilms' tumor suppressor gene, designated *WT1*, has been isolated to date. *WT1* encodes a zinc finger protein and is mutated in a subset of Wilms' tumors and in patients with Denys-Drash syndrome (DDS), an association of Wilms' tumor and severe genitourinary defects. This thesis reports a mutational analysis of *WT1*, detailing the spectrum and frequency of mutations in sporadic Wilms' tumors. Most *WT1* mutations were homozygous and were predicted to cause premature termination of translation, suggesting that tumorigenesis associated with *WT1* involves a two-hit mechanism. The mutational status of *WT1* was determined in patients with variable expressivity of the DDS phenotype in order to assess potential genotype/phenotype correlations. Patients with less severe developmental anomalies had germline mutations predicted to result in truncated WT1 proteins, while those with full manifestation of DDS were characterized by missense mutations in the zinc finger region. This demonstrates that different mutations in *WT1* are associated with specific effects on genitourinary development.

We investigated the biochemical properties of WT1 and assessed the effects of some naturally occurring mutations. A number of studies have provided evidence that WT1 may be involved in RNA metabolism. We used an iterative selection method to identify potential RNA ligands to WT1. Specific, high affinity ligands were isolated. Mutational analysis elucidated structural features necessary for the RNA-protein interaction. These ligands may reflect RNA sequences targeted by WT1 *in vivo*.

Since our studies demonstrated that *WT1* mutations are restricted to a minority of Wilms' tumors, the understanding of mechanisms underlying Wilms' tumorigenesis required the identification of other genes which contribute to this malignancy. We studied the involvement of the *p53* gene tumor suppressor, a critical regulator of abnormal cellular growth and genetic stability. *P53* mutations were restricted to the rare anaplastic subtype of Wilms' tumor, a genetically unstable histological variant associated with poor prognosis and resistance to therapy. *P53* mutations occurred as late events, associated with malignant progression. The association of *p53* mutations with anaplasia allows rationalization of the clinicopathological features of this histological variant, indicating that p53 may regulate genome integrity and chemosensitivity of human cancer cells *in vivo*.

Resumé

Le cancer de Wilms est une tumeur maligne infantile des plus fréquente qui se manifeste au niveau des reins. Cette maladie, apparaît soit de façon sporadique ou héréditaire et est associée avec certains désordres congénitaux. Le cancer de Wilms semble être génétiquement hétérogène en dépit du fait qu'un seul gène suppresseur de tumeur, désigné, *WT1*, a été isolé jusqu'à présent. Le gène *WT1* code pour une protéine à de type 'zinc finger' . Cette dernière est muteé dans un sous-groupe du cancer de Wilms ainsi que chez les patients atteint du syndrome de Denys-Drash (DDS), une maladie qui présente à la fois des symptômes du cancer de Wilms et de sévères anomalies génito-urinaires. Cette thèse décrit une analyse mutationelle du gène *WT1*, détaillant le spectre et la fréquence des mutations au niveau des tumeurs sporadiques de Wilms. La majorité des mutations du gène *WT1* observées sont homozygotes et génèrant une terminaison traductionnelle prémature, suggérant que l'oncogénie associée avec *WT1* implique un mécanisme de type 'two hit'. L'état mutationnel de *WT1* fut déterminé chez des patients ayant une expression variable du phénotype DDS dans le but d'établir des corrélations potentielles entre le génotype et le phénotype. Les patients ayant des anomalies sévères du développement possédaient des mutations constitutives résultant en l'expression d'une protéine WT1 tronquée, tandis que les patients manifestant un phénotype complet du DDS furent caractérisés par des mutations de type faux-sens à l'intérieur de la région 'zinc finger'. Ceci démontre que différentes mutations du gène *WT1* sont associées avec des effects spécifiques sur le développement génito-urinaire.

Les propriétés biochimiques de WT1 furent caractèrisées et les effets de certaines mutations naturelles furent déterminées. De nombreuses études ont dèmontré que WT1 pourrait être impliqué dans le métabolisme de l'ARN. Une méthode de sélection itérative fut utilisée pour identifier les potentiels ligands d'ARN de WT1. Des ligands spécifiques possédant une grande affinité envers la protéine furent isolés. L'analyse mutationelle de ces ligands a permis d'élucider des structures caractéristiques nécessaires à l'intéraction ARN-protéine. Ces ligands pourraient constituer les séquences d'ARN cibleés par WT1 *in vivo*.

Cette étude démontre que les mutations du gène *WT1* sont présentes seulement dans une minorité de tumeurs de Wilms. L'identification de d'autres gènes contribuant à cette tumeur maligne permettra de mieux comprendre les mecanismes fondamentaux responsable de l'oncogénie associée à la maladie de Wilms. Le rôle du gène suppresseur de tumeur *p53* fut donc également étudié dans cette étude. P53 joue un rôle critique dans la régulation de la croissance cellulaire anormale cellulaire ainsi que de la stabilité génétique. Les mutations

présentes dans le gène *p53* sont restreintes à un rare sous-groupe anaplastique de tumeur de Wilms. Ce sous-groupe représente une variante histologique génétiquement instable associée à un prognostic faible et une résistance à la thérapie. Les mutations du gène *p53* apparaissent tardivement et sont associées avec la progression de la tumeur vers un stade malin. La combinaison des analyses mutationnelles de *p53* et des analsyses histologiques des tumeurs anaplasiques rares permet donc une rationalisation des caractèristiques clinico-pathologiques impliqueés chez diffèrents types de tumeurs. Ceci indique que p53 pourrait réguler l'intégrité génomique et la chimiosensibilité des cellules cancéreuses humaines *in vivo*.

Preface

In compliance with the Faculty of Graduate Studies and Research, the following exerpt from the 'Guidelines for Thesis Preparation' is cited below:

> Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis.

> If this option is chosen, connecting texts that provide logical bridges between the different papers are mandatory. The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.

> The thesis must still conform to all other requirements of the 'Guidelines for Thesis Preparation'. The thesis must include: a Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

> Additional materials must be provided where appropriate (e.g. in apprendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

> In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. Supervisors must attest to the accuracy of such statement at the doctoral oral defence. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers.

The studies appearing in chapters 2, 3, 4, 5 and in the appendix of this thesis were previously published in the following journals.

Chapter 2: Varanasi, R., Bardeesy, N., Ghahremani, M., Petruzzi, M.-J., Nowak, N., Adam, M.A., Grundy, P., Shows, T. & Pelletier, J. (1994). Fine structure analysis of the WT1 gene in sporadic Wilms tumors. *Proc. Natl. Acad. Sci. USA* **91**: 3554.

Chapter 3: Bardeesy, N., Falkoff, D., Petruzzi, M.-J., Nowak, N., Zabel, B., Adam, M., Aguiar, M.C., Grundy, P., Shows, T. & Pelletier, J. (1994). Anaplastic Wilms' tumor, a subtype displaying poor prognosis, harbours p53 gene mutations. *Nature Genet.* **7**: 91.

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Chapter 4: Bardeesy, N., Beckwith, J.B., & Pelletier, J. (1995). Clonal expansion and attenuated apoptosis in Wilms' tumors are associated with p53 gene mutations. *Cancer Res.* **55**: 215.

Chapter 5: Bardeesy, N. & Pelletier, J. (1998). Overlapping RNA and DNA binding domains of the WT1 tumor suppressor gene product. *Nucleic Acids Res.* **in press**.

Appendix: Bardeesy, N., Zabel, B., Schmitt, K., & Pelletier, J. (1994). WT1 mutations associated with incomplete Denys-Drash syndrome define a domain predicted to behave in a dominant negative fashion. *Genomics* **21**: 663.

Chapter 2 was a collaborative effort. DNA preparation, SSCP analysis and sequencing, presented in Fig.2, were performed by Ramani Varanasi, Majid Ghahremani, and myself in roughly equal proportions. The analysis of tumor B.M.#7 in Fig. 3 is my work alone. Jerry Pelletier and I prepared the manuscript. The other authors contributed clinical specimens. Chapters 3, 4, 5 and the appendix are essentially my own work. David Falkoff performed some SSCP analysis in chapter 3. Dr. Bruce Beckwith contributed histological analysis for chapter 4. The mutational analysis in the appendix was done myself and Majid Ghahremani assisted in the DNA sequencing. Jerry Pelletier provided supervision throughout the course of each of these studies. All other authors listed provided clinical specimens.

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Original Contributions to Knowledge

Chapter 2. *Proc. Natl. Acad. Sci. USA* (1994) **91**, 3554-3558.

1) Analysed the status of the *WT1* gene in sporadic Wilms' tumors, the first reported mutational scan of the complete *WT1* gene in a large series of tumors.

2) Identified the mutation pattern and incidence of *WT1* mutations in Wilms' tumors. Mutations are present in only a subset of tumors. Most mutations show coincident loss of the wild type allele and are expected result in truncated proteins.

3) Demonstrated a tumor with intragenic mutations on both alleles of *WT1*, showing that this gene fullfills the classical definition of a tumor suppressor gene.

Chapter 3. *Nature Genetics* (1994) **7**, 91-97.

1) Analysed the status of the *p53* gene in Wilms' tumors. In general *p53* mutations are rare in Wilms' tumors.

2) *P53* was demonstrated to be a second gene (the other being *WT1*) whose mutational inactivation contributes to Wilms' tumorigenesis.

3) Identified *p53* mutations exclusively in Wilms' tumors of the rare anaplastic subtype, a Wilms' tumor variant associated with poor prognosis. This establishes mutant *p53* as a molecular marker for anaplasia and suggests that the loss of *p53* may have a causal role in the genetic instability of this histological variant of Wilms' tumor.

4) *P53* loss in anaplasia is *in vivo* evidence that wild type p53 may be critical to therapeutic responsiveness. The absence of *p53* mutations in Wilms' tumors with favorable histology is consistent with their excellent response to treatment.

Chapter 4. *Cancer Research* (1995) **55**, 215-219.

1) *p53* status was analysed in clonal expansion to anaplasia.

2) Demonstrated clonal selection of cells with *p53* mutations. This provides *in vivo* evidence of the clonal evolution model of tumor progression: *i.e.* the selective growth advantage of a rare cell with a critical mutation eventually becomes dominant cell type.

3) Homozygous *p53* mutations are likely to be late events in the progression of Wilms' tumors.

4) Loss of the remaining wild type allele of *p53* appears necessary for the onset of anaplasia.

5) There is reduced a apoptotic index in anaplastic tumor regions providing evidence that *p53* affects the apoptotic potential of human tumor cells *in vivo*.

Chapter 5. *Nucleic Acids Res.* (1998) in press.
1) An RNA selection method was used to isolate specific ligands which bind to WT1.

2) A series of high affinity ligands were identified which may be relevant to WT1 function.

3) Identified determinants in protein structure and RNA sequence necessary for WT1-RNA binding.

4) Found that the RNA ligands can compete with cognate DNA sequence for binding to WT1, hence the binding sites are likely to overlap.

5) The ligands provide molecular tools for the analysis of WT1 function.

Appendix. *Genomics* (1994) **21,** 663-665.

1) The status of the *WT1* gene was analysed in patients with incomplete manifestation of the phenotypes associated with the Denys-Drash syndrome.

2) A specific spectrum of mutations associated with different phenotypes was detected suggesting the existence of complex genotype-phenotype relationships.

3) The identificiation of mutations provides reagents to assess the biochemical origin of phenotypic differences.

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I am very grateful to Jerry Pelletier for his supervision during the course of my PhD. Jerry taught me the importance of considering the wider implications of this work. He also convinced me that many problems in biology can be solved even if current methodologies are insufficient. Jerry's creative spirit and tremendous will to discovery will remain with me as inspirations.

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INTRODUCTION

The term cancer encompasses a heterogeneous class of diseases which vary widely in clinical presentation, histology, incidence and responsiveness to treatment. Despite their diversity, these diseases have been grouped together because of assumptions that they have a shared fundamental character. Advances in molecular biology and related fields have provided a unifying explanation for cancer. That is, direct or indirect damage to genes is responsible for causing cancer, and therefore, it is a genetic disease. The focus of this thesis is an attempt at gaining insights into the molecular genetic basis of neoplasia, in particular the pediatric renal malignancy, Wilms' tumor. The following introduction will provide a brief overview of the historical evolution of ideas regarding the nature of cancer, eventually culminating in contemporary genetic models for this phenomenon. Subsequently there will be a discussion of the two, genetically defined, classes of genes which are central to malignant processes: tumor suppressors and oncogenes. Comparison of the functional properties of the proteins encoded by these genes illustrates that a number of cellular processes may go awry in the induction of neoplasia. A synopsis of hereditary cancer syndromes will follow. These syndromes result from the inheritance of mutated cancer susceptibility genes and have provided valuable insight into the genetic basis of malignancy. The properties of the *p53* tumor suppressor gene will be reviewed. This gene, mutated in at least 50% of all cancers, is a key component of neoplastic processes. The protein encoded by this gene is a critical regulator of abnormal growth and genetic stability. Next, there will be a review of the literature on the biology of Wilms' tumor. Wilms' tumor is of particular interest since it appears to result from defects in the differentiation of kidney cells, and hence this disease involves the association of aberrant embryonic development with the induction of malignancy. Wilms' tumor was originally thought to conform to a simple genetic model, however, current evidence reveals that it has a considerably more complex genetic basis. Several loci have been implicated in the predisposition to Wilms' tumor and one of these genes, the *WT1* tumor suppressor gene, has been isolated. The biochemical properties of WT1 will be reviewed and interpreted with respect to the role of this protein in tumorigenesis. Finally, there will be a discussion of the search for other Wilms' tumor-predisposing loci. Unexpected observations derived from these studies have led to suggestions that the current genetic models to explain the origins of cancer may be incomplete.

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Historical Perspectives on the Nature of Cancer

The terms cancer, neoplasia, and malignancy are generally used interchangeably, and refer to the process whereby a mass of cells causes structural disruption of a tissue and whose persistent growth exceeds, and is uncoordinated with that of the normal tissue. The existence of the clinical phenomenon of cancer has long been known. The evolution of modern scientific methods in the nineteenth century and their application to medicine was integral in the development of current perceptions of malignancy (following section based on Triolo, 1964; 1965; Rowlatt, 1994). Early models employed discoveries by pathologists and embryologists who studied the cellular organization of tissues. An important hypotheses suggested that all animal cells originated from pre-existing animal cells. An extension of this idea declared that all animal cells originate from pre-existing animal cells *of the same kind*; and hence, a tumor is derived from the proliferation of cells from the same general type as those of the tumor. It is this foundation which provides the basis of the current classification of neoplasms as carcinomas or sarcomas depending on their epithelial or mesenchymal origin, respectively. This classification encouraged the appraisal of cancers as substantively related, although heterogeneous, and gave impetus to attempts to account for the nature of this disease. Early models for the origins of neoplasia were highly speculative including hypotheses that irritative stimuli or bacteria may have causal roles. Twentieth century formulations include Waddington's link between developmental aberrations and malignancy, Green's immunological theory of cancer and Haddow's model of tumorigenesis as aberrant 'overhealing' of damaged tissue (all refs. from Rowlatt, 1994).

These descriptive models of the clinical phenomenon of malignancy have today been superseded by the evolution of ideas regarding the mechanistic basis of cancer. Models asserting a genetic origin of neoplasia have gained wide acceptance. On the most simple level these models suggest that gene mutations are the most fundamental changes in tumorigenesis. An early formulation of this idea was advanced by Boveri (ref. in Rowlatt, 1994) in his somatic mutation theory. He suggested that single cells with chromosomal abnormalities were responsible for neoplasia. Early evidence supporting this model was derived from studies of mouse tumor transplants, in which Tyzzer (1913) noted a wide variation in tumor take in mice of the same inbred strain. Since these mice were genetically identical he proposed that the variation must have been caused by novel changes arising in the transplanted tumor. The offspring of mice showing strong tumor take did not inherit susceptibility to successful transplant of the primary tumor, reinforcing the notion that

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acquired changes in the tumor cells conferred the variable tumor growth. The phenomenon of variability was ascribed to somatic mutations, advancing the idea that there are genetic differences between tumor cells and normal cells. Other important observations which supported genetic models include the discovery of the mutagenic nature of carcinogens. H.J. Muller (1948) demonstrated this association when he showed that x-rays, previously recognized as carcinogenic, are mutagenic in fruit flies. Additionally, the two-stage carcinogenesis in experimental mouse skin cancers suggested that an early step in cancer likely involves gene mutation. The treatment with low doses of mutagen (initiation) followed by repetitive application of a nonmutagenic agent (promotion) produced benign, followed by malignant, skin tumors. The interval between the application of the mutagenic initiator and the tumor promoter could be varied with no decrease in the degree of effectiveness. This irreversibility of the initiation step suggested that it involved induction of a permanent genetic change. Muller (1948) and Nordling (1953) proposed that cancer arises due to multiple mutations within the same cell. This concept prevails today, thus, cancer is considered to be a genetic disease which arises from the accumulation of mutations in a cell resulting in the clonal selection of cells with increasing advantages in growth. The first observation of a defective chromosome in cancer was the identification of the Philadelphia chromosome translocation in chronic myelogenous leukemia (Nowell & Hungerford, 1960). The subsequent discovery of recurrent chromosome abnormalities specifically associated with cancer has confirmed the presence of genetic damage in cancer cells.

Cancer Genes

Clinically, neoplasia is manifested as a loss of normal growth control. Under normal growth conditions there is a tight regulation of cell proliferation balanced with natural cell death (Sherr, 1996; Pan et al., 1997). This equilibrium is modulated by the interplay of proteins which enhance or repress cell growth. It follows that neoplasia is associated with aberrations in these pathways of positive and negative control. The advances of molecular genetics have facilitated the discovery of a number of genes which are mutated in human cancers. These genes appear to fall into two general classes: those which are involved in suppressing growth in some manner (tumor suppressors) and those which show growth promoting activity (oncogenes) (Marshall, 1991; Hunter, 1991). The following section reviews the basis of the concepts of tumor suppressor genes and oncogenes and will attempt to evaluate the role of these genes in neoplasia.

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Tumor Suppressor Genes

The concept that the inactivation of genes involved in the negative regulation of cell proliferation may be a critical component in malignancy is derived from multiple lines of evidence, including data from epidemiological analysis of familial cancer, somatic cell genetics experiments and detection of loss of heterozygosity. The discovery of cellular proteins which interact with oncogenic proteins of mammalian tumor viruses subsequently consolidated the models for 'tumor suppressor genes'.

Two-Hit Models for Neoplasia - Epidemiology of Familial Cancers

The emergence of the current notion of tumor suppressor genes was derived in part from attempts to account for the onset of childhood cancers. It is clear that cancer incidence increases exponentially with age (Loeb, 1996). This increase has been attributed to the requirement of an accumulated series of mutations in tumorigenesis. Why then do children with cancers seem to incur such mutations at a much faster rate? Stewart (1961) proposed that a mutation could be inherited and could show no phenotypic manifestation until the occurrence of a second mutation. The inherited mutation would be present in all cells of the body and thus there would be a very large population of cells which would be susceptible to the effects of a second mutation. Given such a large population of susceptible cells the chances of an oncogenic mutation would be very high over a long period of time and hence individuals who inherited the first mutation would develop cancer at an earlier age than people who required two independent somatic mutations. Burch (1962) extended this model to suggest that one or more inherited mutations could substitute for somatic events. He also suggested that the converse may hold: that somatic events may be able to replace germinal events .

These models for childhood cancer were applied by Knudson (1971) in his epidemiological analysis of retinoblastoma, a pediatric eye tumor. Knudson compared bilateral forms (tumors affecting both eyes) with unilateral forms of retinoblastoma. He determined that the bilateral form was associated with an earlier age of onset and with multiple tumors. The bilateral form was found at much higher frequency in a familial setting than in sporadic cases. His statistical analysis suggested that the kinetics of development of bilateral retinoblastoma was consistent with a single rate-limiting event, whereas unilateral cases involved two independent events. Knudson proposed that retinoblastoma involves two sequential mutations. The bilateral cases were postulated to have inherited the first mutation such that it was present in all cells of the body, which

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would subsequently require a single somatic event for tumorigenesis. In the sporadic form, the requirement of two somatic mutations in the same cell accounts for the later onset and development of only a single tumor. Epidemiological analysis showed that a two-hit model could account for a number of cancers that occurred in both hereditary and sporadic forms, including Wilms' tumor (Knudson & Strong, 1972a), neuroblastoma and pheochromocytoma (Knudson & Strong, 1972b). Today, Knudson's work is widely celebrated and two-hit models for malignancy are often referred to as 'Knudson's model' in recognition of his contributions to the field of cancer epidemiology. As we shall see, molecular studies have confirmed the role of two-hit carcinogenesis in a number of malignancies, although Wilms' tumor has a more complex genetic basis.

Somatic Cell Hybrids

The two-mutational statistical model did not address the nature of the mutations. Hence, it was not apparent why two mutations were required for tumor development, neither was it known whether both mutations target the same gene, different alleles of the same gene, or whether they affect two completely distinct genes. Harris et al. (1969) attempted to address whether the phenotype of a cancer cell involved genetic dominance by fusing tumorigenic with nontumorigenic cells. Most hybrid cells grew well in culture and retained many characteristics of the malignant parent cell. These hybrids, however, did not produce tumors when injected into animals. Thus, it was suggested that the tumorigenic phenotype could be suppressed by chromosomes from the nontumorigenic cell, and it was interpreted that tumorigenesis involved a recessive genetic change which could be genetically complemented. It was demonstrated that in some cases the fusion of cells from two different tumors resulted in a nontumorigenic phenotype, while, conversely, hybrids of cells from tumors of the same type retained tumorigenicity (Wiener et al., 1974). The ability of different cancer cells to complement each other suggested that each had incurred a different oncogenic mutation. Non-tumorigenic hybrids between non-tumorigenic and tumorigenic cells were generally stable karyotypically and phenotypically. However, some tumorigenic revertants did appear and these were correlated with the reproducible loss of particular pairs of chromosomes (Stanbridge, 1976). It was proposed that these chromosomes harbor genes that are capable of suppressing tumorigenesis (i.e. 'tumor suppressor genes').

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Loss of Heterozygosity

Genomic instability is a hallmark of neoplasia (Loeb, 1996). Many malignancies show widespread genetic alterations including large-scale events such as chromosomal deletions, duplications and translocations as well as more restricted genetic changes. Large chromosomal changes, which are often easily detectable by cytogenetics or by the use of molecular probes, may be specific tumorigenic alterations or may be random events resulting from general genomic instability. The discovery of the nonrandom loss of chromosomal regions in cancer cells was a further indication of the existence of tumor suppressor genes. The correlation of these genetic loses with tumor development suggested that, in normal cells, these regions must harbor genes which inhibit malignant processes. The first such losses to be discovered were large chromosomal deletions detected cytogenetically in a small proportion of retinoblastomas (Francke, 1976), and Wilms' tumors (Francke *et al.*, 1979) at chromosomes 13q and 11p, respectively. Molecular probes for polymorphic loci throughout the genome have permitted the identification of regions consistently lost in a wide range of cancers (Ponder, 1988). The discovery that the pattern of chromosomal loss is distinct for many cancers supports the notion, derived from the somatic cell fusions, that different tumor suppressor genes are involved in specific cancers. Some regions show loss in several cancers, notably 13q and 17p, suggesting that certain genes may play a role in multiple cancers (ref. in Mannens *et al.*, 1990).

The loss of a chromosomal region in a tumor relative to the constitutional DNA of the same individual is termed loss of heterozygosity (LOH). LOH at a given locus is interpreted to reveal a recessive mutation of the homologous allele loss of the wild type allele. LOH can occur by a number of distinct mechanisms including mitotic nondisjunction (with or without duplication of the remaining chromosome), mitotic recombination, gene conversion, interstitial deletion and chromosome loss (Hansen, 1994) (see Fig. 1).

Properties of Tumor Suppressor Genes

Taken together, the observations discussed above provide evidence for the existence of genes which have an inhibitory effect on malignancy. A number of human tumor suppressor genes have been isolated by molecular cloning (reviewed in Fearon, 1997). Many are heterozygously mutated in the constitutional DNA of patients with the hereditary forms of the respective cancer (see Table 1, pg.12) and sustain somatic mutations in the sporadic forms of the disease. These mutations generally undergo reduction to homozygosity in the tumor itself. The functional inactivation of both alleles

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Figure 1. Mechanisms of Tumor Suppressor Gene Loss in Tumorigenesis.

Mechanisms of tumor suppressor gene loss in tumorigenesis.

A child inherits a chromosome harboring a mutated tumor suppressor gene. The loss of the second allele of this gene may occur somatically by four different mechanims. The homogyzgous mutant genotype results in the development of a tumor.
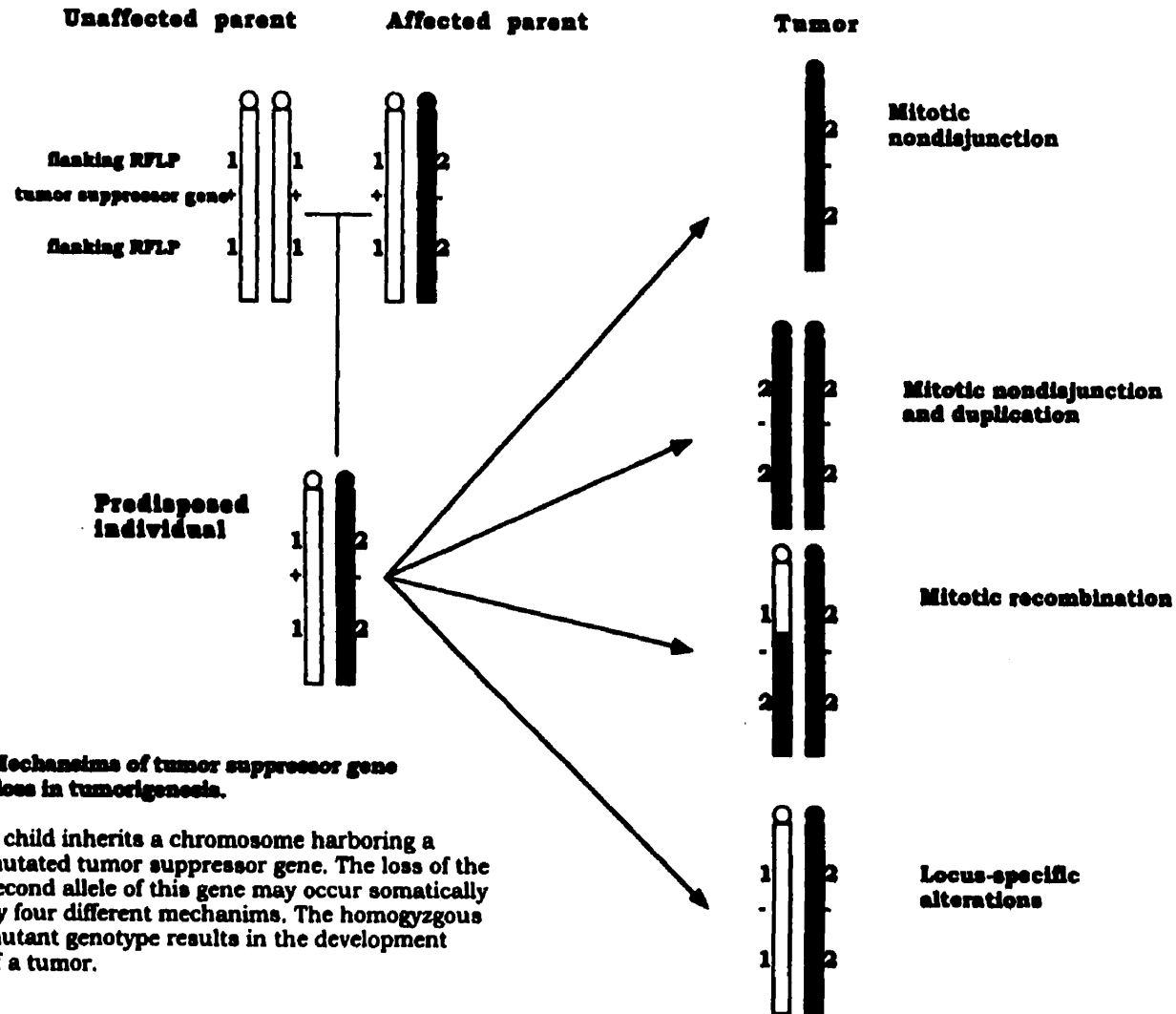
supports the notion that tumor suppressor gene mutations behave recessively within the cell. Mutational inactivation may involve a number of different mechanisms and the distribution of such lesions is characteristic for each tumor suppressor gene (Harris & Holstein, 1993; Fearon, 1997). *P53* generally displays missense mutations, while alterations of *APC* and *RB1* are mainly chromosomal deletions or nonsense mutations. Other means of inactivation of tumor suppressor genes include insertional mutations or complex insertion/deletions (Varanasi *et al.*, 1994) promoter mutations (Sakai *et al.*, 1991), intronic mutations which disrupt splicing (Haber *et al.*, 1990), and gene rearrangements. The p53 protein is functionally inactivated in a subset of sarcomas by the amplification and consequent increased expression of the *Mdm2* gene, coding for a negative regulator of p53 (Oliner *et al.*, 1992). Although it has yet to be conclusively demonstrated, there is some evidence that changes in genomic imprinting (Reeve, 1996) and in RNA editing (Skuse & Cappione, 1997) may be involved in the deregulation of some tumor suppressor genes. Tumor suppressor genes have extremely diverse properties (reviewed in Fearon, 1997). They vary in subcellular localization, in expression profile and in biochemical function. Tumor suppressor gene-encoded proteins include those that localize to the nucleus (*e.g.* *VHL*), are cytoplasmic (*e.g.* *APC*), or are integrated into the plasma membrane (*e.g.* *PTCH*). Examination of the expression profile of tumor suppressor genes shows that some are ubiquitous (*e.g.* *RB1*) while others show highly regulated temporal and spatial expression (*e.g.* *WT1*). The diversity of tumor suppressor genes is also illustrated by their multiform functions. Some of these genes encode DNA-binding transcription factors (*e.g.* *p53*, *WT1*) or may be regulators of transcriptional elongation (*VHL*). Others tumor suppressor gene products function in the negative regulation of the cell cycle (p16), as transmembrane receptors (PTCH), in signal transduction pathways (NF1, APC), and in DNA repair (*e.g.* *MSH* and *PMS* genes). Hence, while each of these genes function must impinge on the fundamental regulation of cellular proliferation, there is apparently no primary biochemical property uniting the tumor suppressor gene products.

Inactivation of Tumor Suppressor Proteins by Tumor Virus Proteins

DNA tumor viruses share a common pathway for the immortalization and transformation of host cells (reviewed in Howley, 1995). The transforming proteins encoded by distinct types of DNA tumor viruses function through interactions with central cellular regulatory proteins, resulting in disruption of the activity of these cellular proteins and release from blocks in cell proliferation. Cellular transformation by adenovirus, type 5 requires expression of the E1A an E1B protein, which interact with the tumor suppressor

proteins RB1 and p53, respectively. Transformation by the polyomavirus SV40 T antigen and the human papillomavirus E6 and E7 proteins is also dependant on their ability to bind and inactivate the cellular RB1 and p53 proteins. The genetic evidence for the existence of tumor suppressor genes discussed above was correlative, since the functional significance of mutations was not directly demonstrated. The discovery that the transforming property of DNA tumor virus oncoproteins depends on their ability to inactivate the RB1 and p53 gene products provides functional evidence that these proteins have critical roles in preventing malignant transformation.

Definition of Tumor Suppressor Genes

Having reviewed the evidence for the existence of genes which have an inhibitory effect on malignancy, we are now in a position to establish a general definition of a tumor suppressor gene. Haber and Harlow (1997), have suggested that a simple, genetic definition of tumor suppressor genes is necessary to provide a unifying principle for classifying these functionally diverse genes and avoid conceptual difficulties. They define tumor suppressors as genes that sustain loss-of-function mutations in the development of cancer. This definition obviates the requirement that reintroduction of a putative tumor suppressor gene into appropriate cancer cells must result in reversion of the tumorigenic phenotype to formally demonstrate its tumor suppressing activity. This assay would result in inappropriate classification of certain genes. For example, tumor suppressor genes encoding DNA repair enzymes, which trigger tumorigenesis by creating a mutator phenotype would be unlikely cause reversion of a tumorigenic phenotype. Conversely, overexpression of genes which inhibit cellular growth by interfering with anchorage-independant growth or tumor take in nude mice, may appear to be tumor suppressor genes using this assay system, and yet may not have critical involvement in tumorigenesis. The problems associated with too broad a definition can been seen in the case of 'oncogenes'. The term oncogene has been used to refer to genes which when ectopically expressed or mutationally activated can result in some type of cellular growth enhancement. However, many of these genes never exhibit mutational activation in human cancers and their relevance to oncogenesis *in vivo* is not established (Weinberg, 1995). Thus, in many cases what has been designated an oncogene *in vitro* may never have authentic involvement in human malignancies (see below).

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Oncogenes

The existence of genes which induce malignancy in a dominant manner was first suggested by studies of animal tumor viruses. Genetic analysis demonstrated the requirement of only one or a few viral genes (oncogenes) for oncogenicity (reviewed in Hunter, 1991; Rosen, 1995). The relevance of these genes to human cancers was not evident, however, since it was not possible to isolate viruses from most clinical tumors and, correspondingly, most cancers did not show an infectious mode of transmission. Great interest in oncogenes accompanied the discovery of cellular counterparts to viral oncogenes. Analysis of the cloned mammalian homologues (proto-oncogenes) demonstrated close relation to the viral genes. The viral genes were distinguished by regulation by strong promoters and, for many, by their 'activated' state coded for by point mutations or truncation relative to the cellular proto-oncogene. A commonly employed technique to identify other dominant transforming genes involved transfections of tumor DNA into host tissue culture cells. Transfected cells which had undergone transformation, defined as loss of contact inhibition, growth in soft agar, and tumorigenicity in nude mice, were interpreted to have been altered by the expression of an oncogene. The search for cellular homologues for viral oncogenes and the use of transfection/transformation assays yielded the identification of over twenty oncogenes (Weinberg, 1995). As in the case of the tumor suppressor proteins, the oncogenes encode proteins with a great diversity of functions (Rubin, 1995).

The absence of mutations in the majority of these genes in human cancers suggests that only a limited subset may be relevant in the pathogenesis of nonviral malignancies (reviewed in Donovan *et al.*, 1994). Overall, the search for cellular oncogenes involved in human cancers through the isolation of homologues to viral oncogenes and transfection/transformation assays failed to match early expectations (Weinberg, 1995). It is likely that the limitations of the former method are due to the peculiarities associated with viral replication and to the artificial conditions presented by the use of NIH3T3 cells in the transfection experiments. These cells are immortal and can become transformed spontaneously, thus they may not present conditions relevant for the study of the carcinogenesis. In spite of these reservations, however, some oncogene alterations do clearly have important roles in malignancies. Oncogenes which have been implicated in human cancer include the *ras* genes which show activating missense mutations in a wide range of human tumors including bladder, breast, lung, and colon carcinomas (Bos, 1989). *Myc* and *N-myc* are overexpressed in a number of tumors due to gene amplification or

translocation (Bishop, 1991). Finally *erbB* and *erbB-2* (*neu*) show amplification in a number of tumors, most notably in 20-30% of primary breast tumors (Meyers *et al.*, 1990). Hematopoietic malignancies are characterized by high frequencies of cytogenetic abnormalities, particular chromosomal translocations. The mapping to these translocation break points has lead to the identification of a large number of dominant transforming genes (reviewed in Look, 1997). Such translocations have been found to result in the aberrant expression of proto-oncogenes (*e.g.* the *c-myc* gene in Burkitt's lymphoma) or in the genesis of gene fusions which produce chimeric proteins with novel oncogenic properties (*e.g.* bcr-abl in chronic myelogenous leukemia).

Common Types of Genetic Lesions in Histologically Related Tumors

As we have seen, chromosomal imbalances and LOH can provide clues to the molecular etiology of a given malignancy. An interesting observation that emerges from studies of cancer genetics is that different malignancies of a related histological type often share a similar type of genetic lesion. For example, leukemias and lymphomas are characterized by chromosomal translocations which result in gene activation or gene fusions which yield chimeric proteins (Look, 1997). Sarcomas (tumors of mesenchymal origin) frequently have chromosomal translocations which produce oncogenic chimeric proteins (Ladanyi, 1995), while carcinomas (tumors of epithelial origin) are usually characterized by tumor suppressor gene loss (Harris & Hollstein, 1993). The differences in the types of genetic lesions detected in histologically unrelated malignancies suggests that they may possess fundamentally different means of controlling genome stability and cell proliferation.

Tumor Suppressor Genes and Oncogenes in Human Malignancies

Multistage Tumorigenesis

It is evident that mutations in cancer disturb two fundamentally different classes of genes, tumor suppressors and oncogenes. We can now attempt to assess the relative role of these genes in human malignancies. Which mutations are responsible for the initiation of neoplasia? Which are associated with the malignant progression of existing tumors?

Knudson's model of pediatric tumors, reformulated with the knowledge of tumor suppressor genes maintains that the rate limiting step for the onset of malignancy is the acquisition of mutations in both alleles of a tumor suppressor gene. In contrast,

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tumorigenesis in the adult and in some pediatric malignancies, likely involves genetic changes at more than one locus. Indeed, there are a number of lines of evidence which suggest that carcinogenesis is a multistep process. Mathematical models to account for the exponential increase of cancer incidence with age predict the requirement of multiple genetic changes for the onset of cancer (Loeb, 1996). In addition, multiple specific genetic changes are observed in human tumors. The progression of cancers often involves a sequence of genetic lesions associated with morphological changes. In the well studied example of colorectal carcinogenesis, *APC* mutations are associated with the formation of benign adenomas, while *p53* mutations occur at the late malignant carcinoma stage (Fearon & Vogelstein, 1990). The *K-ras* oncogene usually displays activating mutations at an intermediate time. There are now a number of cancers for which the association of specific genetic events with progressive stages of carcinogenesis has been documented (*e.g.* Furnari *et al.*, 1995). The progression of multistage malignancy may require homozygous inactivation of a tumor suppressor gene, however, such an event is not the single rate limiting step.

Hereditary Cancer Syndromes

Most cancers are sporadic, that is, they arise in the absence of a family history of the disease. The incidence of most sporadic cancers increases logarithmically with age and in some cases there is a strong environmental component (including diet, toxic exposure, *etc.*). The small role of heredity in the overall occurrence of specific cancers is exemplified by twin studies which show a concordance rate of monozygotic twins which does not differ substantially from that of dizygotic twins (reviewed in Claus, 1995). There is, however, clearly an inherited component in oncogenesis. Epidemiological studies show an increased risk of cancer for individuals with affected family members. A small proportion of cancers (about 1%) occur in the context of clinically defined hereditary cancer syndromes (reviewed in Fearon, 1997). Each syndrome exhibits a specific set of cancer types and some are associated with dysmorphic phenotypes (see Table 1). Most of these syndromes are highly penetrant and familial cases of cancer also show an earlier age of onset compared with sporadic cases of the disease. These patients, therefore, are considered to have inherited predisposing mutations, most likely in a single gene. The availability of pedigrees of affected families and the rise of molecular cloning technology has facilitated the identification and characterization of a number of genes involved in hereditary cancer syndromes. Much of the current knowledge of the genetic basis for cancer is derived from the molecular cloning of these genes.

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Table 1. HEREDITARY CANCER SYNDROMES

Syndrome	Primary tumor	Associated traits	Gene	Gene function
Retinoblastoma, familial	retinoblastoma	osteosarcoma	RB1	cell cycle and transcriptional regulation
Li-Fraumeni syndrome	sarcomas, breast cancer	brain tumor, leukemia	p53	transcription factor, response to DNA damage, apoptosis
Familial adenomatous polyposis	colorectal cancer	duodenal, gastric, & desmoid tumor, jaw osteosarcoma	APC	microtubule binding
Hereditary Non-polyposis colorectal cancer	colorectal cancer	endometrial, ovarian, hepatobiliary and urinary tract cancer	MSH2 MLH1 PMS1 PMS2	DNA mismatch repair
Neurofibromatosis type 1	neurofibroma	neurofibrosarcoma, AML, brain tumor	NF1	GTPase activating protein for ras proteins
Neurofibromatosis type 2	acoustic neuroma meningioma	glioma, ependymona	NF2	linkage of membrane to cytoskeleton?
WAGR syndrome	Wilms' tumor	aniridia, genitourinary defects mental retardation	WT1	transcription factor
Beckwith-Weidemann syndrome	Wilms' tumor	overgrowth, hepatoblastoma, adrenocortical cancer	p57?, IGFII? others	cell cycle regulator growth factor
Breast cancer	breast cancer	ovarian cancer	BRCA1	DNA repair?
Breast cancer	breast cancer	male breast cancer, pancreatic cancer	BRCA2	DNA repair?
von Hippel-Lindau syndrome	clear cell renal cancer	pheochromocytoma, retinal angioma, hemangioblastoma	VHL	regulation of transcriptional elongation?
Hereditary papillary renal cancer	papillary type renal cancer		MET	transmembrane growth factor receptor
Familial melanoma	melanoma	panreatic cancer, atypical moles	p16 CDK4	cyclin-dependent kinase inhibitor cyclin-dependent kinase
Multiple endocrine neoplasia type 2	medullary thyroid cancer	MEN2A:pheochromocytoma parathyroid hyperplasia MEN2B:pheochromocytoma mucosal hamartoma FMTC: thyroid cancer	RET	transmembrane receptor tyrosine kinase for GDNF
Ataxia telangiecstasia	lymphoma	cerebellar ataxia, immunodeficiency	ATM	DNA repair

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Mode of Inheritance

The majority of hereditary cancer genes are inherited in a dominant manner, and are characterized by inactivating mutations (Hansen, 1994; Fearon, 1997). At the cellular level, these mutations are recessive, requiring loss of the wild type allele for tumorigenicity. Thus, most hereditary cancer genes are tumor suppressors. Three syndromes are known to be caused by germline mutations of proto-oncogenes, involving the *RET*, *MET* and *CDK4* genes. In the neoplasms associated with these syndromes the wild type allele is unaffected, and thus the mutations are dominant in the cell (*e.g.* see Moley, 1997). Mutations in other genes are required to cooperate with these activated oncogenes in the development of cancer. A possible explanation for the relative rarity of germline mutation in oncogenes versus tumor suppressor genes may be due to their dominant activity within the cell. Whereas, in most cases, the heterozygous mutation of a tumor suppressor gene causes no phenotype because of the presence of the functional wild type allele, a heterozygously activated oncogene is able to exert its effects dominantly in any cells in which it is expressed. It is possible, therefore, that germline activation of most oncogenes may result in embryonic lethality and thus would not be associated with a hereditary cancer syndrome. Alternatively, oncogenes may not play an initiating role in most cancers.

A small number of hereditary cancer syndromes are inherited in a recessive manner, namely ataxia telangiecstasia, Bloom's syndrome, xeroderma pigmentosum and Fanconi's anemia. All of these involve genes which code for proteins thought to be involved in DNA repair processes (Kinzler & Vogelstein, 1996; Fearon, 1997). With the exception of a possible predisposition to breast cancer in *ATM* heterozygotes, only homozygous individuals show malignant phenotypes in the recessive cancer syndromes. This contrasts with the high penetrance of heterozygous alleles in other syndromes involving germline tumor suppressor gene mutations. These DNA repair genes are unlikely to play direct antineoplastic roles and instead probably control the likelihood of mutations throughout the genome. The DNA damage in homozygotes increases the frequency of mutation in other tumor suppressors and oncogenes which are able to initiate neoplasia (Kinzler & Vogelstein, 1996).

Role of Hereditary Cancer Genes in Sporadic Tumors

All of the hereditary cancers have sporadic counterparts. Knudson's two-hit model for malignancy predicts that the same gene which harbors germline mutations in the

hereditary form of the disease should undergo somatic mutations in patients with the sporadic form. This prediction holds for a number a cancers. All cases of retinoblastoma, for example, show *RB1* mutations (Dunn *et al.*, 1988). Sporadic clear cell renal carcinomas, like those inherited in patients Von Hippel-Lindau disease, show very high incidence of mutations in the *VHL* gene (reviewed in Zbar, 1995). In contrast *BRCA1* and *BRCA2* mutations, which are responsible for most breast cancers arising in a hereditary setting, are rarely present in sporadic cases (Futreal *et al.*, 1994; Wooster *et al.*, 1995). Thus, the genetic etiology of sporadic cancers can differ substantially from that of the corresponding hereditary disease. This may indicate that two-hit models are valid for only a subset of malignancies.

Genetic Heterogeneity of Hereditary Cancer Syndromes

The genetic heterogeneity of cancers is also displayed by a number of hereditary syndromes which predispose to phenotypically indistinguishable tumors but segregate with distinct loci in different families (reviewed in Fearon *et al.*, 1997). In Lynch syndrome (hereditary nonpolyposis colorectal cancer) for example, mutations can be present in any of four DNA mismatch repair genes (*MSH2*, *MLH1*, *PMS1*, *PMS2*) (reviewed in Kinzler & Vogelstein, 1996). There is no clinical distinction between the tumors associated with each mutation. Similarly, familial melanoma is associated with either inactivating or activating mutations in the *p16* or *CDK4* genes, respectively. The proteins encoded by these genes have opposite effects on the cell cycle: p16 inhibits, while CDK4 stimulates, passage through the G1 cell cycle phase (reviewed in Sherr, 1997).

Some hereditary cancer syndromes display wide clinical variability. For instance, Von Hippel Lindau disease (VHL) is associated with a distinct tumor spectrum in different families (Zbar, 1995). All VHL families show predisposition to retinal angiomas and central nervous system haemangioblastomas, whereas the frequencies of renal cell carcinoma (RCC) and pheochromocytoma are variable between families. The genetic basis for these differences has been identified and associated with the nature of the inherited *VHL* mutation. Hereditary medullary thyroid carcinomas are associated with three distinct syndromes: multiple endocrine neoplasia type 2A (MEN2A) and type 2B (MEN2B) and familial, non-MEN medullary thyroid carcinoma (FMTC) (reviewed in Moley, 1997). The syndromes all involve mutations in the *RET* proto-oncogene. MEN2A and FMTC are associated with similar mutations while MEN2B is caused by a different *RET* mutation. These examples demonstrate that different mutations in a cancer susceptibility gene can produce discrete diseases. The observation that distinct syndromes (MEN2A and FMTC)

are associated with identical *RET* mutations suggests that modifier loci may interact with the disease locus and alter the resulting phenotypes.

Other Cancer Susceptibility Genes - Modifier Loci

There are likely to be other cancer susceptibility genes which cannot strictly be considered oncogenes or tumor suppressor genes. (Weinberg, 1995; Perera, 1997). Some of these genes may have functions not directly related to control cellular proliferation and instead control responsiveness to environmental factors. In addition, it would be expected that inherited polymorphisms would be associated with increased susceptibility and thus such genes would be unlikely to show *de novo* somatic mutations associated with malignancy. Mutations in these genes would be likely to show low penetrance since somatic mutations of tumor suppressor genes or oncogenes would be necessary for the development of cancer. Such susceptibility genes could be involved in detoxification or immune surveillance, for example (Weinberg, 1995). A class of susceptibility genes are referred to as modifier loci. These are allelic variants which modulate the phenotype of a separate mutant locus. Modifier genes are likely to be the cause of the variable expressivity of a number of inherited cancer syndromes which cannot be accounted for by specific mutations in the cancer susceptibility genes. Studies of families with *BRCA1* mutations show a lack of correlation between type of mutation and severity of the disease (Neuhausen *et al.*, 1996). There is also a wide clinical variability between familial adenomatous polyposis patients bearing identical mutations in the APC gene (reviewed in Dragani & Manenti, 1997). In mouse models for this disease, bearing a disrupted homologue of the *APC* gene, there is strain-specific severity of colon cancer. The gene responsible for this modifier effect, *Mom1*, has been identified and encodes a secretory phospholipase (Cormier *et al.*, 1997). The low penetrance and absence of tumor-associated mutations of these genes makes them difficult to identify in humans. Effective study requires epidemiological analysis of a large cohort which evaluates the risk factor associated with a candidate polymorphic locus. In the absence of a candidate, identification of modifier loci requires extensive genetic linkage studies of phenotyped pedigrees of individuals carrying specified disease mutations.

The p53 Tumor Suppressor Gene

The *p53* tumor suppressor gene is the most frequently mutated gene in human cancers (reviewed in Greenblatt *et al.*, 1994). *P53* lesions are widely distributed, affecting

nearly every organ and cell type. The prevalence of such mutations is extremely variable between tumor types, ranging from 0 to 60% in major human cancers, and is over 80% in some histological subtypes. Most tumors show small intragenic *p53* mutations and are associated with the loss of the wild type allele. The relative timing of *p53* mutations is variable in the genesis of different tumors suggesting an involvement in tumorigenic initiation in some cases and in progression in others. The wide spectrum of neoplasms showing *p53* involvement is suggestive of a fundamental role in the regulation of cell proliferation. The p53 protein been implicated a wide range of cellular processes including DNA damage repair, senescence, transcription, programmed cell death, cell cycle regulation, and chromosomal stability.

p53 Structure-Function Relationships

The p53 protein has a number of defined domains that are indicative of a role in transcriptional regulation (reviewed in Levine, 1997). There is an amino terminal acidic transcriptional action domain (TAD). This region has been shown to interact with subunits of the TFIID basal transcription factor. The MDM2 protein, a negative regulator of p53, also binds to the TAD and inhibits transcriptional activation (Wu *et al.*, 1993). This region of the p53 gene rarely shows missense mutations in human cancers (Harris & Hollstein, 1993). This may be attributed to the relative amino acid plasticity, with respect to function, of this region (Lin *et al.*, 1994).

The p53 protein displays sequence-specific DNA binding mediated by a core domain (amino acids 102-292). The structure of the p53 core domain bound to DNA has been determined by x-ray crystallography (Cho *et al.*, 1994). The DNA binding domains of most transcription factors are structurally related and can thus be classified into various broad families of proteins (*e.g.* zinc finger class or homeobox class proteins). The DNA binding domain of p53, however, has a unique structure. It is composed of a large B-sandwich, consisting of two antiparallel β sheets which acts as a scaffold for loop-based elements which make contacts with the DNA. Greater than 90% of the missense *p53* mutations in human cancers occur in the DNA binding domain, furthermore, over 40% of these mutations involve six residues (R175, G245, R248, R249, R273, R282) (Harris & Hollstein, 1993). The solution of the structure of the p53 DNA binding domain has allowed the rationalization of this spectrum of p53 mutations (Cho *et al.*, 1994). It is evident that there are two classes of mutations in this domain: those that disrupt residues directly involved in DNA binding, and those that disrupt the overall structural integrity of the domain. R248, the most common naturally occurring mutation, makes direct contact

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with the A/T residues in the minor groove of the DNA binding site. R273 also makes direct contacts with DNA. Mutants at these residues retain the native protein structure, whereas the other common mutations result in denaturation of the protein structure.

The carboxy-terminus of p53 harbors a multifunctional domain (reviewed in Levine, 1997). The basic region nearest the carboxy-terminus is responsible for regulating the sequence-specific DNA binding affinity of p53. DNA binding is activated by a conformational change mediated by acetylation of the basic region. The p300 protein, a cell cycle regulator, catalyses this acetyltransferase activity, potentiating the ability of p53 to activate transcription (Gu & Roeder, 1997). The carboxy-terminus also contains the domain responsible for tetramerization of the protein. The carboxy-terminal domain can catalyze the annealing of single strands of nucleic acids and appears to be involved in DNA repair processes. This region of p53 is rarely mutated in human cancers (Harris & Hollstein, 1993).

p53 Mediates Checkpoint Control in Maintenance of Genetic Stability

The spontaneous mutation rate of normal human cells is low. It has been estimated that during an average lifespan a normal cell will accumulate two to three mutations (Loeb, 1996). In contrast, genetic instability is a characteristic feature of most cancer cells which show multiple mutations ranging from point mutations, to large chromosomal deletions, translocations and aneuploidy. This difference can be accounted for by the loss of controls regulating the cell cycle and DNA damage detection and repair. In normal cells, there is cell cycle arrest at the G1 and G2 checkpoints in response to DNA damage permitting DNA repair enzymes to function preventing the propagation of mutations to daughter cells (Wahl et al., 1997). P53-deficient cells are defective in G1 arrest pathways, and loss of this function is likely to result in selective accumulation of mutations in cancer cells.

Signals Mediating the Activation of p53

p53 levels are low in normal cells due to a short protein half-life (reviewed in Levine, 1997). p53 expression is not necessary for the viability of normal cells and homozygous p53-null mice usually develop normal (although they are highly predisposed to tumors) (Donehower et al, 1992). The role of p53, thus appears to be limited to unusual situations of cell stress. Levels of p53 rapidly increase in response to DNA damage, through an increase in protein half-life. The mechanisms of the induction are not known, however, double-stranded DNA breaks are particularly strong inducers of p53 (reviewed in

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Harris, 1996; Wahl, 1997). This activity may involve direct binding of p53 to the damaged DNA through the carboxy-terminal domain. There is some evidence that the DNA repair machinery can provide signals to p53 indicating that damage has been detected. Cells from ataxia-telangiectasia patients who harbor homozygous mutations in the ATM DNA repair gene show deficient induction of p53 in response to ionizing radiation. Additionally, protein interactions have been detected *in vitro* with a number of DNA repair enzymes (reviewed in Harris, 1996). For p53 activation the DNA damage can be induced by diverse stimuli including gamma and ultraviolet radiation, or chemical agents.

Cancer-Inhibiting Pathways Regulated by p53

The consequences of p53 activation are either arrest in the G1 stage of the cell cycle or apoptosis (programmed cell death) depending on the cell type and the environmental context of the cell under study (reviewed in Levine, 1997). G1 arrest requires the p53 transcriptional activation function. An important transcription target of p53 is the p21 gene which encodes an inhibitor of cyclin-cyclin dependant kinase complexes (El-Deiry *et al.*, 1993). Binding of p21 to these complexes prevents their Rb phosphorylation activity, causing arrest in G1 (Sherr, 1996). These arrested cells can activate DNA repair enzymes before resuming the cell cycle and thus avoid propagation of potentially oncogenic mutations.

P53 has been shown to regulate apoptosis in response to a number of stimuli, most of which involve DNA damage. Normal irradiated thymocytes undergo apoptosis in response to radiation while those from homozygous *p53*-null mice show no response (Lowe *et al.*, 1993a). Oncogene expression, for example of the adenovirus E1A protein, can also result in p53-mediated apoptosis (Debbas & White, 1993). Induction of apoptosis involves the transcriptional activation of a number of genes including *Bax* (Miyashita & Reed, 1995). Bax binds to Bcl2 and disrupts its ability to inhibit apoptosis. The transcriptional activity of p53 may require interaction with the p33^{ING1} growth suppressor protein (Garkavtsev *et al.*, 1998). The activation of apoptosis by p53 is in effective means of eliminating damaged cells that could proceed to malignancy. P53 may also regulate the induction of apoptosis under hypoxic conditions. Hypoxia (low oxygen concentration) is a common feature of solid tumors due to high cell densities with inadequate vascularization, and normally leads to apoptosis. The survival of cells in hypoxic regions correlates with *p53* mutations (Graeber *et al.*, 1996).

It is estimated that between 30 and 70% of malignant tumors have homozygous *p53* mutations (Sidransky & Hollstein, 1996). Germline *p53* mutations cause predisposition to cancer in patients with the Li-Fraumeni syndrome (Malkin *et al.*, 1990). Families with this syndrome are at high risk for developing sarcomas of the soft tissues and bone, and other malignancies including breast cancer (Li *et al.*, 1988). The types of mutations associated with the Li-Fraumeni syndrome are similar to what is observed in sporadic cancers. The cancers associated with this syndrome do not include a number of sporadic cancers in which *p53* is mutated, although there is currently no definitive explanation for this observation. The relative timing of *p53* loss may account for this behaviour such that early loss of *p53* (*e.g.* in the case of Li-Fraumeni syndrome patients) may be incompatible with the development of certain tumor types. Although most *p53* mutations in human cancers result in similar disruption of the protein structure, the specific mutations observed are particular to the cell type and environmental context. Mutagenic agents cause base alterations characteristic of their chemical modifications of DNA. For example skin cancers frequently show CC->TT base substitutions arising from UV light-induced photodimers at pyrimidine dinucleotides. Such mutations are exceedingly rare in cancers not associated with exposure to UV light. The spontaneous amino group hydrolysis of cytosine at 5-meCpG dinucleotides, resulting in cytosine to thymidine transitions, is a very common source of *p53* mutations, constituting 50% of alterations in colon cancers (Harris & Hollstein, 1993).

Other Mechanisms for Inactivation of P53

Gene mutation is not the only mechanism by which p53 can be inactivated during the malignant progression of a cell. As was discussed above, DNA tumor virus oncogenes bind to p53 and prevent its growth repressing functions. In a similar fashion the cellular MDM2 can inhibit p53 activity. In normal cells MDM2 can bind to and down-regulate p53 in an autoregulatory loop (Wu *et al.*, 1993). The aberrant overexpression of MDM2, due to gene amplification of five to 50-fold, results in the development of bone and soft-tissue sarcomas (Oliner *et al.*, 1992). Tumors with *MDM2* amplification do not show *p53* mutations, and it is likely that the tumorigenic effects of such amplifications are due to the resulting functional inactivation of p53. Aberrant cytoplasmic retention may be another means of p53 inactivation. In approximately 30% of breast cancers, p53 does not

translocate to the nucleus and thus cannot function in transcriptional regulation (Moll *et al.*, 1991). The mechanism and pathogenic significance of this activity have yet to be determined.

P53 mutant mouse strains

The gene targeting of *p53* in the mouse to produce homozygous-null animals has created a valuable model system for studying this tumor suppressor gene (Donehower *et al.*, 1992; Jacks, 1996). Most mice deficient for *p53* develop normally and, hence, this gene is not essential for embryogenesis. About 10% of embryos do, however, display neural tube defects due to an unknown mechanism (Sah *et al.*, 1995). The surviving animals all develop tumors within the first six months of life. Malignant lymphomas are the predominant tumor type although a wide range of malignancies are observed (Donehower *et al.*, 1992) Heterozygotes also develop tumors but have a longer latency period. Considerable insight into the biological function of p53 is derived from the study of crosses with *Rb1*-deficient strains (Williams *et al.*, 1994) and by the use of transgenic mice expressing tumor virus proteins which inactivate RB1 and p53 (Howes *et al.*, 1994; Pan & Griep, 1994) To summarize, in the presence of functional wild type p53, embryos lacking Rb1 show widespread apoptosis. Embryos which are also deficient for p53, in contrast, do not display such apoptosis and instead show tumor formation. This is interpreted to indicate that cells lacking the Rb1 are susceptible to DNA damage and p53 is able to initiate an apoptotic pathway to eliminate cells with such damage. In the absence of p53 this pathway is inactivated and tumorigenic mutations are propagated. In a transgenic mouse model system with inactivated *Rb1*, tumor growth was associated with p53 function (Symonds *et al.*, 1994). In the presence of wild type p53 tumors grew slowly, however, stochastic loss of p53 resulted in focal emergence of aggressive tumor nodules. Aggressive tumor development was associated with a decreased level of apoptosis suggesting that p53-dependent apoptosis suppresses tumor growth and progression in this system.

Fibroblasts from p53-deficient mouse embryos have been used to study the role of p53 in mediating the sensitivity of cells to anticancer agents (Lowe *et al.*, 1993b). In the presence of an activated oncogene fibroblasts showed p53-dependent apoptosis in response to ionizing radiation and to a series of unrelated cytotoxic drugs. These results suggest that the resistance of tumor cells to anticancer agents may be due to mutations in *p53*. The study of the therapeutic responsiveness of tumors induced by injection of oncogenically transformed fibroblasts either lacking or expressing p53 showed that this behaviour is consistent *in vivo* since P53-deficient tumors were resistant to treatment (Lowe *et al.*,

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1994). Acquired *p53* mutations were observed in the small proportion of resistant or relapsed tumors which originated from p53-expressing cells. These model systems strongly suggest that the cellular *p53* status is important in determining the therapeutic responsiveness of a tumor (Fischer, 1994). This p53-dependence does not apply to all drugs, however. The cytotoxicity of some therapeutic agents, for example antimicrotubular agents such as Taxol, is independent of the cellular p53 status (Harris, 1996).

Prognostic significance of *p53* mutations

Since p53 regulates genome stability and therapeutic responsiveness, it is consistent to predict that *p53* mutations in tumors should be correlated with adverse prognosis and that such mutations should be more common in relapsed tumors. A variety of tumors confirm these associations including certain lymphomas (Rodriguez *et al.*, 1991) and leukemias (Elrouby *et al.*, 1993; Nakai *et al.*, 1994; Hsiao *et al.*, 1994), breast carcinomas (Andersen *et al.*, 1993), malignant gliomas (Pollack *et al.*, 1997), non-small cell lung cancers (Mitsudomi *et al.*, 1994) and soft tissue sarcomas (Cordon Cardo *et al.*, 1994). Breast cancers show a strong association between *p53* mutations and *de novo* resistance to the anticancer drug, doxorubicin (Aas, 1996). In addition, a number of highly curable cancers rarely display *p53* mutations including acute lymphoblastic leukemia (Gaidano *et al.*, 1991), testicular cancer (Heimdal *et al.*, 1993; Peng *et al.*, 1993) and Wilms' tumor (Bardeesy *et al.*, 1994a). A number of cancers show an association of *p53* mutations with tumorigenic progression. In colon cancer *p53* mutations are a late event, occurring in the malignant carcinoma stage, and absent in early adenomas (Fearon & Vogelstein, 1990). *P53* mutations are also associated with the progression of brain tumors (Sidransky *et al.*, 1992), head and neck carcinomas (Boyle *et al.*, 1993), and leukemias (Wada *et al.*, 1994).

Pediatric Malignancies

Cancer is a relatively rare disease in children. Whereas one in five adults die of cancer, only one in 600 children will develop a malignancy by age 16. The spectrum and incidence of neoplasms affecting each group are completely distinct (reviewed in Israel, 1995). The major tumors in adults, those of the lung, prostate, colon and breast, are almost nonexistent in children. Tumors which affect either age group are clinically and biologically distinct. Adult tumors are derived from fully differentiated tissue which have undergone numerous genetic changes and often show strong correlation with environmental factors (*e.g.* the links between cigarette smoking and lung cancer or between exposure to sunlight

and melanoma. In contrast most pediatric malignancies show minimal environmental effects. The histology of tumors is reminiscent of undifferentiated embryonal cells (see Gonzalez-Crussi, 1984; Israel, 1995). The cells of some tumors form into partially differentiated structures, in what appears to be an aberrant attempt at executing developmental pathways. It thus appears that the cells of pediatric tumors have undergone mutations which prevent the completion of proper differentiation. The primitive origin of these tumors is suggestive of initiating mutations occurring during embryonic life. In consideration of these observations, pediatric malignancies have been thought of as cases of development gone awry and thus provide a link between the processes of development and malignancy (van Heyningen & Hastie, 1992). This correlation is strengthened by the association of several pediatric cancers with dysmorphic syndromes (e.g. see Pelletier, 1991d). The earlier onset of pediatric tumors suggests that fewer mutations are required for malignant transformation compared with adult cancers. These observations indicate that there may be fundamental differences underlying the pathology of cancer in these groups.

Wilms' Tumor

Wilms' tumor or nephroblastoma is a pediatric kidney tumor usually affecting children under age 5. This malignant neoplasm, which arises from primitive metanephric cells, encompasses a spectrum of histological patterns. In the medical literature, descriptions of childhood tumors of the kidney are seen as early as 1814 (reviewed in Gonzalez-Crussi, 1984). Max Wilms' systematic presentation in 1899 of seven new cases of the disease and review of the existing literature eventually earned him the eponymic designation by which the tumor is known today. With an incidence of one in ten thousand, it is the most common intraabdominal solid tumor of childhood (Green, 1997). Wilms' tumors comprise about 6% of all childhood neoplasms, and 97% of those affecting the kidney. Unilateral disease is predominant, although about 8% of patients present with bilateral tumors. Bilateral cases show a significantly earlier age of onset than unilateral cases (2.5 years versus 3.5 years). The male to female ratio is approximately 1:1 for unilateral disease and 0.6:1.0 for bilateral disease (Green, 1997). In both classes, males show an earlier mean age of diagnosis. Wilms' tumor occurs sporadically or in rare hereditary cases, which comprise 1% of patients. Epidemiological studies have indicated that environmental factors have a minimal role in etiology of this malignancy (Breslow et al. 1993). It was originally thought that the incidence of Wilms' tumors was relatively constant between populations and so could be used as an index for comparisons with other cancers. Recent data, however, shows a three to four-fold ratio of maximum and minimum

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rates of Wilms' tumor between populations. This ratio is small in comparison with that of adult epithelial tumors which show ten to twenty-fold variation in incidence between populations. The incidence rate is correlated with ethnic group as opposed to geographic location.

Wilms' tumor as a Problem in Nephrogenesis

Wilms' tumor is thought to arise from cells of the metanephric blastema (Gonzalez-Crussi, 1984). Tumors show a strong histological resemblance to the normal metanephric blastema of the embryo and fetus, and the tumor cells undergo a degree of differentiation often forming glomerular and tubular-like structure which mimic those of the normal kidney. Focal lesions in the kidneys, known as nephroblastomatosis or nephrogenic rests, are interpreted as displaying developmental arrest and are thought to be precursors of Wilms' tumors (Beckwith et al., 1990). Nephrogenic rests appear capable of maturation and regression or of originating malignant tumors. Thus, these lesions are both tumorigenic and malformative. In consideration of these observations, Wilms' tumor is widely regarded as much a problem of developmental biology as of oncology.

Aspects of Kidney Development Relevant to Wilms' Tumorigenesis

The kidney is the final mammalian excretory organ forming from a succession of three embryonic nephric structures (reviewed in Gonzalez-Crussi, 1984). The nonfunctional pronephros forms first, developing simultaneously the precursor ureteric duct. This structure is replaced by the functional mesonephros. The metanephros then forms (at 35 days gestation in the human) in an inductive interaction between the epithelial ureteric bud and the mesenchymal metanephric blastema. Co-culture experiments demonstrate the functional interdependence of the two cells types for differentiation to occur. The metanephric cells condense around the bud. Due to inductive signals, some of these cells form the spheroid nephrogenic vesicle. The cells of this structure gradually lose their proliferative capacity and bend into the 'S-shaped body'. This structure unites with the ampulla derived from branches of the ureteric bud. The cells originating from the metanephric blastema differentiate into nephrons while the cells derived from the ureteric buds form the ureter, renal pelvis, calyces, and collecting tubules. In the process, the blastemal cells undergo a developmentally rare mesenchymal-to-epithelial transition. The development of the kidney is characterized by the progressive loss of proliferative capacity of pluripotent stem cells as they approach terminal differentiation. Alterations in the

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proliferative capacity will disrupt differentiation. Wilms' tumors show the abnormal persistence of blastemal cells which retain their proliferative capacity and varying degrees of differentiative potency. Thus, Wilms' tumors may have histological features of stromal or epithelial components, and in some cases heterologous elements such as muscle or bone.

Given the correlation of the pathogenesis of Wilms' tumor with aberrations in kidney development, it would appear that genes involved in the etiology of this disease would encode regulators of embryonic development. Hence, it is not surprising that Wilms' tumor is often associated with congenital defects occuring in a number of different syndromes.

WAGR syndrome

There is a statistically significant correlation between Wilms' tumor and aniridia (complete or partial absence of the iris) (Miller *et al.*, 1964). The rate of bilateral aniridia among Wilms' tumor patients is 1:73 compared with an incidence of 1:50000 in the general population. In addition, 30% of patients with sporadic aniridia develop Wilms' tumor. Wilms' tumor patients with aniridia show an earlier age of presentation and an increased frequency of bilateral tumors. Most of these patients have cytogenetically detectable deletions of band p13 on chromosome 11, thus indicating the location of a Wilms' tumor susceptibility gene. The deletions are present in a heterozygous state in the constitutional DNA. Boys with such deletions show a high incidence of cryptorchidism (undescended testes) and hypospadias (penile anomaly resulting in misplaced urinary orifice) (Van Heyningen & Hastie, 1992). Patients have increased incidence of kidney or collecting system anomalies such as horseshoe kidney and bifid ureters. Some patients also show mental retardation and cognitive and motor impairment (Matsunaga, 1981). This constellation of abnormalities is referred to as WAGR syndrome, an acronym for Wilms' tumor, aniridia, genitourinary defects and (mental) retardation. The deletions associated with WAGR syndrome indicate that is a contiguous gene syndrome with different phenotypic aspects determined by multiple genes. Genitourinary (GU) defects are more commonly associated with Wilms' tumors than is aniridia suggesting that the gene responsible for these abnormalities is more tightly linked to the Wilms' tumor gene than is the gene for aniridia. It is now apparent that, indeed, the GU defects and Wilms' tumor are due to mutations in the same gene, *WT1*, while a second locus, *PAX-6* is responsible for aniridia (reviewed in Hastie, 1994).

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Denys-Drash Syndrome

The GU anomalies associated with WAGR syndrome are relatively mild. About 2% of Wilms' tumor patients, however, suffer much more severe defects in the GU system in what is known as Denys-Drash syndrome (DDS) (Hastie, 1994; Mueller, 1994). These patients show progressive renal damage culminating in renal failure. Histologically, the nephropathy involves glomerular capillary damage with diffuse mesangial sclerosis (thickening of the endothelial membrane) (Drash et al., 1970). The vast majority of DDS patients with a normal male (XY) karyotype have phenotypically female or ambiguous genitalia (Mueller, 1994). The internal genitalia are often dysgenic (streak gonads or rudimentary testicular tissue) and internal structures are generally inappropriate for the external genitalia (e.g. the presence of internal testes with external female structures).

Beckwith-Weidemann Syndrome

Beckwith-Weidemann syndrome (BWS), is a rare fetal overgrowth syndrome also associated with Wilms' tumor. BWS shows varying expressivity and there are no fixed diagnostic criteria for the disease, but the following features each affect more than 50% of patients: macroglossia (enlarged tongue) and pre- or postnatal gigantism (defined as growth > 90th percentile), exomphalos (umbilical hernia), and characteristic ear creases, and renal abnormalities including nephromegaly (reviewed in Elliot & Maher, 1994). About 10% of BWS patients develop tumors of which Wilms' tumors are most common. Hemihypertrophy (asymmetrical growth) is observed in about 20% of BWS cases and confers increased predisposition to Wilms' tumor (Green et al., 1993). The genetics of BWS are complex and a pathogenic role of imprinted genes is widely recognized (Reeve, 1996; Reik & Maher, 1997). Most cases of BWS are sporadic, however, there are published descriptions of a number of pedigrees which show an autosomal dominant inheritance with incomplete penetrance and varying expressivity (reviewed in Weksberg & Squire, 1996). The penetrance is more complete if the mother is the transmitting parent. Genetic linkage has localized the BWS locus to 11p15.5. A fraction of BWS patients (2 to 3%) have cytogenetic abnormalities of chromosome 11p15 (Elliot & Maher, 1994). Most of these are paternally derived duplications in sporadic BWS patients. A small number of maternally derived inversions or balanced translocations have also been described. These rearrangements map to three distinct regions on 11p15 suggesting that their effect is to exert long-range cis effects on genes in the adjacent region of 11p15. The specific parental origin of these anomalies is a strong indication that imprinted genes are involved in BWS.

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Overexpression of the maternally imprinted *IGF2* gene, located at 11p15, has received considerable attention as a candidate in the etiology of BWS (Reeve, 1996). A more detailed discussion of the genetics of this Wilms' tumor associated syndrome will be presented below.

Perlman Syndrome

Perlman syndrome is a very rare fetal overgrowth disorder with only 11 cases currently described (Grundy *et al.*, 1992). The disease shares some features with BWS. Both conditions frequently present with obstetric complications such as polyhydramnios and both display high birth weights and organomegaly. Perlman syndrome is distinguished by macrocephaly and a typical facies. There is a high neonatal mortality rate and about half of patients develop Wilms' tumor. Umbilical hernia, ear creases and macroglossia, hallmarks of BWS, are not found in Perlman syndrome. Perlman appears to display an autosomal recessive mode of inheritance.

Simpson-Golabi-Behmel syndrome

The Simpson-Golabi-Behmel syndrome (SGBS) is an X-linked overgrowth disorder with facial dysmorphism, polydactyly and multiple variable malformations (Coppin *et al.*, 1997). There may be a predisposition to embryonal malignancy, including Wilms' tumor, associated with this syndrome, and thus there is clinical overlap with BWS and Perlman syndrome. There are reports of four Wilms' tumors out of about 50 documented cases of SGBS (Lindsay *et al.*, 1996; Hughes-Benzie *et al.*, 1996). In one family used in genetic linkage studies, there were two affected SGBS males with tumors, however, a female relative had a Wilms' tumor but was excluded as an SGBS carrier (Xuan *et al.*, 1994). Hence, in this family it appears that predisposition to Wilms' tumor is caused by a separate locus. The three other cases of Wilms' tumor are from separate families although the reports do not address whether there was an associated family history of Wilms' tumor. Documentation of more patients suffering from both SGBS and Wilms' tumor will be necessary to confirm the association. The gene mutated in SGBS has been identified as *GPC3*, encoding the membrane-bound proteoglycan glypican-3 (Pilia *et al.*, 1996). Glypican-3 was originally suggested to be an IGF-2 binding protein. This notion was appealing since it provided a biochemical link between the phenotypically related overgrowth disorders BWS and SGBS. It was suggested an overabundance of IGF-2 was involved in the etiology of both syndromes: by overexpression of the IGF-2 gene in BWS,

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or by deficient IGF-2 sequestration in SGBS. The IGF-2 binding function of glypican-3 has been called into question by Song et al. (1997) who, employing the rat homologue of glypican-3, were unable to detect interaction of these proteins. Instead, the authors identified specific interaction of rat glypican-3 and fibroblast growth factor-2 (FGF-2). The discrepancies of these results may be due to the use of different systems to assay binding activity.

Other syndromes associated with Wilms' tumor

Bloom syndrome is a rare autosomal recessive disease which results in growth failure, sun sensitivity, immune system defects and an increased risk of a range of cancers (reviewed in Ellis & German, 1996). It is characterized by genomic instability and an increased somatic mutation rate which are likely account for the predisposition to cancer in this syndrome. Out of approximately 100 patients with Bloom syndrome who have been reported, three suffered from Wilms' tumor (Cairney *et al.*, 1987). This frequency is about 300 times that seen for Wilms' tumor in the general population, thus, although the numbers are small, it appears the Bloom syndrome patients are at increased risk for the development of Wilms' tumors. The gene mutated in Bloom syndrome, designated *BLM*, appears to encode a DNA helicase which may interact with the DNA replication machinery (reviewed in Ellis & German, 1996). It is likely that mutated *BLM* is not integral in the malignant transformation of Wilms' tumor, but that it predisposes the cell to mutations in other genes critical for this process.

Sotos syndrome is a fetal overgrowth disorder characterized by accelerated growth, minor craniofacial anomalies and intellectual deficits (Cole & Hughes, 1990). A number of associated malignancies have been reported and the estimated risk of cancer is 2% (Hersh *et al.*, 1992). Two cases of Wilms' tumor have been reported out of ten Sotos syndrome associated tumors. Because of the small number of affected patients and the possibility of clinical overlap between fetal overgrowth disorders, it is unclear whether there is predisposition to Wilms' tumor associated with Sotos syndrome.

Chromosomal Changes in Wilms' tumors

The study of genomic alterations in Wilms' tumors using polymorphic DNA probes reveals LOH at chromosome 11p13 (Fearon *et al.*, 1984; Orkin *et al.*, 1984). The finding of LOH at 11p13 is consistent with the observation that patients with WAGR syndrome have 11p13 deletions. Extensive genetic analysis has shown that approximately 35% of

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Wilms' tumors have LOH at 11p13 (Radice *et al.*, 1995; Besnard-Guerin *et al.*, 1996; Grundy *et al.*, 1996; Law *et al.*, 1997). The existence of a Wilms' tumor susceptibility gene at this locus was confirmed by the isolation and characterization of the *WT1* gene (see below). LOH on chromosome 11 is not restricted to 11p13. Most changes on chromosome 11 include much of the short arm spanning from 11p13 to the telomere at 11p15.5 (about 30% of tumors show LOH at both regions) (Grundy *et al.*, 1996). The existence of a second Wilms' tumor locus was identified by the observation of LOH confined to 11p15 in 8-15% of tumors (Mannens *et al.*, 1988; Henry *et al.*, 1989; Jeanpierre *et al.*, 1990; Wadey *et al.*, 1990; Grundy *et al.*, 1996). The application of markers with very tight linkage to the *WT1* gene has permitted the identification of 11p13-restricted LOH in 5-10% of tumors (Wadey *et al.*, 1990; Radice *et al.*, 1995; Besnard-Guerin *et al.*, 1996; Law *et al.*, 1997). When markers adjacent to the *WT1* gene are used a lower rate of 11p13-restricted LOH is observed (Coppes *et al.*, 1992b; Grundy *et al.*, 1996) suggesting that when LOH is limited to 11p13 it may be restricted to the immediate vicinity of the *WT1* gene. Some tumors show LOH at 11p13 and 11p15.5, excluding the intervening region (Besnard-Guerin). Additionally, some WAGR patients, harboring constitutional deletions at 11p13, have Wilms' tumors with LOH restricted to 11p15.5. Based on these observations, it would appear that there may be a cooperative effect of the 11p13 and 11p15.5 loci on tumorigenesis. LOH at chromosome 11p nearly always involves loss of maternal alleles suggesting a role for imprinted genes (Coppes *et al.*, 1992b). A recent study has suggested that there may be a third Wilms' tumor locus on chromosome 11 (Radice *et al.*, 1995). A series of 24 tumors included three showing LOH at both 11p15.5 and 11q23.3, excluding the intervening segment, in addition to six cases displaying loss of the entire chromosome 11. The authors speculate that there may be a synergistic effect of these two regions in tumorigenesis.

Most Wilms' tumors have relatively stable genomes in comparison to adult cancers. Many have normal diploid karyotypes or have a small number of anomalies in chromosome structure and integrity (Soukup *et al.*, 1997) (the unfavorable histological subtype of anaplastic Wilms' tumor is exceptional, with a highly abnormal karyotype). The level of 'background' LOH in Wilms' tumor is also comparatively low (Mannens *et al.*, 1990; Devilee 1991; Maw *et al.*, 1992). LOH mapping of Wilms' tumor revealed that, in addition to 11p losses, there is LOH at chromosome 16q in about 20% of patients (Maw *et al.*, 1992; Grundy *et al.*, 1994). In contrast to allele losses at chromosome 11p there is no bias in parental origin of allele loss at this region. A statistically significant association of 16q LOH and adverse outcome is observed (Grundy *et al.*, 1994). Patients with this alteration show 3.3-fold increase in relapse and 12-fold increase in mortality rates. Some tumors,

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with LOH at 11p and 16q, retained a proportion of 16q alleles while no 11p alleles were detectable, suggesting that 16q loss may be a late event in tumorigenesis, associated with progression not initiation. LOH at chromosome 1p was observed in 12% of tumors (Grundy et al., 1994) suggesting a possible role for this locus. No other loci showed allele loss in more than 10% of Wilms' tumors in LOH studies using probes to each chromosome.

There are a number of descriptions of tumors with translocations involving chromosome 7 as the sole cytogenetic abnormality (Wilmore et al., 1994; Miozzo, 1996). Most of these alterations resulted in monosomy for 7p and trisomy for 7q. Wilmore et al. (1994) identified 7p LOH in two of 24 Wilms tumors. One of these involved a constitutional balanced translocation t(1:7)(q42:p15) and a somatic abnormality deleting 7p alleles of the other chromosome. The tumor was, thus monosomic for 7p (with possible disruption of a gene at 7p15) and trisomic for 7q. This patient had a nephrogenic rest (a nonmalignant precursor of Wilms tumor; Beckwith et al., 1990) in the contralateral kidney suggesting that a constitutional mutation, possibly at the 7p locus, was responsible for the tumor in this patient. Miozzo et al. (1996) found losses of 7p alleles in four of eleven tumors and mapped these changes to a 25 cM region overlapping 7p15. Thus, a potential tumor suppressor gene lies in this region. Gain of 7q alleles occurs in more than 15% of Wilms' tumors (Mertens et al., 1997) and may harbor one or more oncogenes. Other regions showing chromosomal imbalance in greater than 15% of Wilms' tumors include chromosomes 1q21-44, 6p12-q27, 8, and 12 (Mertens et al., 1997). All of these regions undergo chromosomal gains and so may also contain oncogenes.

The *WT1* gene

To date, a single Wilms' tumor suppressor gene, *WT1*, has been isolated. This gene, identified by positional cloning, maps to the 11p13 deletions associated with WAGR syndrome. The characterization of WAGR deletions was accomplished using genomic probes to 11p13 and by studies of hybrid cells containing the abnormal chromosome 11 from WAGR patients (reviewed in Pelletier et al., 1991d). This work permitted the identification and ordering of probes which map within the deleted region. The availability of a sporadic Wilms' tumor, with distinct, but overlapping deletions on either copy of chromosome 11, was instrumental in further definition of the *WT1* locus. A gene falling within the smallest region of overlap of these deletions would be a candidate Wilms' tumor suppressor, assuming that the genetic event resulting in transformation in this tumor was due to the homozygous deletions at 11p13. A genomic clone mapping to this overlapping

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region was identified to be conserved between species and was used to isolate cDNA for *WT1*. The same gene was identified using a chromosome jumping approach (Gessler *et al.*, 1990). Proof that *WT1* is indeed the 11p13 tumor susceptibility gene came from the identification of tumor-specific mutations within the gene (Little, 1992b).

WT1 spans 50 kb on chromosome 11p13 and encodes ten exons (Call *et al.*, 1990). The WT1 protein has four zinc fingers of the Cys_2-Hys_2 class (encoded by exons 7-10) suggesting that it may be capable of sequence-specific DNA binding and play a role in transcriptional regulation. The *WT1* mRNA transcript is 3.1 kb in most WT1-expressing cells except the testis and mesothelium which also express a 2.7 kb transcript (reviewed in Hastie, 1994). The transcript undergoes alternative splicing producing isoforms that lack or contain exon 5, coding for 17 amino acids (Haber *et al.*, 1991). Alternative splicing also occurs at the 3' end of exon 9. The use of an alternative splice donor site nine nucleotides downstream results in the insertion of the codons for lysine, threonine, and serine (KTS) between zinc fingers 3 and 4. These alternative splicing events produce four isoforms which we will designate WT1-/-, WT1+/-, WT1-/+, and WT1+/+ to reflect the presence or absence of the 17 amino acid and KTS insertions. The ratio of these isoforms does not appear to vary temporally nor between tissues and is 1: 2.5: 3.8: 8.3 for WT1-/-: WT1+/-:WT1-/+:WT1+/+, respectively. The ratio of the resulting WT1 protein isoforms is unexplored and thus variations in translational efficiency may potentially cause a lack of correlation between relative mRNA and protein levels. Other WT1 isoforms are generated by the use of alternate translational start codons (Bruening & Pelletier, 1996). Translation initiated from an upstream in frame CUG codon generates protein with molecular masses of 54-56 kDa while initiation from the downstream AUG codon produces proteins of 47-49 kDa. The functional significance and regulation of these alternate isoforms remain undefined.

Studies of the rat *WT1* homologue revealed that adult rat cDNAs have either thymidine or cytosine at position 839 whereas genomic DNA from the same strain of rat has only the thymidine nucleotide (Sharma *et al.*, 1994). Only thymidine, as coded for by the genomic DNA, was present in cDNA from newborn kidney and adult liver. The analogous event was observed in human testis cDNA at the corresponding nucleotide (position 1222). It appears that this phenomenon is due to RNA editing, an enzymatically mediated alteration in mRNA such that its sequence varies from what is encoded in the genome (reviewed in Cattaneo, 1991). Editing can thus change the coding potential of an mRNA; in the case of WT1 editing, leucine is replaced by proline. RNA editing, described for a small number of mammalian genes including the lipid transporter, apoliprotein B (apoB) and the brain glutamine transporter, *GluRB* , can result in dramatically altered

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protein function. The only functional assay reported for the edited WT1 isoform analysed the ability of this protein to act as a transcription factor; a small (25-30%) reduction of transcriptional repression was observed compared with unedited WT1 (Sharma *et al.*, 1994). As it is currently thought that the main role of WT1 is to mediate aspects of embryological development, the observation that editing appears to be absent during embryogenesis in addition to the lack of evidence for a strong effect of the leucine to proline substitution in functional assays has lead to questions regarding the biological significance of *WT1* RNA editing (Hastie, 1994).

The observation of specific hybridization of human *WT1* cDNA probes to Southern blots of genomic DNA from multiple species suggested evolutionary conservation of the *WT1* gene (Call *et al.*, 1990). Partial or complete *WT1* cDNA from the human, mouse, rat, marsupial mouse, alligator, chicken, frog, and zebrafish have been characterized and demonstrate very high conservation at both the nucleotide and amino acid levels (Kent *et al.*, 1995). The *WT1* homologues from all species have more than 80% nucleotide identity and more than 90% amino acid similarity to human *WT1*. The carboxy-terminal zinc finger region is the most conserved, with nearly 100% interspecific amino acid identity. In addition both the +KTS and -KTS isoforms were found in all species. There is also very high conservation throughout the rest of the protein (87% amino acid identity between human and chicken amino-terminal regions). The alternatively spliced exon 5 and the RNA editing site are only found in mammals and thus only two isoforms of *WT1* (-/+KTS) are likely to be produced in lower organisms.

Studies of the expression profiles of the *WT1* mRNA and protein reveal specific spatial and temporal regulation (Pritchard-Jones *et al.*, 1990; Mundlos *et al.*, 1993). *In situ* hybridization studies indicate that WT1 mRNA is expressed at highest levels during embryological development of the kidney. Nephrogenesis involves the development and regression of the pronephros, mesonephros, and metanephros in the process of generating the mature kidney (reviewed in van Heyningen & Hastie, 1992). The final stage of nephrogenesis is initiated by an inductive interaction between cells of the ureteric duct and the blastemal cells of the metanephros. Wilms' tumors arise from the metanephric blastema and thus *WT1* expression is expected in this tissue. Indeed, during genesis of the mature kidney, *WT1* mRNA is first expressed in induced metanephric blastemal cells condensed around the ureteric bud (which does not express *WT1* itself). Expression of *WT1* mRNA increases during the differentiation of these cells, including the formation of the renal vesicle, S-shaped body, finally forming the renal corpuscle, in which *WT1* levels are highest in the podocyte layer of the early glomerulus. In the mature kidney *WT1* mRNA

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expression is greatly diminished. Immunohistochemical detection in most studies showed parallel expression of the WT1 protein (Mundlos *et al.*, 1993; Grubb *et al.*, 1993).

WT1 is highly expressed in the genital ridge, a precursor to the male and female gonad. In the maturing gonad the main sites of expression are the Sertoli cells of the testis and the granulosa cells of the ovary. There is also cell-specific expression in the uterus. Expression in the genital organs continues throughout adult life although levels decrease in the uterus (Zhou *et al.*, 1993) and testis (Pelletier *et al.*, 1991a). *WT1* is also expressed in the endometrium of the female following implantation of the embryo. This tissue undergoes what is known as decidual differentiation which produces a barrier between the implanted embryo and the maternal tissue. *WT1* expression continues in decidual tissue until parturition (Zhou *et al.*, 1993). Spleen stromal cells and mesothelial cells (which line the body cavity and thoracic organs) also express high levels of *WT1* during embryonic development. It has been observed that all of the cells discussed above share common features (Pritchard-Jones *et al.*, 1990; Hastie, 1994). They are all of mesodermal origin and during the time of WT1 expression they undergo a developmentally rare histological switch from mesenchymal cells to epithelial cells. Mesenchymal cells are non-polar cells which are attached to the extracellular matrix which they deposit, while epithelial cells which form linings to organs are polarized cells with tight junctions between them. It has been speculated that WT1 may be an important general regulator of the mesenchymal-to-epithelial transition (Hastie, 1994). A number of other cell types which do not undergo this transition also express *WT1* including cells within the body wall muscle, spinal cord, a small region of the brain (Hastie, 1994), and in the mammary duct and lobule of the adult breast (Silberstein *et al.*, 1997). Low levels of WT1 expression are detected in primitive hematopoietic precursor cells isolated from bone marrow (Baird & Simmons, 1997).

Developmental Defects Associated with *WT1* mutations

The highly restricted pattern of *WT1* expression suggests that this gene contributes to embryological development. This is strongly supported by genetic evidence correlating *WT1* mutations with congenital defects. Mapping of the 11p13 deletions in WAGR syndrome shows that the loci responsible for genitourinary anomalies and for Wilms' tumor are tightly linked (reviewed in Pelletier *et al.*, 1991d). The identification of constitutional heterozygous intragenic *WT1* mutations in two patients with Wilms' tumor and genitourinary abnormalities (hypospadius and undescended testes) established the role of *WT1* in both suppression of malignancy and genitourinary system development (Pelletier *et al.*, 1991b). The mutations in these patients were small deletions causing

frameshifts in the *WT1* mRNA predicted to yield truncated proteins. The genitourinary defects in patients with constitutional small, intragenic *WT1* deletions are the same as in patients with large deletions which eliminate one allele entirely. This suggests the genitourinary defects in these patients are due to a reduced dosage of WT1 protein. The Denys-Drash syndrome (DDS) involves Wilms' tumor associated with severe defects in the development of the genitourinary system resulting in renal failure and pseudohermaphroditism. Analysis of the *WT1* gene in DDS patients reveals that the vast majority have missense point mutations in the zinc finger domain (Pelletier *et al.*, 1991c). In contrast to WAGR syndrome, DDS is not associated with deletions. It is likely that the more severe genitourinary abnormalities associated with DDS are due to dominant negative activity of proteins encoded by the mutant allele. A more complete discussion of *WT1* mutations will follow.

WT1 in murine development

The genitourinary malformations associated with germline *WT1* mutations in the human demonstrate the critical role of this gene as a developmental regulator. Mouse models have been valuable in studies of WT1 function although there is some interspecies differences in the phenotypes associated with *WT1* mutations. The Dickie's Small-eye mouse (Sey^{dey}) is a naturally occurring mutant which has a large chromosomal deletion, equivalent to that found in WAGR patients, deleting the murine *PAX-6* and *WT1* genes (Glaser *et al.*, 1990). Although, when heterozygous, this mutant displays an equivalent phenotype to what is observed for PAX-6 deficiency in the human (*i.e.* absence of the iris), the defects associated with WT1 deficiency are absent (*i.e.* heterozygotes do not develop Wilms' tumors nor genitourinary defects). Hence, hemizygosity of WT1 is not malformative nor oncogenic in the mouse. Sey^{dey} homozygous are not viable and thus cannot be used to investigate the consequences of a *WT1*-null genotype. The generation of *WT1*-null mice by gene targeting methodologies has allowed further elucidation of the involvement of WT1 in developmental processes (Kriedberg *et al.*, 1993). Heterozygotes show no abnormalities and do not develop tumors, however, homozygotes die at 15 days of gestation. Homozygotes show absence of kidneys and ovaries, and in addition have defects in the mesothelium, heart and lungs. The metanephric blastemal cells of the developing kidney, which in the wild type animal express *WT1*, show high rates of cell death due to apoptosis. The heart and lung defects are likely due to abnormalities in the mesothelium which surrounds these organs and which normally expresses *WT1*. The defects detected in *WT1*-null mice demonstrate that this gene is critical to genitourinary

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development consistent with its role in humans. The different phenotypes associated with *WT1* heterozygous mutations reflect interspecific divergences in developmental genetics that have frequently been observed, thus, although gene homologues may be nearly 100% identical between species they can have different effects on the growth of the respective organism (reviewed in Jacks, 1996).

WT1 mutations in Wilms' tumors

From the mapping of WAGR syndrome deletions, genetic linkage studies of BWS and familial Wilms' tumor, and LOH studies, it is has been shown that there are likely to be several loci conferring susceptibility to Wilms' tumor. *WT1* is the only such gene to be isolated to date. Determination of the frequency and spectrum of *WT1* mutations in Wilms' tumor is potentially highly informative in elucidating the genetic basis of this disease and in revealing biochemical properties of the WT1 protein. The status of the *WT1* gene has been thoroughly analysed in Wilms' tumors. It must be stressed that comparison of the results between published studies is complicated by the use of different mutation detection methods. Additionally, some studies bias the selection of tumor specimens according to certain clinical features and thus the detection rates will not reflect what would be observed in unselected series. The following discussion takes these factors into consideration when attempting to summarize the involvement of *WT1* mutations in Wilms' tumorigenesis.

Spectrum of WT1 mutations

Deletion of the *WT1* gene

Large scale deletions or rearrangements of *WT1*, detectable by Southern blot analysis, are rare, occurring in about 5% of tumors (Royer-Pokora *et al.*, 1991; Tadokoro *et al.*, 1992; Nordenskjold *et al.*, 1995). Although these gross deletions can occur somatically it appears that they are most commonly present constitutionally (Nordenskjold *et al.*, 1995; Schumacher *et al.*, 1997), and are associated with the WAGR syndrome. Tumor-associated loss of the remaining wild type *WT1* allele has been determined for a number of cases of WAGR syndrome. Mechanisms for this loss include LOH at chromosome 11p (Dao *et al.*, 1987; Nordenskjold *et al.*, 1995), and intragenic *WT1* mutations including point mutations (Park *et al.*, 1993c) and small insertions (Santos *et al.*, 1993) and deletions (Brown *et al.*, 1992). The loss of both alleles of *WT1* in Wilms' tumor is consistent with its role as a tumor suppressor gene.

Constitutional intragenic *WT1* mutations

Small intragenic *WT1* mutations are detected in about 10% of Wilms' tumors (Varanasi *et al.*, 1994; Nordenskjold *et al.*, 1995; Schumacher *et al.*, 1997) and may be germline or somatic. As discussed above, DDS patients are characterized by constitutional heterozygous mutations. The vast majority of DDS patients have missense mutations in the zinc finger region, particularly affecting exon 9 (see Table 2). More than half of the mutations cause an arginine to tryptophan change at residue 394. The crystal structure of the zinc finger region of EGR1 (which has a high degree of structural homology to WT1 zinc fingers II-IV) bound to DNA demonstrates that the equivalent arginine residue makes direct contact with a guanine nucleotide of the cognate binding site. The prediction that the mutation eliminates DNA binding by WT1 has been confirmed by electrophoretic mobility shift assays (Pelletier *et al.*, 1991c). Mutations in DDS patients have been shown to affect other residues which make direct DNA contacts as well as residues necessary for complexing with zinc, and most have been shown to have disrupted DNA binding (Little *et al.*, 1995). One patient diagnosed with DDS had a G to C transversion in the +1 position of the splice donor consensus sequence in intron 6 causing skipping of exon 6 in tumor RNA and resulting in a premature translational stop codon in zinc finger I. Some Wilms' tumor patients with genitourinary anomalies consistent with WAGR syndrome, but lacking large deletions of chromosome band 11p13, have constitutional intragenic *WT1* mutations. These are generally deletions (Pelletier *et al.*, 1991b) or insertions (Huff *et al.*, 1995) predicted to produce translational frameshifting or nonsense mutations (Schumacher *et al.*, 1997).

Somatic intragenic *WT1* mutations

Somatic mutations in *WT1* have been described less often than germline mutations. Most of these mutations are predicted to result in premature termination of translation and include insertions, deletions, nonsense mutations and intronic mutations which disrupt splicing (see Table 3). Mutations predicted to result in truncated proteins have a broad distribution within the *WT1* gene and have been detected in every exon except the fifth. The somatic mutations which disrupt splicing include a 25 bp deletion causing the in frame deletion of zinc finger III (Haber *et al.*, 1990) and a 14 bp insertion in the intronic region of the exon 7 splice donor site which likely causes exon skipping and translational frameshifting (Santos *et al.*, 1993). Fewer than 20% of somatic mutations in *WT1* are

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Table 2. Germline WT1 mutations.

This table compiles reports of germline WT1 mutations associated with Wilms' tumors. The compillation was based on Jeanpierre *et al.*, (1998) with modifications and additions.

exon: exon which harbors mutation

WT codon , **Mut codon**: wild type codon, mutant codon. Deletion or insertion mutations are indicated by **del** or **ins** followed by the number of affected bases and the position in the codon (**a**, **b**, or **c**, referring to the first, second or third position). For example del29a indicates a 29 base pair deletion including the first base of a codon.

CpG: indicates whether the mutation involves a CpG dinucleotide

WT aa, **Mut aa**: wild type amino acid, mutant amino acid. **fr.**: frameshift mutation.

uni/bi: unilateral or bilateral tumor. **NR** mutation detected in nephrogenic rest. * kidneys removed

LOH: loss of heterozygosity

ext. genit.: external genitalia. **crypt.**: cryptorchidism, **hyposp.**: hypospadius

int.repr.organ: internal reproductive organs

krtp: karyotype

nephr: nephropathy. **MS**: mesangial sclerosis. ·

ref: reference. All references are from Jeanpierre *et al.* (1998) except those marked **MG** which are from personal comunication with Majid Ghahremani.

Table 2. Germline WT1 mutations

exon	codon	WT codon	Mut codon	event	CpG	WT aa	Mut aa	uni/bi	LOH	name	ext.genit.	int.repr.organ	krtp	nephr	ref
1	10	GCG	del29a	stop@120		A	fr.	bi	y	9464	male			no	
1	29	AGC	ins5c	stop@130		S	fr.	uni	y	186523	cryptorchidism		46XY	no18m	
1	123	GGC	del26a	stop@170		G	fr.	bi	y	9200	female			no	
1	137	TGC	del7a	stop@215		C	fr.	uni	y	9184	male	testicular aplasia		no	
2	181	CCC	TCC	C->T	n	P	S	uni	n	9094	female			no	
2	185	CAG	TAG	C->T		Q	stop								MG
3	221	TAC	TAG	C->G	n	Y	stop	bi		CN	cryptorchidism			MS?	
3	s.d.	gta	gaa	del. exon3			fr.								
4	224	GAC	del17a	stop@228		D	fr.	bi	y	PG	crypt./hyposp.		46XY	no	
5	253	GGG	GCG	G->C	n	G	A	uni	n	9614	female			no	
6	271	TAC	TAG	C->G	n	Y	stop								MG
6	271	TAC	TAG	C->G	n	Y	stop	bi	y	WT10	cryptorchidism	Sertoli only gonad		no 8y	
6	274	GAT	ins1b	stop@275		D	fr.	bi	y	PM/3	ambiguous	atrophy	46XY	MS	
6	276	CAC	del1b	stop@306		H	fr.	uni		SL	female	ovaries	46XX	MS15y	
6	292	GGT	del1a	stop@306		G	fr.	uni	y	TS	crypt./hyposp.			no3y	
7	301	CGA	TGA	C->T	y	R	stop	bi		HDWT7	female			no	
7	301	CGA	TGA	C->T		R	stop	uni		HDWT6	female			no	
7	313	TCG	TAG	C->A	n	S	stop	uni	n	9561	male			no	
7	313	TCG	TAG	C->A	n	S	stop	bi	y	9318	female			no	
7	316	GAG	TAG	G->T	n	E	stop								MG
7	322	CCC	ACC	C->A	n	P	T								MG
7	330	TGC	TAC	G->A	n	C	Y	NR		KJ	female		46XX	MS	
8	355	TGT	TAT	G->A	n	C	Y	uni		KS	female	ovaries	46XX	yes	
8	360	TGT	TAT	G->A	n	C	Y	•		R6	ambiguous	testes	46XY	MS	
8	360	TGT	GGT	T->G	n	C	G	uni	n	3	female			yes	
8	362	CGA	TGA	C->T	y	R	stop								MG
8	362	CGA	TGA	C->T	y	R	stop								MG
8	362	CGA	TGA	C->T	y	R	stop								MG

Table 2. Germline WT1 mutations

exon	codon	WT codon	Mut codon	event	CpG	WT aa	Mut aa	uni/bi	LOH	name	ext.genit.	int.repr.organ	krtp	nephr	ref
8	362	CGA	TGA	C->T	y	R	stop	uni	y	Z-2368	female				
8	362	CGA	TGA	C->T	y	R	stop	bi	y	4	ambiguous	testes	46XY	MS	
8	362	CGA	TGA	C->T	y	R	stop	uni		WIT29	female				
8	366	CGT	CAT	G->A	y	R	H	no			female		46XY	MS	
8	366	CGT	CAT	G->A	y	R	H	no			female	dysgenic	46XY	MS	
8	366	CGT	CAT	G->A	y	R	H	no		SV	female	streak/dysgenic testis	46XY	MS	
8	366	CGT	CAT	G->A	y	R	H	uni		LVH	ambiguous		46XY	yes	
8	373	CAC	CAA	C->A	n	H	Q	bi		WT5100	female	gonadoblastoma/streak	46XY	yes	
8	373	CAC	CAG	C->G	n	H	Q	no		5	hypspadius		46XY	yes	
8	377	CAT	TAT	C->T	n	H	Y	*		D10	female	streak gonads	46XY	yes	
8	377	CAT	CGT	A->G	n	H	R	uni		D1	ambiguous			MS	
9	386	AAA	ins1c	stop@406		K	fr.	no			crypt./hyposp.			yes	
9	390	CGA	TGA	C->T	y	R	stop	uni		HDWT8	crypt.			yes	
9	390	CGA	TGA	C->T	y	R	stop	bi	y	NP57	normal			no(11m)	
9	394	CGG	TGG	C->T	y	R	W	uni		MM	female	ovaries	46XX	yes	
9	394	CGG	TGG	C->T	y	R	W	no		AK	female		46XY	MS	
9	394	CGG	TGG	C->T	y	R	W	uni		A1	female	dysgenic	46XY	MS	
9	394	CGG	TGG	C->T	y	R	W	NR*			hypospadias		46XY	MS	
9	394	CGG	TGG	C->T	y	R	W	no		85-583	female		46XY	yes	
9	394	CGG	TGG	C->T	y	R	W	?		802669				yes	
9	394	CGG	TGG	C->T	y	R	W	no		802646				yes	
9	394	CGG	TGG	C->T	y	R	W	uni		IV				yes	
9	394	CGG	TGG	C->T	y	R	W	uni		I			46XY	yes	
9	394	CGG	TGG	C->T	y	R	W	no		TQ	ambiguous		46XY	yes	
9	394	CGG	TGG	C->T	y	R	W	uni	y	12	female			no(7y)	
9	394	CGG	TGG	C->T	y	R	W	*		D5	ambiguous	testes	46XY	yes	
9	394	CGG	CCG	G->C	n	R	P	uni		JK	female		46XY	MS	
9	394	CGG	TGG	C->T	y	R	W	uni		LW	ambiguous	streak gonads	46XY	MS	

Table 2. Germline WT1 mutations

exon	codon	WT codon	Mut codon	event	CpG	WT aa	Mut aa	uni/bi	LOH	name	ext.genit.	int.repr.organ	krtp	nephr	ref
9	394	CGG	TGG	C->T	y	R	W	uni	y	RS	ambiguous	dysgenic testes	46XY	MS	
9	394	CGG	TGG	C->T	y	R	W	uni	y	MW	female	streak/dysgenic gonad	46XX	MS	
9	394	CGG	TGG	C->T	y	R	W	uni		SE	female	ovaries	46XX	MS	
9	394	CGG	TGG	C->T	y	R	W	uni		PL	ambiguous	normal/dysgenic testis	46XY	MS	
9	394	CGG	TGG	C->T	y	R	W	bi		AU	female		46XX	MS	
9	394	CGG	TGG	C->T	y	R	W	uni	y	CB	ambiguous	dysgenic testes	46XY	MS	
9	394	CGG	TGG	C->T	y	R	W	no		AM	ambiguous			MS	
9	394	CGG	TGG	C->T	y	R	W	bi		D1	female	ovaries	46XX	MS	
9	394	CGG	TGG	C->T	y	R	W	*		D3	ambiguous		46XY	MS	
9	394	CGG	TGG	C->T	y	R	W	no		AK	female	dysgenic	46XY	yes	
9	394	CGG	TGG	C->T	y	R	W	uni		LB	female	ovaries	46XY	MS	
9	394	CGG	TGG	C->T	y	R	W	*		HD/7	female		46XY	MS	
9	394	CGG	TGG	C->T	y	R	W	uni		MA1	ambiguous		46XY	MS	
9	396	GAC	AAC	G->A	y	D	N	no		802629	ambiguous		46XY	yes	
9	396	GAC	AAC	G->A	y	D	N	bi	y	2	female		46XX	yes	
9	396	GAC	AAC	G->A	y	D	N	uni		WY	ambiguous	L.Wolffian/no Mullerian	46XY	MS	
9	396	GAC	GGC	A->G	n	D	G	*		BE	female		46XX	MS	
9	396	GAC	AAC	G->A	y	D	N	uni		D5	female		46XX	MS	
9	396	GAC	AAC	G->A	y	D	N	uni		D2	ambiguous		46XY	MS	
9	396	GAC	AAC	G->A	y	D	N	uni		SS/12	male		46XY	MS	
9	398	CTG	CCG	T->C	n	L	P	uni		YI	female		46XX	MS	
9	401	CAC	TAC	C->T	n	H	Y	uni			female		46XX	MS	

Table 3. Somatic WT1 mutations.

This table, modified from Jeanpierre *et al.* (1998), compiles the somatic WT1 mutations detected in Wilms' tumors.

See legend for Table 2.

LOH: loss of heterozygosity. **del**: somatic mutation is associated with a germline deletion of the other WT1 allele. * patient has two independent somatic mutations affecting both alleles. ^ somatic mutation is associated with a germline point mutation affecting the other WT1 allele.

ref.: all references are from Jeanpierre *et al.* (1998) unless otherwise indicated.
1 Miyagawa *et al.* (1998).
2 Varanasi *et al.* (1994).
3 Brown *et al.* (1992).

Table 3. Somatic WT1 mutations

exon	codon	WT codon	mut codon	event	CpG	WT aa	mut aa	name	LOH	clinical	karyotype/sex	ref
1	27	CCT	del 4b	stop @ 88		P	fr.	266672	n			
1	77	AGC	del 34c	stop @ 78		S	fr.	802649	del	WAGR	46XX, del11p13	
1	115	CCT	del 19c	stop @ 211		P	fr.	802501	del	WAGR	46XY,del11p13	
1	125	GCC	del 5a	stop @ 128		A	fr.	S87-877	y		female	
2	152	GTC	del 5c	stop @ 177		V	fr.	WT12A	del	dev. delay	46XY,del11p13	
2	154	TTC	TCC	T -> C	n	F	S	D.B.	n		female	
2	161	GGT	ins 4c	stop @ 179		G	fr.	M.W.	y		female	
2	181	CCC	TCC	C -> T	n	P	S	BT1	n		46XY	
3	194	TCG	ins 7b	stop @ 224		S	fr.	B.M.#7	y*			
3	201	GGC	GAC	G -> A	n	G	D	WT/201	del	WAGR	46XY,del11p13	
4	238	TGG	TGA	G-> A	n	W	stop	WT5	del	WAR	female	
6	272	GAG	ins 4c	stop @ xx		E	fr.	9177	n		male	
7	301	CGA	TGA	C-> T	y	R	stop	9385	y		male	
7	301	CGA	TGA	C -> T	y	R	stop	WT40	y			1
7	302	CGT	del 1a	stop @ 306		R	fr.	S86-1334	del	WAGR	46XY,del11p13	
7	307	GCC	del 16a	stop @ 375		A	fr.	9394	y		female	
7	311	GTA	del 5c	stop @ 314		V	stop	WT24	y			1
7	312	CGG	ins 10c	stop @ 316		R	fr.	GOS 543	del	WAGR	del11p13	
7	338	TCC	TAC	C -> A	n	S	Y	K.K.#33	n			
8	362	CGA	TGA	C -> T	y	R	stop	B.M.#7	y*			
8	362	CGA	TGA	C -> T	y	R	stop	B.T.#53	y			
8	362	CGA	TGA	C -> T	y	R	stop	GOS 157	del	WAGR	del11p13	
8	362	CGA	del9b/ins2	stop @ 378		R	fr.	A.H.#20	n			2
8	366	CGT	TGT	C -> T	y	R	C	WT10	n			
8	372	AGA	ins 2a	stop @ xx		R	fr.	9561	y^		male	
8	373	CAC	TAC	C -> T	n	H	Y	S87-52	y		male	
8	385	TGT	del7a/ins18	stop @ 390		C	fr.	M.F.#88	y			2
9	390	CGA	TGA	C-> T	y	R	stop	WT2A		WAG	46XY	
9	390	CGA	TGA	C -> T	y	R	stop	D.J.#11	y			

Table 3. Somatic WT1 mutations

exon	codon	WT codon	mut codon	event	CpG	WT aa	mut aa	name	LOH	clinical	karyotype/sex	ref
9	390	CGA	TGA	C -> T	y	R	stop					
10	433	CGC	ins 1b	stop @		R	fr.	Wit-24	y		male	
10	433	CGC	del 2b	stop @		R	fr.	Wit-26	n		female	
	63		del226a	stop@145		Q	fr.	WT7	del	WAGR	46XY,del11p13	3

missense mutations. The small number of somatic missense *WT1* mutations include those which are located in the zinc finger region and are expected to affect DNA binding as well as three which map to the putative transcriptional repression domain. One of these mutations, causing a glycine to aspartic acid substitution, is capable of altering the transcriptional properties of WT1 *in vitro* (Park *et al.*, 1993c). This patient has WAGR syndrome due to a germline deletion of the other *WT1* allele and thus the missense mutation cannot cause dominant-negative effects with respect to WT1 function.. Since the tumor associated with this mutation appears clinically unexceptional (*i.e.* cannot be differentiated from tumors with null mutations) it is possible that the missense mutation merely creates a null allele and thus is equivalent to nonsense mutations. Despite the diversity of mutations detected in *WT1* there is no known association of specific mutations with distinct clinical properties of Wilms' tumor. In contrast, specific *WT1* mutations cause dramatically different abnormalities in the development of the genitourinary system.

Other means of disrupting WT1

A number of tumorigenic *WT1* mutations which disrupt splicing have been discussed. Some reports have suggested that in Wilms' tumors there may be other means by which WT1 splicing is disrupted in addition to conventional mutation of the gene. Haber *et al.* (1993) detected aberrant splicing such that exon 2 sequences were absent from a proportion of WT1 transcripts in Wilms' tumor cell lines derived through passaging as xenographs in nude mice. The cause of the altered splicing could not be determined since no mutations in the genomic DNA were observed. The authors suggested that the disruption of a regulatory gene could be responsible for the splicing abnormality and this could represent a Wilms' tumor suppressor locus. Since no analysis was done on primary tumors the *in vivo* relevance of the altered splicing was not clear and subsequent analysis of primary Wilms' tumors has determined that aberrant splicing of exon 2 is unlikely to be an important component of Wilms' tumorigenesis (Gunning *et al.*, 1996). Gunning *et al.* (1996) also addressed whether RNA editing was relevant to Wilms' tumor pathology. In a series of 15 tumors they failed to detect edited transcripts and concluded that this is not a frequent mechanism of altering WT1 function in tumorigenesis. Simms *et al.* (1995) employed quantitative PCR to show a small, but consistent increase in the ratio of exon 5+ transcripts to exon 5- transcripts in primary Wilms' tumors, although no mechanism for this alteration could be determined by mutational analysis of the flanking introns. It remains to be established whether the change in splicing of exon 5 is important to Wilms' tumor pathogenesis. Such a change in splicing could be secondary to changes in cell physiology

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associated with malignancy or may reflect the splicing pattern associated with the stage of differentiation cell progenitor of the tumor, and thus may not contribute to neoplastic transformation.

Noncoding, regulatory regions of a gene are potential targets of inactivating mutations. A small number of such mutations have been reported for other cancers. For example in two cases of familial retinoblastoma, the disease segregated with single base changes in the promoter region of the *RB1* gene (Sakai *et al.*, 1991). The mutations disrupted binding of the transcription factors Sp1 or ATF and likely reduced transcription of the gene. Studies of the proximal promoter (Grubb *et al.*, 1995; M. Discenza, unpublished observations) and the 5' and 3' untranslated regions of *WT1* (Varanasi *et al.*, 1994) have failed to detect alterations in these sites in Wilms' tumors and thus mutational inactivation of the *WT1* promoter is unlikely to play a major role in Wilms' tumorigenesis.

Association of *WT1* mutations with LOH at chromosome 11p

Models of tumor suppressor gene function predict that both alleles must be inactivated for tumorigenesis. In 'classical' models for tumor suppressor genes the loss of function mechanism involves the unmasking of a mutated allele by loss of the the wild type allele. This loss may be due to a *de novo* deletion or point mutation affecting the wild type allele or to somatic recombination detectable as LOH. As expected, most Wilms' tumors with mutated *WT1*, display coincident loss of the remaining wild type allele. In a series of 22 patients with constitutional intragenic WT1 mutations, 18 showed tumor-associated loss of the wild type *WT1* allele (see Table 4). Likewise out of 27 tumors with somatic *WT1* mutations 20 had lost the wild type allele. Of these 11 showed LOH, 8 were hemizygous due to a constitutional deletion of one WT1 allele, and one tumor showed two independent mutations affecting either allele. It is evident that *WT1* mutations are generally associated with allele loss at the *WT1* locus. The degree of allele loss in these tumors is considerably higher than what is observed for Wilms' tumor in general (about 30% show 11p13 LOH; Grundy *et al.*, 1996).

It is necessary to account for the significant number of cases of Wilms' tumors with heterozygous mutations (*i.e..* those in which the wild type allele appears to be retained). First of all, such observations need not call into question the notion that *WT1* is a tumor suppressor gene whose inactivation leads to tumorigenesis. There is potential for a mutated allele to behave in a dominant negative manner and, thereby, disrupt functioning of the normal allele. In addition, it is conceivable that specific mutations in a tumor suppressor gene may induce malignancy in a dominant gain-of function manner (*i.e.* behaving as an

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oncogene) though the generation of an altered protein with novel properties. The detection of a wild type allele in a tumor (*i.e.* a heterozygous mutation) may suggest that either of these two mechanisms may be at work. In analyses of tumor tissue, however, there is significant risk of 'contamination' by normal tissue (*e.g.* due to vascularization). Many studies employ PCR in the detection of LOH at a disease locus and since this technique is very sensitive, the presence of even a small quantity of normal tissue could make it appear that the normal allele is retained in a tumor. It is also possible that patients with apparently heterozygous mutations may have a second mutation in the other *WT1* allele which eluded detection, either due to analysis of only small portions of the gene (Little *et al.*, 1992B; Coppes *et al.*, 1993a; Huff *et al.*, 1995) or to limitations of the detection methods (Sheffield *et al.*, 1993; Bardeesy & Pelletier, 1995b) Thus, apparently heterozygous mutations need not indicate a dominant or dominant negative mechanism of WT1-induced malignancy. With respect to genitourinary development, however, it is clear that WT1 mutations have a dominant or dominant negative mode of behaviour (see below).

It is of note that out of five tumors with missense *WT1* mutations located outside of the zinc finger region, four retain the wild type allele, while the other case arose in a patient with a germline deletion of *WT1*. This may indicate that these mutations produce proteins which disrupt function of the protein encoded by the remaining wild type allele. Conversely, it is possible that some of the germline and somatic missense *WT1* mutations observed are, in fact, rare neutral polymorphisms and are not responsible for the malignancy in these patients. This is somewhat less likely for somatic mutations since their appearance in tumor cells indicates clonal selection of cells containing this change. Since non-zinc finger missense mutations are rare, confirmation of their significance to Wilms' tumor pathology requires further documentation of such changes. One Wilms' tumor which appears to retain the wild type allele has a deletion spanning an exon-intron boundary which causes an in-frame splicing defect, removing sequences coding for exon 9 (Haber *et al.*, 1990). The protein has defective DNA binding (Drummond *et al.*, 1994) and may be capable of behaving in a dominant negative manner. The expression of this protein, however, was not demonstrated in the Wilms' tumor from which it was derived, nor was the presence of contaminating tissue ruled out, hence this hypothesis remains untested. There does not appear to be any distinction in the clinical presentation of tumors with different *WT1* mutations. For example, tumors with complete absence of *WT1* due to deletions appear to resemble those with missense or nonsense mutations. Given the preponderance of mutations which produce grossly truncated proteins, this may suggest that, in terms of tumorigenesis, most, if not all, authentic *WT1* mutations behave as null alleles (*i.e.* in a loss-of-function manner).

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It is notable that a number patients with constitutional 11p13 deletions have tumor-associated, intragenic *WT1* mutations as opposed to LOH at this locus. Indeed, it appears that this is the most common means of inactivating the remaining *WT1* allele in patients with such deletions. Earlier studies noted this lack of 11p13 LOH in WAGR patients, and due to detection of LOH at 11p15 suggested that there may be cooperation between heterozygous loss of *WT1* and LOH at 11p15 (Henry *et al.*, 1989). It is now apparent that such failure to detect allele loss in these patients is due to the occurrence of small intragenic mutations and it does not reflect the existence of a wild type allele. These observations raise an interesting question: Why are constitutional deletions of *WT1* generally associated with a somatic intragenic *WT1* mutation in tumorigenesis while small constitutional mutations are usually accompanied by LOH? Glaser *et al.* (1989) have proposed that large scale deletions affecting both homologues of a chromosome may be rare because they delete genes essential to the viability of the cell. The detection of homozygous deletions of *WT1* in tumor cells demonstrates that *WT1* itself is not essential for cell survival (ref. in Pelletier, 1991d), however, adjacent genes may be essential. Glaser *et al.* (1989) speculate there may be evolutionary pressure in the chromosomal positioning of tumor suppressor genes such that they tend to be located adjacent to cell-lethal genes. The proximity of these lethality genes to the tumor suppressor locus would limit the size of chromosomal deletions which could be sustained in this region and thus would exert a protective effect against malignancy. The lower rate of LOH at 11p in tumors with large constitutional 11p13 deletions versus the rate in tumors with other *WT1* mutations is supportive of such a model (also see below, discussion of oncogenicity of *WT1* mutations).

WT1 mutations in the progression of Wilms' tumor

Most malignant tumors are thought to arise from a normal cell through a series of preneoplastic lesions involving a multistep accumulation of genetic changes to tumor suppressors and oncogenes. The mutations may occur in a specific order, with each genetic change associated with a tumor stage (Fearon & Vogelstein, 1990). It is of importance to address relative timing of *WT1* mutations in the progression of Wilms' tumor. Nephrogenic rests are foci of abnormally developing renal cells which are found in a substantial proportion of fetal kidneys (Beckwith *et al.*, 1990). Normally these lesions regress and are undetectable in kidneys after one year of age. Approximately one third of kidneys affected by sporadic Wilms' tumors, however, contain these structures, as do nearly 100% of kidneys of bilateral Wilms' tumor patients. It is presumed that these lesions are preneoplastic precursors to Wilms' tumor and thus are useful in the study of

progressive genetic changes in the development of this malignancy. Two essential questions that can be addressed by analysis of these specimens. First of all, at what point does *WT1* become mutated in sporadic tumors (*i.e.* do nephrogenic rests have *de novo* mutations or are these changes restricted to the malignant tumor)? Secondly, at what stage is the wild type *WT1* allele lost? To date, there is a single report analysing the status of *WT1* in nephrogenic rests and the associated tumors (Park *et al.*, 1993b). Two of 19 specimens had *WT1* mutations consistent with a role for this gene in a subset of Wilms' tumors. In both specimens *WT1* mutations were absent in the corresponding constitutional DNA. One nephrogenic rest and associated tumor had a homozygous mutation in exon 2 creating a premature stop codon. In the second specimen, there was a missense, phenylalanine to serine, mutation at codon 154. This mutation was heterozygous in both the nephrogenic rest and tumor. The observations indicate that *WT1* mutations are likely to be early events in the generation of some nephrogenic rests. The results are not concordant with respect to loss of the wild type allele in nephrogenic rests, however. This may be due to differences in mutation type. The heterozygous mutation results in an amino acid change while the homozygous change creates a truncated protein. As we have seen above, the small number of non-zinc finger missense *WT1* mutations on record are generally associated with retention of the wild type allele, possibly reflecting dominant negative activity. The patient with this missense mutation showed malformations suggestive of Beckwith-Wiedemann syndrome (BWS), which predisposes patients to Wilms' tumor and maps to chromosome 11p15. This is the only BWS-associated Wilms' tumor case on record to have a *WT1* mutation and given that it is a rare N-terminal missense mutation, this example may not be representative. In general heterozygous mutation of *WT1* cannot be the rate limiting step in the genesis of nephrogenic rests since although DDS and WAGR patients have constitutional *WT1* mutations, and thus every cell has mutant WT1, nephrogenic rests comprise only a fraction of renal cells in these individuals. The description of a homozygous mutation in a nephrogenic rest, absent in the germline appears to support the notion that loss of wild type *WT1* is involved in the development of this lesion. If this is the case, mutations in other genes must be required to transformation of these cells to malignant tumor cells. Thus it appears that Wilms' tumor involves a multistep genetic pathway. The generation of nephrogenic rests appears to provide an expanded population of target cells which could incur additional mutations at high frequency resulting in malignant tumors. Future studies will be needed to confirm these observations. In particular, nephrogenic rests from patients with germline *WT1* mutations should be analysed for loss of the wild type allele. This would establish unequivocally that such losses are the rate-limiting step in the development of these structures.

The apparent degeneracy of *WT1* mutations with respect to tumorigenesis is strongly contrasted by the diverse effects of such mutations of genitourinary development. As was discussed above, WAGR syndrome, characterized by mild developmental defects is associated with *WT1* mutations which delete one allele or result in a severely truncated proteins. In contrast DDS patients, who have very severe genitourinary abnormalities, generally display missense mutations in the zinc finger region. It appears likely that DDS associated mutations produce proteins which disrupt functioning of the remaining wild type allele (*i.e.* they are dominant negative) while WAGR associated mutations create null allele and the resulting reduced dosage of WT1 protein is responsible for the developmental aberrations. It is notable that *WT1* mutations are recessive at the cellular level with respect to tumor suppression but dominant with respect to genitourinary development.

There appears to be variable expressivity of *WT1* mutations in their effects on genitourinary development. In contrast to males, females with 11p13 deletions do not show defects in reproductive organs. This is suggestive of different roles for WT1 in the development of the reproductive system in either sex. More interestingly, DDS patients of the same chromosomal sex with identical *WT1* mutations may have quite different anomalies of their internal reproductive organs. For example, in individuals with XY karyotype and the [394]R-W mutations the Mullerian or Wolffian structures may be alternatively present or absent, and gonads may be streak, dysgenic or normal (Pelletier *et al.*, 1991c). Such variable expressivity may indicate the influence of background genetic factors which modify the observed phenotypes. There are two reports of individuals with constitutional *WT1* mutations identical to those found in DDS patients but lacking the developmental anomalies associated with DDS. In one case an unaffected father with a constitutional *WT1* mutation transmitted the mutant allele to a son who developed DDS (Coppes *et al.*, 1992a). The other report described a Wilms' tumor patient with no developmental anomalies who displayed a constitutional mutation identical with the most common mutation in DDS patients (Akasaka *et al.*, 1993). The absence of the DDS phenotype in these patients may be due to the genetic background or it may be due to somatic mosaicism. Somatic mosaicism is the occurrence genetically different populations of cells within an organism and may result from a mutational event occurring early in embryogenesis. Cell lineages descending from the mutant cell will inherit this genetic alteration whereas other cells will be wild type at this locus. In such a case, alternate cell populations may make different contributions to various tissues. For example, a given cell

lineage may contribute to hematopoiesis but not nephrogenesis, or may contribute some varying fractions of cells to different tissues. Thus, although a mutation may appear to be present constitutionally by detection in blood cells, there may be a substantial population of cells which have only wild type alleles. In the case of a patient who is mosaic for a *WT1* mutation, such a population of cells in the appropriate organs could be sufficient for normal development to proceed. There is at least one report of a Wilms' tumor patient with constitutional mosaicism for a chromosome 11p13 deletion (Little *et al.*, 1992c). In addition, a small number of Wilms' tumor patients show 11p LOH in normal tissues (Chao *et al.*, 1993). The result is mosaic individuals with varying proportions of homozygous and heterozygous cells in different tissues. Mosaicism may also occur at the level of gene imprinting such that monoallelic expression may be detectable in some organs. A defect in imprinting could result in the silencing of one allele in a given cell population. Indeed, mosaic imprinting of *WT1* has been observed although its pathogenic significance has not been demonstrated (Jinno *et al.*, 1994; Mitsuya *et al.*, 1997). In the case of an individual with a constitutional *WT1* mutation, the transcriptional silencing of the mutant allele by imprinting would be protective to genitourinary development. Since the patient under discussion is female no anomalies to the reproductive organs would be expected to accompany reduced WT1 dosage. An alternative explanation for the variable expressivity is that embryonic development may involve stochastic processes and thus there may be some random variation in the consequences of *WT1* mutations.

Oncogenicity of *WT1* mutations

A final issue regarding phenotypic effects of constitutional *WT1* mutations is their penetrance with respect to oncogenicity. In particular it is notable that the frequency of Wilms' tumor in DDS patients is greater than 90% whereas as only about 50% of patients with WAGR syndrome 11p13 deletions develop tumors (ref. in Coppes *et al.*, 1994). Since *WT1* mutations are recessive with respect to tumorigenesis, the determining factor in penetrance is likely to be the probability of somatic loss of the wild type allele. As was discussed above, it appears that there are constraints on allele loss associated with 11p13 deletions because of the possibility of elimination of genes required for cell viability. Thus, tumorigenesis associated with 11p13 deletions may require more specific somatic genetic alterations such as intragenic *WT1* mutations. In contrast, there are likely to be degrees of freedom permitted in the elimination of the wild type *WT1* from cells containing missense mutations, such as somatic recombination events, large deletions, as well as point mutations in *WT1*. Alternatively, this apparent discrepancy in penetrance may simply

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reflect diagnostic bias. Since the clinical criteria for DDS specify the association of intersex disorders, renal failure and Wilms' tumor, children who do not display such tumors may be differentially diagnosed despite the presence of *WT1* mutations. If such is the case the reported penetrance of Wilms' tumor associated with DDS may be inappropriately high.

Molecular mechanisms for *WT1* gene mutations

Mutations in cancers are genetic alterations which have eluded detection by the DNA repair machinery and have been selected during malignant progression because they confer a growth advantage such that daughter cells with this alteration gradually overgrow the rest of the cell population. Mutations are comprised of spontaneous nucleotide alterations arising during normal cellular metabolism, such as DNA polymerase errors, and changes due to the activity of mutagens. Hence, the types of mutation which disrupt a cancer susceptibility gene are reflective of the etiology of the cancer. Precursor cells may show particular sensitivity to specific mutational mechanisms due to the relative efficiency of their DNA repair processes, to exposure to mutagens, or to local environmental factors. The types of mutation observed in a cancer susceptibility gene will also reflect the properties of the gene product encoded by the mutated gene. Genes encoding proteins which enhance growth may be amplified or have activating point mutations at specific residues while tumor suppressor genes may be deleted or have other inactivating mutations. In addition, specific germline mutations in a tumor suppressor gene may predispose to different tumor types (see above).

Review of a set of 33 somatic intragenic *WT1* mutations in Wilms' tumors revealed 16 point mutations, 9 deletions, 6 insertions and 2 deletion/insertions (see Table 3). The frequency of point mutation at a given residue will reflect its mutability and/or its functional importance. Of the point mutations, 12 are cytosine to thymidine transitions, mostly occurring in the context of CpG dinucleotides. Cytosine residues in these dinucleotides undergo higher rates of mutation compared with other bases since they are usually methylated to 5-methylcytosine which is susceptible to spontaneous deamination to yield thymidine (Laird & Jaenisch, 1996). The mutagenicity of this event is compounded by the relatively low efficiency of cellular repair processes for T-G mismatches (Loeb, 1996). Cytosine to thymidine transitions are common events in tumorigenesis and usually reflect spontaneous versus environmentally induced carcinogenesis. There is no specific mutational 'hot spot' in *WT1* in sporadic tumors although there have been three descriptions of C to T transitions at position 1084 (codon 362). The distribution of these mutations is likely determined to a great extent on the methylation status of the particular

residue and by whether the mutational event arises in a sequence context which will produce a stop codon. There is definitively a hot spot for constitutional WT1 mutations associated with DDS (see Table 2). More than 50% of DDS patients display a C to T mutation at position 1180, encoding an R394W substitution. The high frequency of this mutation may indicate that this cytosine residue is methylated during gametogenesis and thus is more susceptible to mutation.

It is of note that a number of the deletion mutations are flanked by short repeat sequences, occur within the context of repetitive sequences and are within five nucleotides of a CCTG sequence (Huff et al., 1995). The latter motif has been identified to be associated with novel deletions in mammalian DNA. Large tandem repeats are hypermutable, likely due to slipped mispairing during DNA replication, and expansion of such elements is observed in a number of congenital disorders, including fragile X syndrome (Fu et al., 1991) and myotonic dystrophy (Brook et al., 1992). Although the repetitive elements in WT1, located in exon 1, are much smaller, they may be susceptible to DNA replication errors and thus be mutational 'hotspots'. In general, short deletions or insertions occur at runs of two or more identical bases or at tandem two to eight base pair motifs (Greenblatt et al, 1996). The likely mechanism, slipped mispairing, involves misalignment of the template DNA strands during replication. Depending on whether the nucleotides excluded from pairing are on the template or primer strand determines whether a deletion or insertion, respectively, results. When repeats mispair with an adjacent motif, the intervening sequence may be looped out and deleted (Krawczak & Cooper. 1991)

Parental origin of inherited WT1 mutations

Genetic lesions at chromosome 11p show parental origin effects. LOH studies of Wilms' tumors using polymorphic markers to chromosome 11 showed that the maternal allele was lost in 52 of 53 tumors with 11p LOH (compiled in Coppes et al., 1992b). In addition, 7 of 8 patients with de novo constitutional deletions of chromosome band 11p13, the deletions were of paternal origin (Huff et al., 1990). This parental-specific allele loss may reflect an increased mutation rate of gametogenesis in the male. The increased mutation rate during spermatogenesis cannot account for tumors in which causative mutations are somatic. The other factor accounting for this preferential loss of maternal alleles is likely the involvement of imprinted genes, most likely at 11p15 since this region shows an abundance of such genes. Studies of children who inherited 11p13 deletions from parents carrying balanced translocations of chromosome 11 suggest that it is unlikely that imprinting of WT1 itself is involved. Four of five of these WAGR patients inherited the

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abnormal chromosome from their mothers (Huff *et al.*, 1990). In addition, studies of WT1 show biallelic expression in most tissues (see below).

Molecular Function of the WT1 Protein

DNA binding specificity of WT1

The zinc finger motif found in WT1 suggests a DNA binding function and consequently most studies of this protein have attempted to identify transcriptional regulatory properties. Consistent with a role in DNA transcription, early studies showed that the protein, when expressed via plasmid transfection, is localized to the nucleus, and this was corroborated for the endogenous protein (Pelletier *et al.*, 1991a; Mundlos *et al.*, 1993). Selection experiments with a randomized population of double stranded oligonucleotides identified a guanine-rich DNA element recognized by bacterially expressed recombinant WT1 protein (Rauscher *et al.*, 1990). Due to the homology of WT1 zinc fingers II to IV with the three zinc fingers of EGR1 the investigators suggested that the true WT1 binding site might be GCGGGGGCG (*i.e.* the cognate EGR1 site) although this exact sequence was not selected. Only the -KTS isoform of WT1 showed strong binding to the G-rich motifs. Zinc finger I is dispensable for binding to the EGR1 site (Drummond *et al.*, 1994). Other investigators used selection methods to refine the sequence requirements necessary for high affinity WT1 binding and to identify possible ligands for the +KTS isoforms. Nakagama *et al* (1995) employed WT1-KTS synthesized in rabbit reticulocyte lysates to identified highest affinity binding for the sequence GCGTGGGAGT in selection a experiment using a pool of genomic DNA. Drummond *et al.* (1994) demonstrated that WT1+KTS isoforms could bind to regions in the insulin-like growth factor promoter by DNA footprint and methylation interference analysis although binding affinities were about ten-fold weaker than for WT1-KTS. The binding site for WT1+KTS contained the core EGR1 site, and, in contrast to WT1-KTS, all four zinc fingers were required for binding. Bickmore *et al.* (1992) used a whole genome PCR method to identify targets for the WT1-KTS and +KTS isoforms. The -KTS isoforms recognized sequences with repeats of the nucleotides GT. Another element, GGAGAGGGAGGA was recognized by both WT1 isoforms, although the specificity of binding to WT1+KTS was not clear. This target site requires zinc finger I-III of WT1 for complex formation (Little *et al.*, 1996) in contrast to other binding sites which require zinc fingers II-IV. Studies of the regulation of the platelet-derived growth factor (*PDGF*) promoter identified a novel WT1-KTS-binding sequence containing the (TCC)n motif (Wang *et al.*, 1993). It has not been demonstrated whether the

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interaction occurs with the cytosine/thymidine-rich strand or its complement, however, based on structural information on the binding of zinc fingers to DNA (Pavletich & Pabo, 1991; Drummond *et al.*, 1994) it appears more likely that direct protein contacts are made with the guanine residues, hence it may be more appropriate to refer to this motif as (GGA)n.

It is not clear which binding sites for WT1 have relevance to the physiological function of this protein. There is limited data on the DNA binding of the endogenous cellular WT1 and it is difficult to make comparison between the relative affinities for binding sites identified in different reports due to the use of different protein sources (*e.g.* *in vitro* translated versus bacterially expressed WT1) (reviewed in Licht, 1996). *In vitro* cotransfection experiments have shown that WT1 can regulate transcription through several of the apparent DNA binding sites when positioned adjacent to the transcriptional start site of a reporter gene construct. The artificially high levels of WT1 expression in these experiments, however, could produce transcriptional effects that are not relevant to the protein function *in vivo* (see below). The finding that WT1 can recognize guanine-rich binding sites is consistent with predictions based on X-ray crystallographic and other structural studies of the related Cys_2-His_2 class zinc finger protein Zif268 (EGR1) (Pavletich & Pabo, 1991; Nardelli *et al.*, 1991). Members of this very large group of proteins are characterized by conserved motifs of 28-30 amino acids which are usually present as tandem repeats (reviewed in Berg, 1992). This small, independently folding motif has the consensus amino acid sequence (Tyr,Phe)-X-Cys-X_{2-4}-Cys-X_3-Phe-X-X^a-X_2-X^b-Leu-X-X^c-His-X_{3-5}-His which ligands a zinc ion to the cysteine and histidine residue. The three amino acids residues per zinc finger (marked X^a, X^b and X^c) make direct contacts with one nucleotide each, and that the binding is in the 3' to 5' orientation (i.e. the carboxy-terminal zinc finger in a series binds to the 5' end of a DNA sequence). These residues are the primary determinants of the site preference of the protein. X-ray crystallography of the Zif268 (EGR1) zinc fingers bound to DNA has indicated that arginine residues at these positions make two hydrogen bonds with guanine nucleotides and thus when present, confer much of the binding specificity (Pavletich & Pabo, 1991). The predicted contacts for the WT1 zinc fingers include two arginines in zinc fingers II and IV and one arginine in zinc finger III, and thus the binding of WT1 to guanine-rich binding sites conforms to expectations. In the absence of X-ray crystallographic data on WT1 it is not possible to readily account for the altered binding specificity due to the insertion of the alternatively spliced +KTS amino acids. Additionally, no studies provide satisfactory evidence for an important role for zinc finger I in DNA binding. It remains possible that

zinc finger I and the +KTS isoform have different biochemical functions not related to DNA binding.

Role of WT1 in transcriptional regulation

A number of studies have assessed the ability of WT1 to regulate transcription from promoters which contain EGR1 binding sites. In cotransfection assays, WT1 has been shown to repress the activity of a CAT reporter construct driven by an artificial promoter containing three EGR1 binding sites (Madden *et al.*, 1991). Only the WT1-KTS isoform was capable of transcriptional repression. The body of subsequent studies of potential targets transcriptionally regulated by WT1 have focussed on known genes involved in growth regulation (*e.g.* oncogenes, growth factors and receptors) which harbor G-rich or TCC elements in their promoters. Candidates for WT1 regulation were chosen based on their presumed relevance to the biology of Wilms' tumors or on their more general roles in embryonic development. Cotransfection experiments using such promoters elements flanking reporter genes has shown that WT1 can modulate the transcription of a number of growth regulatory genes *in vitro*. The significance of this data is not clear since in most cases the endogenous gene has not been shown to be regulated by WT1. The following table lists some of the genes for which there is evidence of transcriptional regulation by WT1.

Table 4:
Transcriptional Regulation by WT1: Potential Target Genes

Promoter	Effect of WT1	Reference
IGF2	repression	Drummond *et al.*, (1992)
	activation	Nichols *et al.*, (1995)
PDGF-A	repression	Wang *et al.*, (1992)
	repression	Gashler *et al.*, (1992)
	repn./actvn.	Wang *et al.*, (1993)
IGF1R	repression	Werner *et al.*, (1993)
EGF-R	repression	Englert *et al.*, (1995)
RAR-a	repression	Goodyer *et al.*, (1995)
PAX-2	repression	Ryan *et al.*, (1995)
G-protein a_{i-2}	repression	Kinane *et al.*, (1996)
syndecan-1	activation	Cook *et al.*, (1996)
bcl-2	repression	Heckman *et al.*, (1997)

IGF2 is an appealing candidate for WT1 regulation since 1) overexpression of *IGF2* has been implicated in Wilms' tumor malignancy (see above), 2) it has potential WT1

binding sites in its promoter region and 3) a reciprocal expression pattern between *IGF2* and *WT1* is observed in the kidney. These observations are suggestive of transcriptional repression of *IGF2* by WT1. Cotransfection experiments with the P3 promoter of *IGF2* indicate a strong repression of transcription by WT1 (Drummond *et al.*, 1992). Effective repression was dependent on the presence of WT1 binding sites on both sides of the site of transcriptional initiation. However, there are no reports to date which demonstrate a convincing repressive effect on the endogenous *IGF2* gene. In addition many Wilms' tumors show high levels of expression of both IGF2 and WT1 indicating that the *IGF2* promoter can be active in the presence of WT1 protein. Nichols *et al.* (1995) have reported that WT1 can induce the expression of endogenous *IGF2* in a Wilms' tumor cell line indicating that the transcriptional properties of WT1 are cell type-dependent. The platelet-derived growth factor-A chain (PDGF-A) is another growth factor which may be regulated by WT1. WT1 represses transcription from the PDGF-A promoter through binding sites located both 5' and 3' to the transcriptional start site (Wang *et al.*, 1992; Gashler *et al.*, 1993). The elimination of either binding site results WT1-KTS-mediated activation (Wang *et al.*, 1993), suggesting that the promoter context affects the transcriptional properties of this protein.

A small number of studies have demonstrated the WT1-responsiveness of genes from their endogenous promoters or *in vivo* binding of WT1 to these promoters. Cook *et al.* (1996) have demonstrated WT1-mediated transcriptional activation of the endogenous *syndecan-1* promoter. This gene encodes a cell surface proteoglycan whose induction is coincident with epithelial differentiation in the kidney, an embryonic process in which WT1 has been implicated. Englert *et al.* (1995) infer that WT1 transcriptionally represses the epidermal growth factor receptor (EGFR) since protein levels are reduced in response to WT1 expression and since the EGFR promoter is repressed in cotransfection assays. Unfortunately, no data is presented to show an effect of WT1 expression on EGFR mRNA levels. *In vivo* footprinting demonstrated the binding of cellular WT1 to regulatory sites in the *c-myb* and *bcl-2* proto-oncogene promoters (McCann *et al.*, 1995; Heckman *et al.*, 1997). Cotransfection experiments indicate that WT1 represses these promoters. Interestingly, WT1 is unable to bind to the *bcl-2* allele disrupted by a chromosomal translocation in follicular lymphoma cells. The inability of WT1 to bind to the translocated allele may contribute to the oncogenic overexpression of *bcl-2*.

Although most promoters regulated by WT1-KTS are unaffected by WT1+KTS, there are some promoters which appear to be responsive to both isoforms (Wang *et al.*, 1995; Hewitt *et al.*, 1996). The weak affinity for DNA binding in vitro of WT1+KTS and the high levels of expression needed to generate a transcriptional effect lead to questions

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regarding the significance of this data and thus it is not possible to currently ascertain whether WT1+KTS regulates transcription *in vivo*.

Several studies have addressed the structural requirements for the transcriptional activity of WT1. The results are essentially consistent between the studies and will be summarized briefly. There is an N-terminal minimal repression domain mapping to amino acids 84-124 (Madden *et al.*, 1993; Wang *et al.*, 1993). A central domain (amino acids 181-250) mediates transcriptional activation. Deletion of exon two (amino acids 149-189) converts WT1 from a repressor to an activator of the *EGR-1* promoter (Haber *et al.*, 1993). The 17 amino acid stretch encoded by exon 5 may also be a repression domain (Wang *et al.*, 1995). A small number of missense WT1 mutations naturally occurring in Wilms' tumors have been reported to convert WT1 from a transcriptional repressor to an activator (reviewed in Licht, 1996). Paradoxically, these mutations map to the transcriptional activation domain of WT1 but they disrupt the repressive function of this protein. This behaviour may be due to global changes on protein conformation effected by these mutations.

As we have seen, full length WT1 shows complex transcriptional behaviour alternately repressing or activating dependent on the promoter and cell type under study. Little is known, however, about the mechanistic basis for the transcriptional regulatory function of WT1. This behaviour may be due to cofactors which modulate the transcriptional activity of WT1. Evidence for the existence of interacting proteins was first suggested by cotransfection of WT1 expression constructs encoding truncated WT1 proteins. The expression of amino acids 85-179 can inhibit the transcriptional repression or potentiate the transcriptional activation functions of wild type WT1 suggesting that this region binds to factors responsible for WT1-mediated repression (Wang *et al.*, 1995) . This region also mediates the self-association of WT1 *in vitro* and in yeast two-hybrid systems (Reddy *et al.*, 1995; Moffett *et al.*, 1995). The binding is likely to be weak or transient since WT1 complexes have not been observed in coimmunoprecipitations from transfected cells. Coexpression of full length WT1 proteins which are incapable of DNA binding (*e.g.* zinc finger mutants) inhibits the transcriptional functioning of the wild type protein suggesting that these mutants either sequester factors necessary for wild type WT1 activity or directly bind to wild type WT1 forming non-functional complexes.

A potential modulator of WT1 transcriptional activity has been identified using a yeast two-hybrid screen of a HeLa cell cDNA library (Johnstone *et al.*, 1996). Par-4, originally identified to be induced during apoptosis in the prostate, binds to the WT1 zinc finger region. Coexpression of par-4 augments WT1-mediated repression. Additionally the ability of WT1 to transactivate other promoters is inhibited by par-4 expression. Par-4 does

not contain a DNA binding domain and it is thought that the transcriptional repression function of this protein requires recruitment to DNA elements by binding to WT1. In stably transfected cells, par-4 can relieve WT1-mediated growth suppression. Tissue and temporal specific regulation of par-4 levels may be an important means of regulating the transcriptional function of WT1.

P53 may also be a transcriptional cofactor for WT1. WT1 and p53 can be co-immunoprecipitated under mild conditions in extracts from transfected cells or from Wilms' tumors (Maheswaran *et al.*, 1993). In the Saos-2 osteosarcoma cell line, lacking endogenous p53, WT1 activated the EGR1-CAT reporter gene. However, when p53 was coexpressed, the level of WT1-mediated activation was reduced. The authors suggest that WT1 may be a transcriptional activator in cells lacking endogenous wild type p53, whereas it is a repressor in cells expressing endogenous p53 expression. It is now clear that this behaviour is not a general property of WT1. For example, WT1 represses the epidermal growth factor receptor promoter (EGFR) in the p53-deficient Saos-2 and HeLa cell lines (Englert *et al.*, 1995a) and thus the significance of the interaction remains undefined.

A report by Reddy *et al.* (1995), demonstrated that the transcriptional effects of WT1 can be modulated by the choice of expression vector. When WT1 was expressed from the very strong cytomegalovirus (CMV) promoter, it repressed the activity of an EGR1-CAT reporter construct. Conversely expression of WT1 from the weaker β-actin or rous sarcoma virus (RSV) promoters resulted in transcriptional activation. The difference in activity cannot be accounted for by the relative quantity of expressed WT1 protein since cotransfection of the CMV expression vector (lacking a cDNA insert) was able to convert WT1, expressed from an RSV promoter, into a repressor of transcription. It appears that the CMV promoter sequesters factors which are required for the activating function of WT1, in the absence of which it behaves as a repressor of transcription. This study, however, does not completely account for WT1 activity since there are several examples of promoters which show cell-specific activation by WT1 independently of the expression vector employed (*e.g.* Hamilton *et al.*, 1995; Wang *et al.*, 1995). The development of WT1-responsive *in vitro* transcription assays would be of great use in more fully exploring these issues.

A small number of transcription factors have well characterized dual functions as transcriptional activators and repressors and may provide models applicable to the study of WT1. The *Drosophila melanogaster* zinc finger protein Krüppel activates transcription when present in low concentrations, whereas at higher concentrations it is a repressor (Sauer & Jäckle, 1993). This behaviour is due to the concentration dependant dimerization of the protein. At low concentrations, monomeric Krüppel recruits the basal transcription

factor TFIIB to the promoter and thus activates transcriptional initiation (Sauer *et al.*, 1995). Conversely, dimeric Krüppel is unable to interact with TFIIB and instead binds to the the basal factor TFIIEβ. This interaction may cause transcriptional repression by masking surfaces of TFIIEβ necessary for interaction with other factors in the assembly of the transcriptional preinitiation complex.

There is also precedent for a transcriptional repressor requiring DNA binding sites flanking both sides of the transcriptional start site as is observed for WT1 repression of the *PDGF-A* promoter. The binding of the *Drosophila even-skipped* (*eve*) protein to an upstream site in the *Ultrabithorax* promoter recruits binding of a second *eve* molecule to a lower affinity binding site 3' to the transcriptional start site (TenHarmsel *et al.*, 1993; TenHarmsel & Biggin, 1995). It appears that transcription is repressed by the bending of DNA in the region spanning the transcriptional start site inhibiting the binding of the general transcription factor TFIID.

Another means by which the transcriptional function of WT1 may be regulated is protein phosphorylation. It has recently been demonstrated that the WT1 protein can be phosphorylated by protein kinase A (PKA) both *in vitro* and in transfected cells (Ye *et al.*, 1996; Sakamoto *et al.*, 1997). The sites of phosphorylation have been mapped to Ser-365 and Ser-393 which are in zinc fingers 2 and 3, respectively. Phosphorylation inhibits WT1 DNA binding *in vitro*. Additionally, transfection of PKA or the activation of this protein by chemical inducers results in inhibition of the DNA binding and transcriptional repression properties of transfected WT1. Activation of PKA also results in a small increase in cytoplasmic retention of WT1, although the proportion is small relative to the amount of nuclear WT1 (Ye *et al.*, 1996; Sakamoto *et al.*, 1997). It is unknown whether the cytoplasmic WT1 is indeed phosphorylated. It is possible that the PKA-mediated phosphorylation of WT1 may allow the regulation of WT1 through extracellular signals. Sakamoto *et al.* (1997) speculate that the function of WT1 in sexual development require modulation by sex hormones which signal via the cAMP/PKA pathway.

Regulation of the *WT1* Promoter

Investigation of the transcriptional regulation of the *WT1* promoter is of considerable interest given the highly specific temporal and spatial expression of *WT1* mRNA during development. Immunohistochemical studies have shown that WT1 protein expression parallels that of the WT1 message suggesting that *WT1* regulation is not predominantly translational (Mundlos *et al.*, 1993; Grubb *et al.*, 1994). Structurally, the WT1 promoter region in both the mouse and human is characterized by a high G-C content

and the absence of TATA and CCAAT boxes (Pelletier *et al.*, 1991a; Hoffmann *et al.*, 1993; Gessler & Bruns, 1993a). These features are more commonly found in housekeeping (ubiquitously expressed) genes, however, some other promoters of genes showing tissue specific expression have similar organization including that of the hematopoietic *CD43* (Kudo & Fukada, 1995) and the placental and muscle-expressed *TEF-1* (Boam, *et al.*, 1995). Promoters of this structure show regulation by Sp1. Cohen *et al.* (1997) used a series of deletion construct of the 5'-flanking region to show that the proximate promoter region contains an enhancer element which contributes much of the transcriptional activity of the *WT1* gene. This region conferred activity to a reporter construct in a non-*WT1* expressing cell line suggesting that it is not involved in the tissue-specific regulation of *WT1*. This specific regulation may involve the competitive binding of a protein complex to sites adjacent to Sp1 elements in the *WT1* promoter (Discenza *et al.*, 1997).

Members of the paired-box (*PAX*) family of transcription factors have been implicated in embryogenesis and tissue-specific gene regulation. The *Pax-3* gene is mutated in the splotch mouse (Epstein *et al.*, 1991) and in Waardenberg syndrome in the human (Tassabehji *et al.*, 1992) and *Pax-6* mutations are responsible for the small eye (Hill *et al.*, 1991) and aniridia phenotypes (Ton *et al.*, 1991) in the mouse and human, respectively. The *PAX-2* and *PAX-8* genes are attractive candidates for activators of *WT1* expression during nephrogenesis. Expression of both genes precedes that of *WT1* in the condensed mesenchyme. *Pax-2* homozygous mutant mice lack kidneys, ureters and genital tracts, consistent with a role for this gene in urogenital system development (Torres *et al.*, 1995). Dehbi *et al.* (1996a; 1996b) showed that transfection of *PAX-2* and *PAX-8* gave strong activation of reporter constructs containing the proximal promoter of *WT1* and also of the endogenous *WT1* gene. The results showing regulation of *WT1* expression by Pax-2 and Pax-8 were corroborated and extended in other studies (McConnell *et al.*, 1997; Frazier, *et al.*, 1997). In summary the data are consistent with an important role of PAX-2 and PAX-8 in regulating WT1 expression in the human and mouse. It is possible that tight control of WT1 mRNA levels is attained by a regulatory loop since WT1 may repress transcription of *PAX-2* and possibly the *WT1* promoter itself (see below) occur, resulting in sharp reduction of WT1 mRNA levels. Since, PAX-2 and PAX-8 appear important in the activation of *WT1* expression, the loss of dysfunction of these genes may result in loss of *WT1* expression. Given the tumor suppression function of WT1 such a lack of expression would be expected to be tumorigenic, and thus *PAX-2* and *PAX-8* are candidate tumor suppressor genes. Mutational analysis of these genes in a series of Wilms' tumors has, however, failed to reveal any mutations (Dehbi & Pelletier, 1996b).

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In vitro cotransfection experiments in the 293 adenovirus-transformed fetal kidney cell line have indicated that WT1 may negatively regulate its own promoter (Rupprecht *et al.*, 1994). In investigations of the regulation of *WT1* in hematopoietic cells, Frazier *et al.* (1994) identified an enhancer which increases *WT1* transcription in K562 erythroleukemia cells but not in the non-hematopoietic cells tested. The enhancer is located 3' to the *WT1* gene and can mediate *WT1* induction through interaction with the GATA-1 zinc finger transcription factor (Wu *et al.*, 1995). A second hematopoietic enhancer was identified in intron 3 of the *WT1* and can result in transactivation of *WT1* through the binding of GATA-1 or c-Myb (Zhang, *et al.*, 1997).

Although the experiments described above may be useful in the preliminary analysis of the *WT1* promoter, they are generally performed in under nonphysiological conditions in cells which do not normally express *WT1*. The use of cells in which WT1 expression is altered in response to the induction of differentiation may provide more biologically relevant clues to the regulation of *WT1*. Very strong induction of *WT1* expression is seen in F9 and P19 embryonal carcinoma cells which have been chemically stimulated to differentiate (Kudoh *et al.*, 1996; Scharnhorst *et al.*, 1997). Identification of the signalling pathways responsible for this induced expression may provide useful clues to the regulation of *WT1* during development. Ultimately, however, transgenic mouse experiments are needed for a deeper understanding of the complex tissue- and temporal-specific regulation of this promoter under physiological conditions. A potential source of information suggestive of critical regions in the *WT1* promoter would be the identification of mutations in this region in Wilms' tumors. However, analysis of 1154 bp upstream of the *WT1* start site and the first 83 bp of the 5'-UTR failed to detect any tumor-specific sequence alterations (Grubb *et al.*, 1995).

Possible role for WT1 in RNA metabolism

Most studies of the functional properties of WT1 have addressed its properties as a DNA binding transcription factor and have focused on the -KTS isoform since it appears to have higher affinity binding to DNA. Recent studies, showing that the more abundant +KTS isoform has different localization within the nucleus, are suggestive of a distinct function for WT1+KTS (Larsson *et al.*, 1995). In immunofluorescence studies of transfected cells, WT1+KTS shows a speckled nuclear distribution against a diffuse staining background. In these 'speckles' there is colocalization with splicing factors. In contrast, WT1-KTS is restricted to nuclear domains, colocalizing with transcription factors. The treatment of fixed cells with RNAse A causes WT1+KTS to show a different

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distribution, similar to that of WT1-KTS in untreated cells. Treatment with DNase I does not change the localization of WT1+KTS. Conversely, in DNAse I treated cells, WT1-KTS relocalizes to speckles but is unchanged by RNAse A treatment. Mutations or deletions of the WT1 zinc fingers increase the colocalization of WT1 proteins with splicing factors (Larsson *et al.*, 1995; Englert *et al.*, 1995b). These data suggest that DNA binding directs WT1 to nuclear domains, in the absence of which, WT1 localizes to speckles. The inhibition of DNA binding by PKA-mediated phosphorylation of WT1-KTS may divert this isoform to function in RNA metabolism (Sakamoto *et al.*, 1997). Intact cellular RNA is necessary for a speckled pattern of WT1 suggesting that either WT1 must bind to RNA or that it must interact with another RNA-binding protein to be directed to speckles. Evidence for WT1-RNA interaction was suggested by mobility shift studies using *Igf2* RNA as a probe (Caricasole *et al.*, 1996). Both isoforms of the WT1 zinc fingers were able to bind exon 2 of *Igf2* but not to other RNA sequences used as controls. Curiously the sequence to which the WT1 zinc fingers apparently bind overlaps an element whose DNA sequence can bind to WT1, however, DNA binding involves contacts with the guanine-rich strand (Drummond *et al.*, 1994), whereas it is the complementary, cytosine-rich sense strand of Igf2 RNA which binds to WT1 proteins. Protein modelling by homology has suggested that the amino terminus of WT1 may possess an RNA recognition motif (RRM) [Kennedy *et al.*, 1996] based on structural rather than sequence homology to RRM-containing proteins. This technique assigns a three-dimensional structure to an unknown protein based on structural information from members of the same family and is based on the notion that structurally conserved regions are located at the inner core of proteins where alteration of the topology of the peptide chain would have dramatic effects on the conformation of the protein. The proposed structure of amino acids 20-107, consisting of a series of B-sheets and a-helices composing a B-platform with exposed aromatic side chains, is homologous that of characterized RRMs. There is currently no experimental evidence for the interaction of this domain of WT1 with RNA. In summary the observations discussed above and the data which will be presented in Chapter 5 are suggestive of a role for WT1 in RNA metabolism, however, such a function has yet to be directly demonstrated *in vivo*.

Tumor Suppression Assays

Cell fusion experiments were instrumental in the development of the concept of tumor suppressor genes. As discussed above, the tumorigenicity of malignant cells can be suppressed by fusion with microcells containing specific chromosomes, and this is interpreted as a demonstration that these chromosomes harbor genes which prevent

malignant transformation (*i.e.* tumor suppressor genes). Tumor suppression assays, in modified systems, are now being used to study the molecular genetics of Wilms' tumor, for both fuctional analysis of WT1 and as a mapping technique to elucidate an 11p15 Wilms' tumor locus. These studies are based on assumptions which may not be entirely tenable and invite commentary and cautious interpretation.

The first growth suppression studies of Wilms' tumors involved transfer of a normal human chromosome 11 into the G401 renal tumor-derived cell line and resulted in reduced growth and suppressed tumorigenicity in nude mice (Weissman *et al.*, 1987). These effects were interpreted to be the result of genetic complementation of the lesion in the G401 cells. Subsequent studies, employing the transfer of chromosomes 11 with deleted regions (Dowdy *et al.*, 1991; Hink Reid *et al.*, 1996), found that the telomeric p15 region was responsible for suppressing the tumorigenic phenotype of G401 cells. A second type of tumor suppression study has been used to directly test the growth regulatory properties of WT1. In these experiments, high levels of WT1 are ectopically expressed in tumorigenic cell lines and the resulting effects on tumorigenicity are evaluated. Haber *et al.* (1993) reported that transfection of any of the four major isoforms of *WT1* could reduce colony formation of a cell line derived from a Wilms' tumor. The magnitude of this reduction, however, was extremely variable between experiments. McMaster *et al.* (1995) reported that transfection of the G401 cell line with *WT1* resulted in suppressed tumorigenicity and that the WT1-/+ isoform was more potent than WT1+/-. Cell clones showed wide variability in *in vivo* growth characteristics and in tumorgenicity. Menke *et al.* (1996), using adenovirus-transformed baby rat kidney cells, found that WT1-/+ increased the latency period of tumor formation in nude mice, while WT1+/- and WT1+/+ had no effect and WT1-/- potentiated tumorigenicity. Tumors which eventually developed from the WT1-/+ transfectants showed loss of WT1 expression suggesting that loss of WT1 was necessary for tumor formation.

Studies of the effect of WT1 expression on cell growth have also indicated that it may play a role in the regulation of apoptotic pathways. Englert *et al.* (1995a) reported that in transfections of the osteosarcoma cell lines U2OS and Saos-2 (which possess and lack endogenous wild-type p53 respectively), only the WT1+/- isoform suppressed colony formation. Using an inducible system for studying WT1 in these cells, it was shown that WT1+/- caused growth inhibition and apoptosis, and that sustained expression of WT1 was required for maximal effects. The WT1-induced apoptosis was independent of p53. Decrease in levels of the epidermal growth factor receptor (EGFR) was associated with induction of WT1 expression, whereas as constitutive expression of EGFR restored cell viability. Using a similar expression system as above, but with lower levels of WT1

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synthesis, it was shown instead that WT1 inhibited p53-mediated apoptosis (Maheswaran *et al.*, 1995). This effect was correlated with an increased stability of p53 protein, an activity which mapped to the first two zinc fingers of WT1. In M1 myeloblastic leukemia cells expression of WT1+/-, but not WT1 +/+, causes apoptosis (Murata *et al.*, 1997). This behaviour is somewhat surprising since many leukemic cells express WT1 (Miwa *et al.*, 1992b; Menssen *et al*, 1995) and the reduction of WT1 levels in the HL60 and K562 leukemic cell lines using antisense oligonucleotides results in apoptotic cell death (Algar *et al.*, 1996). In both the Hep3B and Hep2G hepatoblastoma cell lines (which lack and express wild type p53, respectively), WT1-/- and WT1+/- induced apoptosis (Menke *et al.*, 1997), whereas as the +KTS WT1 isoforms did not. In another study (Kudoh, *et al.*, 1993), stable transfection of any of the four *WT1* splice forms in monkey kidney CV1 cells resulted in suppression of colony formation. The strongest and weakest effects were associated with the WT1+/- and WT1-/+ splice forms, respectively. It was also demonstrated that microinjection of the WT1+/+ and WT1+/- isoforms, but not WT1-/+ or WT1-/-, caused arrest in the G1 phase of the cell cycle. The apparent growth inhibitory activity of WT1 may involve a role of this protein in the regulation of DNA replication. Anant et al. (1994) reported that transfection of any of the four WT1 splice forms in COS-1 cells results in a decrease of replication of a plasmid containing the SV40 origin of replication. Deletion analysis indicated that the inhibitory activity required the zinc finger region and portions of the N-terminal 180 amino acids. WT1 proteins were shown to be able to form complexes with the 21 bp GC-rich repeat element of the SV40 origin. In contrast to binding of WT1 to transcriptional targets, it is the first three zinc fingers and not the fourth which mediate the interaction.

These tumor suppression and apoptosis studies are of considerable interest, however, their physiological relevance may be questionable. With respect to tumor suppression assays, it is possible that the growth suppressing genes which have been introduced are unrelated to the original tumorigenic genetic lesions and that a new differentiation program may be initiated by these genes (Marshall, 1991). Secondly, it is not clear that all tumorigenic pathways are reversible. Malignant progression involves a series of mutations whereby a normal cell is altered to show abnormal growth (Fearon & Vogelstein, 1990). It does not necessarily follow that reintroduction of one of these genes into a cancer cell will correct the phenotype since the cellular environment is radically altered compared with a normal cell.

These experimental systems involve expression of WT1 out of its developmental context, often in cells derived from tissues which never express WT1, it is not clear how to relate the observed activity with *in vivo* function The expression of *WT1* in tissue culture

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cells does not cause differentiation although it clearly performs this function *in vivo*. It is apparent, therefore, that WT1 has particular physiological requirements for being fully functional, and thus its *in vitro* effects on growth cannot be fully relevant to its native activity. Most experiments involve transfection of *WT1* and the resulting abnormally high levels of ectopic expression of this gene, normally under stringent regulation, can yield artifactual effects on cell growth. Some of the reports discussed above involve constitutive expression of WT1 and this may result in selection for mutated cells, since several studies have noted that cells in culture appear to select for extinction of WT1 expression (*e.g.* Haber *et al.*, 1992). Thus, in some cases and we cannot be sure that cells retain WT1 do not have mutations in WT1 or a other loci to permit growth. Finally, there is concern regarding the G401 cell line employed in some Wilms' tumor suppression studies. The origins of this cell line are not certain and one report has suggested that it is, in fact, derived from a rhabdoid tumor of the kidney and not a Wilms' tumor (Garvin, 1993).

Role of *WT1* in Other Cancers

Second Malignancies Associated with Wilms' tumor

Several tumor suppressor genes play roles in multiple cancers (see above). Since *WT1* is expressed in tissues other than the kidney, it is potentially involved in tumorigenesis in these tissues as well. The development of second malignant neoplasms (SMN) in bilateral Wilms' tumor survivors or in DDS or WAGR syndrome patients (that is, in patients with constitutionally heterozygous mutations) would provide evidence of the involvement of *WT1* in other tumor types. Observations of such second cancers must be interpreted with caution, however, because survivors of pediatric tumors in general are at an increased risk of developing second malignancies due to exposure to the mutagenicity of radiation therapy and chemotherapy. Hence it is often unclear whether SMN are caused by pleiotropic effects of the gene responsible for the first tumor or whether they result from the mutagenic effects of the therapeutic agents. In the case of retinoblastoma, however, it is evident that mutations in RB are associated with development of SMN. Patients treated with for bilateral or familial retinoblastoma are known to have an increased risk for second cancers (Meadows, *et al.*, 1985). Assuming that the basic principle of the two-hit cancer model applies to *WT1* (*i.e.* familial/bilateral patients inherit a mutated allele and lose the second in the tumor; sporadic cases involve two somatic mutations), an analogous higher incidence of SMN might be expected for familial/bilateral versus unilateral Wilms' tumor. To date, however, there has been no evidence of such an increase (Coppes, *et al.*, 1991b),

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although Wilms' tumor survivors in general have a significantly greater risk of developing another cancer than the general population (Breslow et al., 1989). Most of these SMN are myeloid leukemias or sarcomas arising in the radiation field. Such a spectrum is typical of that for patients receiving cytotoxic therapy. Some specific exceptional examples of SMN in Wilms' tumor survivors will be discussed below.

The clinical rarity of SMN unrelated to the mutagenic effects of the cancer therapy in Wilms' tumor survivors suggests that WT1 mutations may not be strongly predisposing to other types of malignancy. It should be emphasized, however, that the survival from Wilms' tumor was relatively rare until the 1960's and thus the numbers of patients reaching adulthood was significantly reduced. Current studies are hampered by a relatively short patient follow-up. It remains possible that adult cancers may be more commonly observed as Wilms' tumor survivors reach more advanced ages.

Mutational Analysis of WT1 in other Cancers

A number of studies have analysed the mutational status of WT1 in different tumor types. Most of these studies have focus on tumors affecting cell types which show WT1 expression. The detection of high levels of WT1 expression in the mesothelium (lining of inner body cavities) lead Park et al. (1993a) to analyse WT1 in tumors derived from these cells. The majority of malignant mesotheliomas are associated with a history of asbestos exposure. No WT1 mutations were found in over 70 such specimens (Park et al., 1993a; Kumar-Singh et al., 1997). Park et al., (1993a) detected a homozygous missense mutation in a single case of multicystic peritoneal mesothelioma, a rare condition which pathologists consider to reflect nonmalignant hyperplasia rather than neoplastic transformation (Kumar-Singh et al., 1997). The significance of this unique case of a WT1 mutation in a mesothelial neoplasm remains unclear. The mutation, which changes a serine to glycine at codon 273, has never been described in a Wilms' tumor. It appears to alter the transcriptional properties of WT1 in vitro such that the protein converted from a transcriptional repressor to an activator. Although it is evident that WT1 mutations are not involved in the malignant transformation of most mesotheliomas, the generalized overexpression of WT1 in mesotheliomas may be of use in the differential diagnosis of this tumor versus other histologically related pleural malignancies. There have been four reported cases of malignant mesothelioma in survivors of Wilms' tumor, and one of these did not involve asbestos exposure (Austin et al., 1985). It remains possible that WT1 may play a role in the etiology of rare mesotheliomas without asbestos exposure.

LOH at 11p in ovarian tumors and the high levels of WT1 expression seen in the developing ovary is suggestive of a role for the WT1 tumor suppressor gene in this malignancy. No WT1 mutations, however, were detect in a total of more than 40 epithelial ovarian tumors in two studies (Bruening *et al.*, 1993; Viel *et al.*, 1994). Most of these tumors were found to express WT1 mRNA by Northern blot analysis. These studies also failed to detect WT1 mutations other female reproductive tract cancers including small numbers of endometrial and fallopian tube tumors.

The potential role of WT1 in leukemia has been investigated. Early studies showed that WT1 is expressed in leukemic cell lines and in primitive leukemias although expression in normal hematopoiesis was not defined in these studies (Call *et al.*, 1990; Miwa *et al.*, 1992b; Miyagi *et al.*, 1993). Due to the high level of WT1 expression detected in the majority of leukemias, it was proposed that the WT1 gene transcript could be used as a 'pan-acute leukemia marker' (Menssen *et al.*, 1995). Recently, there have been a number of reports which demonstrate WT1 expression within the normal hematopoietic system (Inoue et al.., 1997; Mauer *et al.*, 1997; Baird & Simmons, 1997). Expression is restricted to CD34+ mononuclear bone marrow cells, which are early progenitors of the blood system. WT1 is downregulated the K562 and HL60 myeloid leukemia cells lines following induction of terminal differentiation (Phelan *et al.*, 1994; Seikya *et al.*, 1994), suggesting that the differentiation process in these cells may require the elimination of WT1 expression. To investigate the role of WT1 expression in myeloid leukemia cells, a WT1 antisense oligonucleotide was added to cell cultures (Algar *et al.*, 1996). The oligonucleotide, which spanned the translation initiation site, was effective in specifically diminishing WT1 protein levels, and in two of three cells lines caused marked reduction in cell proliferation accompanied by apoptotic cell death. The authors suggest that in some leukemia cells WT1 is necessary in the maintenance of viability. The reduction in WT1 levels was not accompanied by cellular differentiation. This contrasts with the association of reduced WT1 levels with the differentiation of K562 and HL60 cells and suggests that if WT1 is involved in this process, it alone is insufficient to induce differentiation. A final observation which would appear to question an essential role for WT1 in hematopoiesis is the absence of defects in this system in the *Wt1* knock-out mouse (Kriedberg *et al.*, 1993).

There is evidence that WT1 may behave as a tumor suppressor gene in some leukemias. Leukemias may be more common in relatives of Wilms' tumor patients (Hartley *et al.*, 1994a) and comprise 20% of the secondary malignancies in Wilms' tumor survivors (Moss *et al.*, 1989). A Wilms' tumor patient with constitutional deletion of 11p13 later developed a secondary leukemia in which the remaining WT1 allele was mutated. Analysis of WT1 in a series of acute leukemias revealed mutations in three of 20 acute myeloblastic

leukemias (AML) and one of three biphenotypic leukemias. The mutations were all predicted to result in production of truncated proteins. The mutations appeared heterozygous, although in one leukemia only the mutated allele was expressed indicating that the detection of the wild type allele at the DNA level was due to the contamination of normal cells. Another specimen had independent mutations affecting either WT1 allele and so there was no wild type WT1 in the these leukemic cells. The other two specimens showed expression of wild type WT1 mRNA. Since bone marrow and lymphocytes express very low levels of WT1 it is likely that this normal mRNA originated from the leukemic cells. This indicates that either the mutations are heterozygous or that the mutations are only present in a fraction of the cells and thus a late event in leukemogenesis. Since patients with constitutional WT1 mutations do not show strong predisposition to leukemias heterozygous mutation of WT1 must not be sufficient for the development of leukemia. In addition, the low frequency of leukemias in these patients suggests that mutations in other genes are necessary and are not common events. A second study failed to detect WT1 mutations in 33 cases of acute lymphocytic anemia or in 15 cases of AML. Although it appears that WT1 mutations may be important in some leukemias, it is evident that such mutations are not common and further studies will be needed to definitively identify the spectrum and frequency of such lesions.

It may seem difficult to rationalize how WT1 apparently behaves as a leukemic tumor suppressor despite the observation that abrogation of its expression is associated with loss of viability in leukemia cell lines. Such behaviour is in fact not unusual for tumor suppressor genes. For example, gene knockout in the mouse of both the Rb and WT1 tumor suppressor genes are characterized by wide wide spread apoptosis of the retinal and nephric anlage, respectively (Jacks, 1996; Kriedberg et al., 1993). Such an apparently contradictory behaviour is probably reflective of the temporal effects of the genes in tissue of different developmental stages.

WT1 has also been analysed in a small series of gonadal tumors known as sex cord stromal (SCS) tumors (Coppes et al., 1993b). These tumors are histologically mixed, containing cells resembling Sertoli cells, Leydig cells and granulosa cells, the normal counterparts of which are known to highly express WT1. None of 15 SCS tumors were found to harbor mutations in the zinc finger region of WT1, suggesting the WT1 is unlikely to be involved in the development of these tumors.

In conclusion, with the exception of certain leukemias, there is currently no molecular evidence that WT1 performs a critical tumor suppressing role in a cancer other than Wilms' tumor. This is consistent with the lack epidemiological data showing the association of other cancers with germline WT1 mutations in WAGR and DDS patients.

Oncogenic, Balanced Translocation of WT1 in an Abdominal Cancer

Desmoplastic small round cell tumor (DSRCT) is a rare, aggressive abdominal neoplasm, which generally affects young males. Gene rearrangements are a common feature of sarcomas such as DSRCT. Accordingly, cytogenetic studies identified the recurrent translocation, t(11, 22)(p13, q12) in this malignancy (Ladanyi & Gerald, 1994). Molecular characterization of these translocations revealed rearrangement of the *EWS* and *WT1* genes. The rearrangement results in fusion of the first seven exons of *EWS* to the final three exons of *WT1*, which encode zinc fingers 2 to 4 (Ladanyi & Gerald, 1994; Gerald *et al.*, 1995). Wild type *EWS* encodes an RNA binding protein, however, the RNA binding motif is absent from the chimeric transcript. *EWS* translocations with transcription factors also occur in several other malignancies including Ewing's sarcoma and clear cell sarcoma (reviewed in Ladanyi, 1995). In each case the result is the production of a chimeric protein fusing the amino terminal portion of EWS with the DNA binding domain of a transcription factor. The reciprocal transcripts are not detected. The amino terminus of EWS can behave as a transcriptional activation domain (Kim *et al.*, 1998a). Promoters of genes transcriptionally repressed by native WT1 may instead be activated by the chimeric protein (Karnieli *et al.*, 1996; Lee *et al.*, 1997). EWS-WT1[-KTS] displays ten-fold enhanced DNA binding to the cognate WT1 site *in vitro* (Kim *et al.*, 1998a). The expression of EWS-WT1[-KTS], but not EWS-WT1[+KTS], in NIH3T3 cells causes tumorigenic transformation. Cells show faster growth and higher saturation density, foci formation on monolayers, anchorage independent growth in soft agar, and induce tumor formation in nude mice (Kim *et al.*, 1998b). In summary, the oncogenic effect of the t(11;22) translocation is mediated by the EWS-WT1[-KTS] isoform, coding for a chimeric protein which acts in a gain of function manner. The [-KTS] isoform of WT1 has been shown to bind DNA at high affinity and since the EWS amino terminus has a well characterized transcriptional activation function when bound to DNA, it seems likely that the oncogenicity of the chimeric protein involves transactivation of target genes.

Familial Wilms' Tumor

LOH studies and genetic mapping clearly indicate the existence of multiple Wilms' tumor loci. Early studies suggested that familial Wilms' tumor is not linked to *WT1*. More recently, however, it has become apparent that a small number of familial cases indeed involve *WT1* mutations (Pelletier *et al.*, 1991b; Kaplinski *et al.*, 1996). A familial Wilms'

tumor gene has been mapped to chromosome 17 (Rahman *et al.*, 1996). This gene, designated *FWT1* (familial Wilms tumor gene) was originally found to segregate with Wilms' tumor susceptibility in a large French-Canadian pedigree. Subsequent mapping showed definitive segregation of *FWT1* with inherited Wilms' tumor in two pedigrees and possible linkage in six others (Rahman *et al.*, unpublished). Linkage to *FWT1* was ruled out in five families indicating the existence of at least one other susceptibility gene. Analysis of Wilms' tumors from one family showing linkage to *FWT1* showed a general absence of loss of heterozygosity in the vicinity of this locus (Rahman *et al.*, 1997). The single tumor with LOH surprisingly involved loss of the allele segregating with the disease. The authors interpret the absence of LOH to indicate that *FWT1* is not a tumor suppressor gene, but a dominant oncogene. The authors account for the loss of the mutated FWT1 allele in tumor by suggesting that the putative *FWT1* oncogene is involved in the initiation of malignancy but is dispensable in the maintenance and progression of the tumor. If the authors' interpretations are correct, *FWT1* would be a rare example of a cancer susceptibility syndrome caused by the inheritance of an activated oncogene. It should be noted that the reported LOH analysis is confined to tumors from a single family. It remains possible that the mutation in this family is exceptional. If the mutation behaves in a dominant-negative manner to abrogate function of the wild type allele it could appear to be an oncogene. There are examples of dominant mutations in well defined tumor suppressor genes which cause disruption of the normal allele, for example the *p53* tumor suppressor gene, was originally identified to be an oncogene (Lane & Benchimol, 1990). Only later did it become apparent that complete elimination of p53 resulted in malignancy, thus qualifying it as a tumor suppressor gene. There are also apparently oncogenic dominant mutations in *WT1* (see above), however, with respect to both *p53* and *WT1* the majority of mutations behave recessively as a loss of functional protein. LOH analysis of more families showing linkage to *FWT1* will be necessary to resolve the genetic nature of this locus.

Genomic Imprinting and Wilms' tumor

Mendellian genetics predicts that the phenotypes associated with inheritance of chromosomes is independent of the sex of the transmitting parent. A number of experiments, however, have demonstrated the lack of equivalence of paternal and maternal genomes (reviewed in Lalande, 1997) and resulted in models describing the epigenetic marking of regions of the genome, designated as genomic imprinting. The generation of gynogenetic and androgenetic zygotes (derived from two female and two male pronuclei, respectively) by pronuclear transplantation results in developmental failure. Gynogenetic

embryos have grossly normal embryogenesis but die due to malformations of the extraembryonic structures. Androgenetic embryos, conversely, show normal extraembryonic tissue but malformations of the embryo. The generation of mice with uniparental disomy (inheriting both copies of a chromosome segment from a single parent) for many regions of the genome resulted in the identification of ten region which show imprinting effects (i.e. developmental defects in uniparental disomic animals). A number of imprinted genes have been characterized in both the mouse and human and they appear to be clustered in chromosomal subregions, including those at chromosomes 11p15 and 15q11-q13 which are associated with human diseases.

A number of lines of evidence suggest a role for genomic imprinting at chromosome 11p15 in the development of Wilms' tumor. Complete discussion of this complex and fascinating aspect of Wilms' tumor genetics is beyond the scope of this thesis. The following section, however, will review salient aspects of this field and attempt to integrate this information into a more general discussion of the genetics of Wilms' tumor.

As we have seen, Beckwith-Weidemann syndrome (BWS) is an overgrowth syndrome, characterized by variable expressivity of a number of malformations and predisposition to Wilms' tumor and other malignancies. Most cases are sporadic although familial cases do exist, showing linkage to 11p15.5. The genetics of BWS, associated with paternal duplications of 11p15 (either paternal trisomy or isodisomy), maternal 11p15 translocations and increased penetrance of maternally transmitted mutant alleles, is characteristic of a role for imprinted genes (reviewed in Reik & Maher, 1997). In addition changes in the expression pattern of imprinted genes are commonly observed in BWS patients. A number of imprinted genes clustered at 11p15.5 have been implicated in BWS, namely insulin-like growth factor 2 (IGF2), H19, cyclin-dependant kinase inhibitor 1C (CDKN1C, p57^{KIP2}) and potassium channel A9 (KCNA9).

IGF2 encodes a paternally expressed fetal growth factor. IGF2 shows aberrant biallelic expression (or doubled paternal expression in cases with paternal uniparental disomy) in 80% of BWS patients. In addition four of six children with somatic overgrowth but no other anomalies showed biallelic IGF2 expression (Morrison et al., 1996). Transgenic mice which overexpress Igf2 develop most of the symptoms of BWS (Sun et al., 1997). Although no tumors are observed observed in these transgenic mice, a role for the overexpression of this mitogen in the development of Wilms' tumors cannot be discounted since embryonic kidney tumors are exceedingly rare in the mouse. In Wilms' tumorigenesis in humans there are a number of mechanisms which result in IGF2 overexpression. Maternal LOH at 11p15 occurs in 40% of Wilms' tumors and the resulting paternal disomy at 11p15 doubles the dosage of expressed IGF2 alleles (reviewed in

Reeve, 1996). Additionally, the maternal allele of *IGF2* is aberrantly expressed in 74% of Wilms' tumors, a phenomenon which has been designated loss of imprinting (LOI) (Rainer *et al.*, 1993; Ogawa *et al.*, 1993). LOI is detectable in the normal kidney cells adjacent to Wilms' tumors which show biallelic *IGF2* expression (Okamoto *et al.*, 1997). These observations suggest that increased expression of *IGF2* may increase the proliferation of precursor cells to Wilms' tumors potentiating the accumulation of tumorigenic mutations. Correspondingly, epigenetic changes affecting *IGF2* occur as an early event in Wilms' tumorigenesis (Okamoto *et al.*, 1997).

H19 is a maternally expressed gene adjacent to *IGF2* which produces an mRNA transcript that is not translated. The *H19* and *IGF2* promoters are controlled by DNA methylation and enhancer competition such that on either chromosome the genes show reciprocal expression (*i.e.* only H19 is expressed from the maternal chromosome and only *IGF2* is expressed from the paternal chromosome). Methylation changes at 11p15 and the associated biallelic *IGF2* expression and absence of *H19* expression is observed in Wilms' tumor (Taniguchi *et al.*, 1995) and in BWS (reviewed in Reik & Maher, 1997). As with *IGF2*, epigenetic changes affecting *H19* occur as an early event in Wilms' tumorigenesis (Okamoto *et al.*, 1997; Cui *et al.*, 1997). The direct role of the *H19* in regulating *IGF2* expression is demonstrated by the biallelic *IGF2* expression and somatic overgrowth in mice with germline disruption of the maternal *H19* gene (Leighton *et al.*, 1995). This supports the role of relaxation of imprinting in BWS.

CDKN1C codes for a maternally expressed cyclin-dependent kinase inhibitor which is a negative regulator of cell proliferation. Homozygous-null mutation of *Cdkn1c* in the mouse results in a number of anomalies which are characteristic of BWS including abdominal wall defects and adrenal cytomegaly (Zhang *et al.*, 1997). The sum of the anomalies associated with *Igf2* overexpression and with *Cdkn1c* accounts for most of the features observed in BWS, although no tumors develop in the mouse. About 5% of BWS patients have *CDKN1C* mutations (Hatada *et al.*, 1996; Lee *et al.*, 1997). The status of *IGF2* expression in these patients is unknown. Extensive analysis of *CDKN1C* in Wilms' tumors failed to detect any mutations in this gene (Orlow *et al.*, 1996; O'Keefe *et al.*, 1997).

KCNA9 is paternally expressed and encodes a potassium channel. Mutations in this gene are associated with cardiac arrythmia and congenital deafness (reviewed in Mannens & Wilde, 1997). Most maternal translocations of 11p in BWS disrupt *KCNA9*, however, BWS patients develop neither arrythmia nor deafness, thus translocations of this gene produce completely different phenotypes than point mutations. Multiple transcripts are produced from the *KCNA9* locus including some which do not appear to be translated.

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Disruptions of this gene are associated with biallelic expression of *IGF2*, thus it is possible that transcription from this locus can regulate *IGF2* expression, perhaps by a similar enhancer competition mechanism as is observed with *H19*.

Biallelic expression of *IGF2* is the most common molecular alteration associated with BWS and overexpression of *Igf2* produces most of the BWS phenotype in a dosage-dependent manner in the mouse. Reik and Maher (1997) have suggested that the molecular pathology of BWS is likely due to heterogeneous mutations but that they all act through similar pathways to produce overexpression of a single principle phenotypic effector (*i.e.* *IGF2*).In the context of this model, it is unlikely that the 11p15 locus encodes a tumor suppressor gene. Instead growth disturbances associated with increased *IGF2* expression enhance the possibility of the accumulation of tumorigenic changes at other loci. Consistent with the role of *IGF2* overexpression in Wilms' tumorigenesis is the observation that the Simpson-Golabi-Behmel syndrome (SGBS) is predisposing to Wilms' tumor. This syndrome has considerable clinical overlap with BWS and the gene mutated in SGBS,*GPC3*, may encode a protein which sequesters IGF2 extracellularly. Mutations in *GPC3* may result in increased effective levels of IGF2. The phenotypes of both of these diseases may, therefore, be due to excess IGF2. The phenotypic heterogeneity of these diseases may be due to relative IGF2 levels.

The relationship of the complex alterations in imprinting at chromosome 11p15.5 and the role of WT1 in the development of Wilms' tumor is unclear. In a significant proportion of tumors with WT1 mutations there is LOH that includes both 11p13 and 11p15 and a subset of these tumors have LOH exclusively at 11p15. This may indicate a cooperative role of WT1 and loci at 11p15 in tumorigenesis. Alternatively, the LOH at both loci may simply be due to their physical proximity and synteny on the short arm of chromosome 11. The observation that allele loss at 11p nearly always involves the maternal allele in Wilms' tumors does not necessarily support the existence of a cooperative role for *WT1* mutations and imprinting changes since*WT1* mutations are only observed in a subset of tumors and in addition a large proportion of the mutations are present constitutionally on the paternal chromosome (possible due to higher mutation rates during spermatogenesis). A clear demonstration of the association of WT1 mutations with imprinting changes will require the identification of both mutations and epigenetic changes in the same tumor.

CHAPTER 2

This chapter describes a thorough mutational analysis of the entire *WT1* coding region and untranslated regions in a large series of sporadic Wilms' tumors. Previous to this work there was considerable genetic evidence linking the *WT1* gene to predisposition to Wilms' tumor although there were few descriptions of intragenic *WT1* mutations. It was also evident that Wilms' tumor could involve other loci, particularly when associated with the familial form of the disease and with the Beckwith-Weidemann syndrome. The extent of involvement of *WT1* in the sporadic form of the disease was unknown. Wilms' tumors show extensive histological and clinical heterogeneity. It was of interest to determine whether *WT1* mutations were associated with any specific histological subtypes or whether they had prognostic significance. The nature of mutations was also of interest in potentially giving clues about the function of the WT1 protein. Mutations could indicate the existence domains or residues of critical importance. In addition determining whether certain mutations were heterozygous versus homozygous suggests a potential of a dominant or dominant-negative mode of behaviour for such mutations.

Fine Structure Analysis of the WT1 Gene in Sporadic Wilms' Tumors

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ABSTRACT

Molecular genetic studies indicate that the etiology of Wilms' tumor (WT) is complex involving at least three loci. Germline mutations in the tumor suppressor gene, WT1, have been documented in children with WTs and urogenital developmental anomalies. Sporadic tumors constitute the majority (>90%) of WT cases and previous molecular analyses of the WT1 gene have focused only on the DNA binding domain. Using the single strand conformational polymorphism (SSCP) assay, we analyzed the structural integrity of the entire WT1 gene in 98 sporadic WTs. By PCR-SSCP we find that mutations in the WT1 gene are rare, occurring in only six tumors analyzed. In one sample, two independent intragenic mutations inactivated both WT1 alleles, providing a singular example of two different somatic alterations restricted to the WT1 gene. This case is consistent with the existence of only one tumor suppressor gene at 11p13 involved in the pathogenesis of WTs. Our data, together with the previously ascertained occurrence of large deletions/insertions in WT1, define the frequency at which the WT1 gene is altered in sporadic tumors.

INTRODUCTION

Wilms' tumor (WT), an embryonal malignancy of the kidney, occurs most frequently in children under the age of 5 years, affecting ~1 in 10,000 individuals (1). A genetic predisposition is observed in 5-10% of cases. The WAGR syndrome [an association of Wilms' tumor, aniridia (absence or malformation of the iris), genitourinary malformations, and mental retardation (2)] is associated with constitutional deletions within chromosome 11 band p13 (3). These cytogenetic abberations define a cluster of closely linked genes, that control development of the kidney, iris, urogenital tract, and brain. Molecular genetic analysis of 11p13 interstitial deletions in patients with WAGR syndrome and sporadic WTs, led to the identification of a WT suppressor gene, WT1 (4,5).

The WT1 gene spans ~ 50 kbp and contains 10 coding exons, two of which are alternatively spliced to yield 4 mRNA species (for a review, see ref. 6). The protein isoforms have several characteristics of transcription factors, including nuclear localization (7), a proline/glutamine rich NH_2-terminus, and a nucleic acid binding domain consisting of four zinc fingers of the Cys_2-His_2 variety (4,5). WT1 mRNA expression is predominant in components of the urogenital system, spleen, and mesothelial cells (7, 8). Mice in which the WT1 gene is homozygously deleted fail to activate the developmental program of the urogenital system, consistent with a role for WT1 in formation of this system (9). The

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WT1 gene product is thought to regulate gene expression during mesenchymal to epithelial cell differentiation (8).

Reports of allelic loss restricted to 11p15 in sporadic WTs, as well as lack of linkage to 11p in a few familial cases of WT, suggest the existence of additional predisposing loci, playing initiating roles in tumor development (see ref. 6 for a review). Additionally, we have recently reported that p53 mutations are associated with progression to anaplasia in WTs, a histological subtype associated with poor prognosis (10). These observations suggest that the genetics of WTs is more complex than originally proposed and may provide a multi-step model for tumorigenesis more broadly applicable to adult cancers than Knudson's two-hit mutational model (11). Althought intragenic alterations and point mutations in a few sporadic and hereditary WTs have provided molecular evidence that WT1 is a tumor suppressor locus (12-20), the frequency with which WT1 is altered in sporadic WTs is not known since a fine structural analysis of the entire gene has thus far not been reported. We designed twenty-one oligonucleotide primer pairs to the WT1 gene to enable us to amplify the coding and (5' and 3') untranslated regions (UTRs). Using the single strand conformational polymorphism (SSCP) assay (21), we scanned the WT1 gene in 98 sporadic tumors for point mutations, small deletions, and rearrangements. We find somatic mutations in the DNA binding domain of WT1 in 6% of tumors analyzed. Three of these tumors have also lost the wild-type allele; whereas a fourth has inactivated both WT1 alleles by two independent intragenic mutational events. Our results are: 1) consistent with the involvement of only one 11p13 tumor suppressor locus required for WT etiology, 2) identify a mutational "hotspot" within the WT1 gene, and 3) define the frequency at which the WT1 gene is altered in sporadic WTs.

MATERIALS AND METHODS

Tumors and DNA Preparation. DNA analyzed in this study was prepared from ninety-eight tumors. All samples were single Wilms' tumors from unilateral sporadic patients. None of these patients showed any stigmata of the WAGR, Beckwith-Wiedemann, or Denys-Drash syndrome (6). The family history in all instances was negative for WTs. The wild-type DNA used as a negative control was from blood of unaffected normal males. Constitutional DNA was prepared from blood obtained after informed consent from the patients. Isolation of constitutional DNA and tumor DNA were performed as previously described (14). Preparation of plasmid DNA, restriction enzyme digestion, agarose gel electrophoresis of DNA, DNA ligation, and bacterial transformation were carried out using standard methods (ref. 22 and references therein).

PCR-SSCP Analysis. To detect possible point mutations, small deletions, or rearrangements in genomic DNA from tumors, sequences from the ten exons of WT1 were amplified by the polymerase chain reaction (PCR) and analyzed by SSCP (21). Twenty-one pairs of oligonucleotides were designed to enable coverage of the WT1 coding region, as well as the 5' and 3' UTRs (Fig. 1). Prior to the PCR, one oligonucleotide was radiolabeled with T4 polynucleotide kinase and $[\gamma\text{-}^{32}P]ATP$ (NEN). The PCR was performed using a DNA thermocycler (MJ Research) with 50 ng of genomic DNA in a total volume of 20 ul. Thirty-five cycles of amplification were performed; each consisting of 1 min at 94°C, 1 min at the optimally determined annealing temperature for each oligonucleotide pair, and 1 min at 72°C. DMSO was added to a final concentration of 10% when amplifying segments from the first WT1 exon. After the PCRs, aliquots of 2 ul were mixed with 8 ul of sample buffer [95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF] and boiled for 5 min. Samples (2 ul) were loaded onto 8% polyacrylamide (50:1, acrylamide:bisacrylamide) gels, and electrophoresis was performed in 1 x TBE (90 mM Tris/90 mM boric acid/ 2.5 mM EDTA) buffer at 30W in the cold room. The gels were dried on filter paper and exposed to Kodak X-OMAT film at -70°C for 12-24 hr with an intensifying screen.

Exons whose PCR products demonstrated mobility shifts by SSCP analysis were amplified with unlabelled oligonucleotides, purified on low-temperature melting agarose gels, and cloned into pKS II+ (Stratagene). Recombinant clones were sequenced by the chain termination method using Sequenase polymerase (U.S. Biochemical) (23). No less than 6 clones were sequenced to ensure against Taq polymerase artifacts and to determine the sequence of both alleles.

RESULTS

To determine the frequency of small deletions, insertions, or rearrangements within the WT1 gene in sporadic WTs, we undertook a structural analysis of the entire gene in 98 tumors. The WT1 coding region, as well as the (5' and 3') UTRs, were scanned for mutations by PCR-SSCP analysis of DNA isolated from sporadic tumors. The 5' end of the human WT1 gene has been recently defined and four sites of transcription initiation cluster within a 32 bp region, ~390 bp upstream from the initiator ATG (24). Our oligonucleotides span 3017 bp of predicted cDNA sequence, with 675 nucleotides of 5' UTR being contributed from exon 1. This essentially covers the entire gene, with the exception of introns and 298 nucleotides encompassing the polymorphic $(CA)_n$ repeat within the 3' UTR (Fig. 1). The WT1 mRNA transcript migrates as a 3.1 kb species when analyzed by Northern blotting (4).

PCR-SSCP analysis of each product was performed twice - alternating the identity of the radiolabeled oligonucleotide primer. Six tumors were thus identified with intragenic WT1 mutations within the DNA-binding domain (Fig. 2A-C). None of the other WTs analyzed in this study showed evidence for WT1 mutations, either within the coding or (5' and 3') UTRs. Two WTs demonstrated homozygous mobility shifts within WT1 zinc finger III (Fig. 2A). One tumor harbors a nonsense mutation (D.J.#11), whereas the other has suffered a deletion/insertion (M.F.#88), both of which are predicted to result in the production of truncated WT1 polypeptides. This alteration, coupled with loss of the wild-type allele, inactivates the WT1 locus. Both mutations are somatic since the mobility shifts observed with tumor DNA are not present in constitutional DNA isolated from the affected individuals (Fig. 2A). Three of the sporadic tumors analyzed harbor mutations within zinc finger II (Fig. 2B). Two of these samples demonstrated apparent heterozygous changes within exon 8 (B.M.#7 and A.H.#20; Fig. 2B), whereas the mutation detected in tumor B.T.#53 is homozygous, due to the absence of a normal allele conformer (compare the first and last lanes in Fig. 2B). In addition, six clones containing PCR products from tumor B.T.#53 yielded only sequence of the mutant allele (data not shown). Two samples, B.M.#7 and B.T.#53, suffered the same nonsense mutation (a C to T transition); whereas the third sample (A.H.#20) contains a deletion/insertion resulting in a frameshift mutation. Sample K.K.#33 demonstrated a mobility shift by PCR-SSCP analysis within zinc finger I (Fig. 2C). This tumor has a missense mutation predicted to convert a serine to a tyrosine (Fig. 2C). All of these alterations are somatic as none of the constitutional DNA from the affected individuals demonstrated mobility shifts by PCR-SSCP analysis.

In addition to a mutation in exon 8 (Fig. 2B), tumor B.M.#7 demonstrated a mobility shift when exon 3 was analyzed by PCR-SSCP (Fig. 3A). The WT1 gene has

suffered a 7 nucleotide insertion in exon 3, predicted to cause translational frameshifting. To determine if the alterations within exon 3 and 8 of tumor B.M.#7 were on different haplotypes, we isolated RNA from this tumor specimen. Complementary DNA was prepared and used to amplify the WT1 coding region between exons 2 and 9. Clones containing these amplified products were isolated and characterized by sequencing (23). Complementary DNA products harboring exon 8 mutations did not have an exon 3 insertion and visa versa. In this analysis, 7 clones demonstrated exclusively an exon 8 alteration, and 3 clones only had an exon 3 mutation. Five clones had a wild-type configuration, consistent with contamination of the tumor by normal tissue.

DISCUSSION

Mutations in the WT1 gene from a small set of WTs have provided molecular evidence that this gene plays an initiating role in WT pathogenesis. Southern blotting analysis of genomic DNA from large collections of sporadic WTs reveal WT1 rearrangements or deletions in ~10% of tumors (5, 17, 25, 26). Northern blotting analysis of WTs expressing WT1 mRNA have failed to reveal deletions or rearrangements (12, 27, 28). Previous studies aimed at identifying intragenic WT1 mutations, have restricted their analysis to the WT1 zinc fingers, which constitute <15% of the entire gene (19, 20). In these studies, four sporadic tumors out of 67, analyzed by chemical cleavage mismatch or PCR-SSCP, were identified as harboring WT1 mutations (19, 20).

We have analyzed the fine structure of the complete WT1 gene in 98 unilateral sporadic WTs. This was a comprehensive study aimed at identifying the frequency and sites of mutations within the WT1 gene in sporadic WTs. The most common area altered was found to be the zinc fingers - with lesions occurring in 6% of the tumors analyzed. Mutations in the WT1 DNA binding domain have been reported for a small number of hereditary WTs (14, 16, 18). The last three zinc fingers of WT1 are similar to the zinc fingers of some transcriptional regulators named early growth response gene 1 (EGR1), EGR2, Sp1, Krox-20, and Krüppel, all of which bind to guanidine-rich DNA sequences (4, 5). The majority of the zinc finger mutations described herein are predicted to cause early termination of translation, disrupting the nucleic acid binding domain and causing loss of function. Tumor K.K.#33 harbors a missense mutation within zinc finger I converting ^{338}Ser to Tyr. This change occurs adjacent a histidine residue involved in Zn^{++} chelation and is likely to affect DNA binding. Confirmation of this awaits the identification of WT1 binding sites which can accommodate a function for the first WT1 zinc finger in DNA recognition. Studies of WT1 DNA binding with the EGR-1 consensus motif ($^{5'}$GCGGGGGCG$^{3'}$) only require zinc fingers II, III, and IV and are inadequate to determine the contribution from zinc finger I on DNA sequence recognition (29).

The observation that some tumors have undergone reduction to homozygosity for a mutated allele (e.g.- D.J.#11 and M.F.#88 [Fig.2A]) whereas others are apparently heterozygous (e.g.-A.H.#20 [Fig. 2B]) raises an interesting issue. Although we cannot exclude the possibility of contamination by normal tissue in some of our specimens, different WT1 mutations may produce macromolecules with quite varying biological properties (e.g.- altered mRNA stability, protein half-life, etc...), as documented for p53 mutations (for a review, see ref. 30). Thus, some tumors with WT1 mutations, may produce an altered WT1 product capable of behaving in a dominant-negative fashion (for example, because of increased protein half-life), negating the requirement to lose the

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second allele. Whereas other WT1 mutations may result in a loss of WT1 function (e.g. by destabilizing protein or mRNA half-life). These tumors may then require somatic loss of the remaining wild-type allele to attain complete inactivation of WT1. This hypothesis is currently under investigation.

Our results, together with previous studies documenting large scale insertions, deletions, or rearrangements within the WT1 gene (5, 17, 25, 26), suggest that ~6-15% of sporadic WTs have WT1 mutations. We have not detected WT1 mutations in the majority of sporadic cases analyzed, despite having scanned the entire gene. We cannot exclude the possibility of a mutational "hotspot" within WT1 yielding a false negative result by PCR-SSCP analysis. However, we do not favor such an argument given that each amplified strand of the WT1 gene was analyzed individually, our PCR products are within the optimal size range for mutational detection by SSCP analysis (31), and the high mutational detection rate of PCR-SSCP (31). Likewise, a common intron mutation affecting RNA processing would not be detectable by our PCR-SSCP assay but should have been previously identified by Northern blot analysis of the WT1 transcript in WTs (12, 27, 28). Direct sequencing of the WT1 gene from several sporadic tumors is required to definitively address these issues. Repression of WT1 gene expression in sporadic tumors by epigenetic modifications, such as DNA methylation, is not a method by which WT1 expression is extinguished since both the maternal and paternal alleles are expressed at approximately equal levels in WTs (32).

What defines the subclass of WTs which harbor WT1 mutations? Beckwith et al. (33) have proposed a classification scheme for WTs based on the distribution and histology of the precursor nephrogenic rest lesion. Intralobar nephrogenic rests are observed in association with hereditary WTs and genetic lesions at 11p13; whereas perilobar nephrogenic rests are found in association with possible genetic defects at 11p15. The prevalence of intralobar nephrogenic rests in unilateral WTs is ~17% (33). Tumors B.M.#7, D.J.#11, B.T.#53 contain regions of intralobar nephrogenic rests. Not all nephrogenic rests harbor WT1 mutations since other samples from our collection containing such rests did not demonstrate SSCP mobility shifts. In addition, Park et al. (34) analyzed 19 WTs containing foci of nephrogenic rests and observed WT1 mutations in only 2 specimens.

Approximately 50% of sporadic WTs show allelic loss restricted to 11p15, whereas both 11p13 and 11p15 show allelic loss in ~30% of WTs (6). This suggests a role for both 11p13 and 11p15 in pathogenesis of WTs and it is conceivable that either locus at 11p13 or 11p15 can predispose to tumorigenesis, with the 11p15 gene altered in the majority of sporadic WTs. Allelic loss or deletions at 11p13 in tumors harboring WT1 mutations has

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always been interpreted as a second "hit" inactivating the remaining copy of WT1 (15, 17-20). An alternative interpretation is that these deletions represent a requirement for unmasking a recessive lesion at a nearby second tumor suppressor gene on 11p13 necessary for tumor progression. Support for the existence of such a locus comes from LOH studies in bladder cancers (35) and a report of a metastasis repressor gene(s) between 11p11 and 11p13 in prostate cancers (36). However, tumor B.M.#7 provides a unique example of a WT in which both copies of the WT1 gene have been inactivated by intragenic mutations. This specimen has suffered two intragenic mutations, one on each 11p haplotype and both restricted to the WT1 gene. It is unlikely this tumor arose from two independent clonal events, each suffering a WT1 lesion, given the rarity of Wilms' tumors and relatively low frequency of WT1 mutations in sporadic tumors. The molecular genetics of tumor progression for this specimen are consistent with the existence of only one 11p13 tumor suppressor gene involved in pathogenesis of WTs.

ACKNOWLEDGMENTS

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FIGURE LEGENDS

Figure 1. Schematic Diagram of the Genomic Organization of WT1 and Oligonucleotide Primer Pairs used to Amplify the Coding Region and (5' and 3') UTRs. The 10 exons of the WT1 gene are represented by boxes. The open boxes represent the alternatively spliced exons, whereas the dotted boxes symbolize the 4 zinc fingers. The remaining WT1 exons are shown as blackened boxes. The ATG and TGA codons are in italics and indicate where the start and stop of translation occur. Each exon is numbered and the position of the $(CA)_n$ polymorphic repeat within the 3' UTR is indicated. The oligonucleotide primer pair used to amplify specific regions of WT1 is represented by a bi-directional arrow under the exon segment they amplify. The primer names, their sequence, annealing temperature, and size of the PCR product generated are tabulated below the WT1 gene. * These PCRs were supplemented with DMSO to a final concentration of 10%.

Figure 2. PCR-SSCP analysis of the WT1 Zinc Finger Domains. The PCR products obtained after amplification from DNA isolated from blood (B) or tumor (T) of each affected individual was analyzed in parallel on a 8% non-denaturing polyacrylamide gel as describe in the Materials and Methods. The PCR products from an unaffected individual was included as an internal standard for the normal allele. The mobility shifts are indicated by arrowheads. Open circles represent the normal alleles. A schematic diagram of the WT1 region analyzed, as well as the nucleotide sequence of the mutations is shown to the right of the SSCP analysis. The asterisks, cross, and small cap are used to delineated the mutated amino acid and tumor sample affected. A) Mutational analysis of zinc finger III in tumors D.J.#11 and M.F.#88. WT1 exon 9 was amplified with INT-1 and INT-2, with INT-1 radiolabeled for the analysis shown. B) Mutational analysis of zinc finger II in tumors B.M.#7, B.T.#53, and A.H.#20. Oligonucleotide INT-3 was radiolabeled when amplifying from tumors B.M.#7 and B.T.#53; whereas oligonucleotide INT-4 was kinased for the analysis of tumor A.H.#20. C) SSCP mobility shift in zinc finger I of tumor K.K.#33. For the amplification, oligonucleotides INT-5 and INT-6 were used, with INT-5 radiolabeled to allow detection of the PCR product. The PCR product from tumor DNA (T) is compared to the product from DNA of a normal (N) individual. Subsequent analysis of tumor versus blood DNA from this individual showed that this mutation was not constitutional (data not shown).
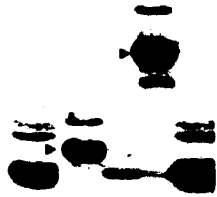
Figure 3. Mutational Analysis of Tumor B.M.#7. A) PCR-SSCP of exon 3 from tumor B.M.#7 revealed a mobility shift indicated by an arrowhead. For the analysis presented, oligonucleotide INT-15 was radiolabeled. DNA for the amplification reaction was isolated

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from: N, a normal individual; B, blood; or T, tumor from the affected child. B) Predicted genomic organization at the WT1 locus of tumor B.M.#7. RNA was isolated and converted to cDNA as previously described (16). For the reverse transcription and amplification reactions, the following oligonucleotides were used: Oligonucleotide 103 ($5'$GTCCCCGAGGGAGACCCC$^{3'}$) which anneals to exon 10 was used to prime the reverse transcription reaction. Oligonucleotide 301 ($5'$TCTGACAACTTGGCCACCGAC$^{3'}$) which anneals to exon 9, and oligonucleotide DHJP.025 ($5'$CCTTCGACGGGACGCCC$^{3'}$) which targets exon 2, was used to amplify the WT1 cDNA between exons 2 to 9. Following cloning of the PCR products, 14 clones were sequenced with oligonucleotides 301 and DHJP.025. Three clones showed a mutation in exon 3 and a normal exon 8 sequence. Seven clones had alterations restricted to exon 8 (N. Bardeesy and J. Pelletier, unpublished data). The deduced genomic organization of the WT1 gene in tumor B.M.#7 is diagrammed with asterisks representing mutations.
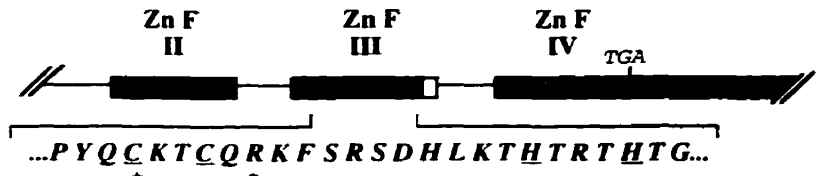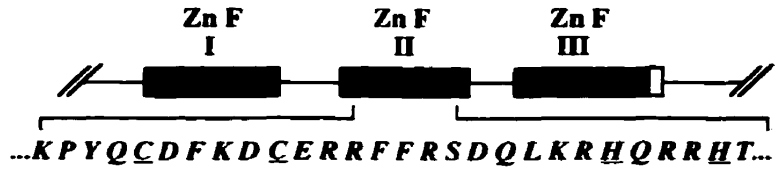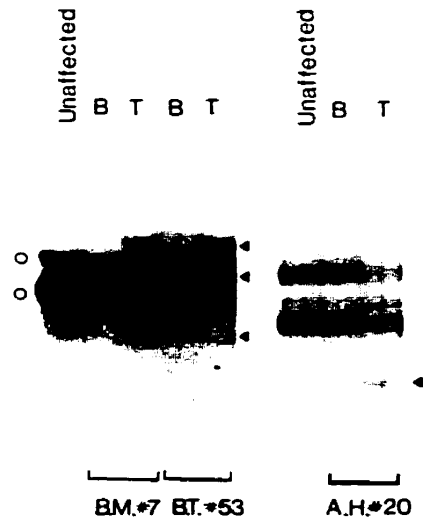
Fig. I

Primer Pair	Primer Name	Sequence	Annealing Temperature [°C]	Exon Scanned	PCR Product Generated (bp)
A*	HWT-2 / HWT-2i	5'GCCGCTCCATTCACTCAG3' / 5'CCCACACTGGCGAAGGCC3'	55	Exon 1 (5' UTR)	206
B*	HWT-3 / HWT-3i	5'CTGAGTGAATGGAGCGGC3' / 5'GGGTGAATGAGTAGGTGG3'	55	Exon 1 (5' UTR)	204
C*	HUTR-2S / HUTR-2A	5'GGGGCTCTCCGCAACCCG3' / 5'GCTCCGGGACACACGTGGG3'	55	Exon 1 (5' UTR)	167
D*	UTR-1 / HWT-4	5'CAGGACCCGGCTTCCACG3' / 5'CCCATTTGCTGAGGCTCAGACC3'	55	Exon 1 (5' UTR)	206
E*	EX-1(I) / EX-1(II)	5'CGGGTCTGAGCCTCAGC3' / 5'ACCCGTAAGCCGAAGCG3'	54	Exon 1	167
F*	EX-1(III) / EX-1(IV)	5'GGGCGCCGGTGCTGGAC3' / 5'TCAGGCACTGCTCCTCG3'	54	Exon 1	168
G*	EX-1(V) / A1	5'CAGGAGCCGAGCTGGGG3' / 5'CTCCCCGGCCTACTTAC3'	54	Exon 1	231
H	A2 / S2	5'CCGTCTTGCGAGAGCACC3' / 5'CTAATTTGCTGTGGGTTAGG3'	58	Exon 2	262
I	INT-16 / INT-15	5'CAGCTGTCTTCGGTTC3' / 5'GGGTCTGCGTCTCGG3'	56	Exon 3	157
J	INT-14A / 106AR	5'CAGTTGTGTATATTTGTGG3' / 5'CCCTTTAAGGTGGCTCC3'	55	Exon 4	130
K	INT-11 / INT-12	5'CGCCATTTGCTTTGCC3' / 5'CCACTCCCCACCTCTTC3'	55	Exon 5	116
L	INT-10 / INT-9	5'CCTTTTTCCCTTCTTTG3' / 5'CCTTCCGCTGGGGC3'	56	Exon 6	153
M	INT-6 / INT-5	5'GCTTAAAGCCTCCCTTC3' / 5'CTTGAACCATGTTTGCCC3'	54	Exon 7	231
N	INT-4 / INT-3	5'GAGATCCCCTTTTCCAG3' / 5'GTGTCGACGGTCGTTAC3'	56	Exon 8	176
O	INT-2 / INT-1	5'CTCACTGTGCCCACATTG3' / 5'CAATTTCATTCCACAATAG3'	58	Exon 9	211
P	INT-7 / 103	5'CCTGTCTCTTTGTTGC3' / 5'GTCCCCGAGGGGAGACCCC3'	55	Exon 10	187
Q	102 / RG01	5'CTTCAGCTGTCGGTGGCCA3' / 5'GTGAGGAGGAGTGGAGAG3'	55	Exon 10 (3' UTR)	196
R	RG03 / MG01	5'CCCAGGCAGCACAGTGTG3' / 5'GGACATGATCAGCTATG3'	55	Exon 10 (3' UTR)	289
S	MG02 / RG11	5'CTACTGTAAGAAGAGCC3' / 5'CTTCAGCTGCTTGAAATGCATG3'	55	Exon 10 (3' UTR)	322
T	RG06 / DHJP.024	5'AGCAGGATGTAGTTCTG3' / 5'CCATTTGTGCAAGGAGG3'	55	Exon 10 (3' UTR)	252
U	401(S) / WB-1	5'CCGTTGCTTGAAATTACTGTG3' / 5'GAATAGACTTTAATTGAGAGC3'	55	Exon 10 (3' UTR)	177

2Ⓐ

B T B T Unaffected

```
                              ZnF        ZnF        ZnF
                               II         III        IV    TGA
        ═╪═──────████████──████──□──████████──═╪═

        └─────────────────────────────────────────┘
        ...P Y Q C K T C Q R K F S R S D H L K T H T R T H T G...
                  +       *
```

D.J. #11 M.F #88

```
* D.J. #11    ...CAGCGAAAGTTC...
              ...Q   R   K   F...
                        ↓
              ...CAGTGAAAGTTC...
              ...Q    ***

+ M.F.#88     ...CAGTGTAAAACTTGT...
              ...Q   C   K   T   C...
                         ↓
              ...CAGCGAACTTGTAAACTGTAACTTGT...
              ...Q   R   T   C   K   L   ***
```

2®

ZnF I   ZnF II   ZnF III

...K P Y Q C D F K D C E R R F F R S D Q L K R H Q R R H T...

^
+
*

Unaffected   B T B T   Unaffected   B T

^ B.T.#53   ...TGTGAACGAAGGTTT...
+ B.M.#7   ...C   E   R   R   F...

↓

...TGTGAATGAAGGTTT...
...C   E   ***

* A.H.#20   ...GAACGAAGGTTTTCTCGT...
...E   R   R   F   F   R...

↓   36 nt

...GAACACCTCGTT.........GTGTGA...
...E   H   Q   V.....G ***

B.M.#7 B.T.#53   A.H.#20

2c

N T

Zn F
I

Zn F
II

...$\underline{C}$ A Y P $\underline{C}$ N K R Y F K L S $\underline{H}$ L Q M $\underline{H}$...
*
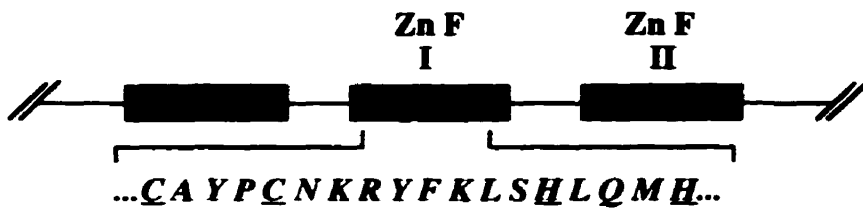
KK#33

* K.K.#33   ...AAGCTGT$\underline{C}$CCACTTA...
...K   L   S $\underline{H}$ L...

↓

...AAGCTGT$\underline{A}$CCACTTA..
...K   L   Y   H   L...

3 A.

N B T

Exon 3   Exon 4   Exon 5

...Q Q Y S V P P P V...

B.M.#7

* B.M.#7   ...TACTCGGTGCCG .....AGCAGTGAC...
            ...Y   S   V   P ...... S   S   D...

                        77 nt.

...TACTCGGGGGGCTGGTGCC .....AGCAGTGAC...
...Y   S   G   A   G   A ...... Q   ***

B.

Exons: 3   4   5   6   7   8   9
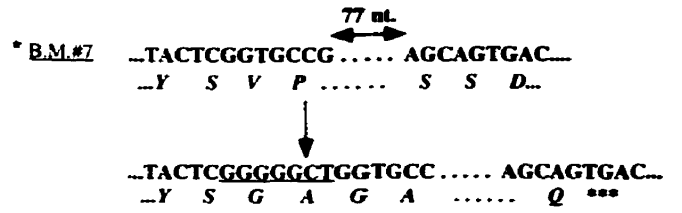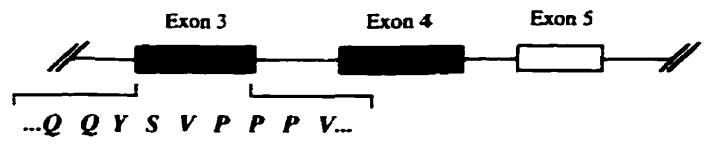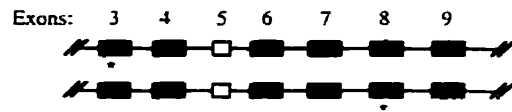
CHAPTER 3

Our initial molecular genetic studies of Wilms' tumors analysed the involvement of the *WT1* gene in this malignancy. These data are consistent with the role of *WT1* mutations with a subset of Wilms' tumors and but also suggest the involvement of other loci in Wilms' tumor pathogenesis. There was evidence of Wilms' tumor susceptibility loci at chromosome 11p15 and 16q but neither had been isolated (they remain uncloned at the time of this writing) and thus only the only gene known to be mutated in Wilms' tumors was *WT1*. A more complete understanding of the molecular nature of Wilms' tumor required the identification of other genes which harbor mutations associated with the development of this malignancy. The high frequency of *p53* mutations in a broad range of human cancers made this gene an attractive candidate for involvement in Wilms' tumor. In addition there were two observations which to specifically implicated a role for *p53* in Wilms' tumorigenesis. Non-random chromosomal alterations in the proximity of the *p53* locus were observed in some Wilms' tumors. Secondly, there was the curious observation that, when overexpressed, WT1 and p53 proteins could associate in vitro. In consideration of these observations we decided to perform a mutational analysis of the *p53* tumor suppressor gene in Wilms' tumor. It was hoped that *p53* mutations, if found, would be associated with specific subtypes of Wilms' tumor and thus would help account for the histological heterogeneity of this cancer.

Anaplastic Wilms' Tumour - A Subtype Displaying Poor Prognosis Harbours p53 Gene Mutations

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(Tumour Suppressor/ Anaplastic WT/ Cancer Genetics/ Chemoresistance)

Running Title: P53 Gene Mutations in Anaplastic Wilms' Tumours.

Number of Figures: 6

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Summary

The genetics of Wilms' tumour (WT), a pediatric malignancy of the kidney, is complex. Inactivation of the tumour suppressor gene, *WT1*, is associated with tumour etiology in ~10-15% of WTs. Chromosome 17p changes have been noted in cytogenetic studies of WTs, prompting us to screen 140 WTs for *p53* mutations. When histopathology reports were available, *p53* mutations were present in eight of eleven anaplastic WTs, a tumour subtype associated with poor prognosis. Amplification of *MDM2* , a gene whose product binds and sequesters p53, was excluded. Our results indicate that *p53* alterations provide a molecular marker for anaplastic WTs.

Introduction

Wilms' tumour (WT) affects approximately 1 in 10,000 individuals, occurring most frequently in children under the age of 5 years[1]. A genetic predisposition to WT is observed in several syndromes with congenital birth defects: a) the WAGR syndrome [a complex of *W*ilms' Tumour, *a*niridia, *g*enitourinary malformations, and mental *r*etardation][2], b) Denys-Drash syndrome (DDS) [an association of WT, intersex disorders, and renal nephropathy][3,4], and c) Beckwith-Wiedemann syndrome (BWS) [a fetal overgrowth syndrome associated with predisposition to pediatric malignancies, including WT][5] . Individuals with WAGR syndrome suffer heterozygous constitutional deletions within chromosome 11 band p13[6]. The WT suppressor gene (*WT1*) from 11p13 has been cloned[7,8], and germline mutations within *WT1* are associated with DDS[9,10], as well as some components of the WAGR spectrum of anomalies[11]. Intragenic mutations in 10-15% of sporadic and hereditary WTs provide molecular evidence that this gene is a tumour suppressor locus. Reports of allelic loss restricted to 11p15 in sporadic WTs, as well as linkage of BWS to 11p15, suggest the existence of a second tumour suppressor locus distinct from 11p13 (for a review, see ref. 12). Lack of 11p linkage in some WT families, as well as allelic loss at 16q24 in 20% of informative sporadic WTs, indicate the presence of additional predisposing loci[12,13]. These observations suggest that the genetics of WTs is complex and may provide a multistep model for tumour pathogenesis more broadly applicable to adult cancers than Knudson's "two-hit" mutational model[14].

Cytogenetic analysis of WTs has highlighted large-scale genetic lesions responsible for tumour progression[12], identifying non-random structural alterations at 17p. The *p53* tumour suppressor gene, located at 17p13, is currently documented as the most frequently mutated gene in human malignancies. Mutations in the p53 gene cluster within four "hotspots" in the conserved regions encoding amino acids 118-280 (refs. 15,16). P53 gene

mutations are thought to release cells from the normal state of growth inhibition and inactivate a *p53*-dependent apoptotic program[17,18]. This cell death program is activated when cells containing normal p53 are exposed to chemotherapeutic agents, but blocked in cells lacking p53 (ref. 19). These observations, derived from *in vitro* cell culture experiments, suggest that resistance to chemotherapy in some malignancies may correlate with the presence of *p53* mutations. Conversely, malignancies sensitive to the action of cytotoxic agents are expected to harbour wild type *p53*. WTs provide a model for testing this hypothesis, since most cases (>85%: favorable histology - Stages I and II) are cured by current regimens, and thus are predicted not to harbour *p53* mutations[20].

To determine if p53 gene mutations are associated with pathogenesis of WTs, we analyzed the *p53* coding exons in 140 sporadic and hereditary WTs for small deletions, insertions, or rearrangements by the single-strand conformational polymorphism (SSCP) assay[21]. Of the histologically defined tumors we studied, *p53* mutations were restricted to anaplastic WTs, an uncommon subtype associated with poor prognosis[22]. The majority of sporadic WTs analyzed did not harbour p53 gene mutations, suggesting that such lesions may provide a molecular marker for anaplastic WTs.

Results

Expression of the p53 gene in WTs.

To determine whether the p53 gene is expressed in WTs, we performed northern blot analysis on RNA isolated from seven sporadic tumours (Fig. 1). All tumours (lanes 1 to 7), as well as normal kidney tissue (lane N.K.), express significant amounts of the 2.8 kb *p53* transcript. Although only a small number of samples were investigated, these results demonstrate that *p53* expression is not extinguished in WTs. Our results confirm an earlier report documenting expression of p53 in a single WT[21].

Analysis of the p53 gene in sporadic and hereditary WTs.

In our initial study, 136 tumours were examined for deletions, insertions, or base-pair substitutions within the *p53* coding region spanning exons 2 to 11 (Fig. 2). The exons were amplified (see Methodology) and the PCR-SSCP analysis performed twice - alternating the identity of the radiolabeled oligonucleotide primer. Five tumours displayed mobility shifts (Figs. 3 and 4) of which four of occurred in patients with no documented family history of cancer (Fig. 3).

Patient R.F.#57 developed a unilateral WT with no associated congenital abnormalities or family history of renal cancer. The tumour contained epithelial and stromal cells with diffuse anaplasia. A mobility shift was identified in tumour DNA when exon 10 of *p53* was analyzed (Fig. 3a). The mutant allele appeared heterozygous and nucleotide sequencing revealed a point mutation at a CpG dinucleotide at codon 342 (Fig. 3b). This mutation converts a cytosine to a thymine, resulting in an arginine becoming a stop codon. The nonsense mutation is not present in DNA isolated from lymphocytes of patient R.F.#57 (Fig. 3a), and therefore must have arisen *de novo* either in the tumour or late in embryogenesis. Tumours C.D.#79 and N.L.#4 both show features of diffuse anaplasia and mobility shifts were detected when *p53* exons 5 and 10 were respectively analyzed (Fig. 3a). Tumour C.D.#79 has a homozygous mutation which converts a lysine to an arginine at codon 139 (Fig. 3b). Tumour N.L.#4 has an adenine to guanine transition converting a glycine to arginine at codon 354 (Fig. 3b). A mobility shift was also observed in exon 7 of the p53 gene when DNA from tumour X.X.#27 was analyzed (Fig. 3a). Nucleotide sequencing revealed a point mutation at codon 248, changing a guanine residue to an adenine resulting in the conversion of an arginine to a glutamine (Fig. 3b). We do not know the histological subtype of this sample (no pathology report was available) nor could we determine if this mutation was present in the germline of the affected individual since constitutional DNA was not available.

Patient T.J.#46 had hemihypertrophy at birth and developed bilateral WTs containing multifocal, highly anaplastic cells, at the age of 2 years (Fig. 4a). There is a strong history of early onset cancer in this individuals' family including cervical cancer and a glioma affecting the mother, adrenal cancer in a sibling, and a second WT affecting the fathers' half sister (Fig. 4a). Along with tumour DNA, constitutional DNA from the affected child and both parents were analyzed by PCR-SSCP. The results indicate that tumour T.J.#46 harbours a p53 mutant allele, also present in the heterozygous configuration in constitutional DNA from the affected individual and her mother (Fig. 4b). This mutation is an adenine to guanine transition causing a Met246Val substitution.

P53 mutations are associated with anaplastic WTs.

We obtained histopathology reports for 99 tumours analyzed in this study. Of these, five were classified as diffuse anaplastic WTs - tumours (N.L.#4, P.G.#18, R.F.#57, B.U.#71, C.D.#79) and two were focal anaplasic malignancies - tumours (T.J.#46 and A.I.#34). We detected p53 mutations in four of these samples (N.L.#4, T.J.#46, R.F.#57, C.D.#79). None of the other WTs, without anaplasia, harboured p53 mutations. These results suggested a possible correlation between the anaplastic subtype and the presence of p53 mutations. In cases of focal anaplasia, the anaplastic cells will only represent a small proportion of the entire tumour bed and consequently detection of p53 gene mutations in these samples would require genotyping a small cell population. This may account for our failure to detect p53 mutations in some of the anaplastic tumours (see below). We therefore sought to strengthen the correlation between anaplasia and the occurence of p53 mutations by increasing our sample size and focusing on tumor areas with predominantly anaplastic cells. DNA was prepared from parrafin-embedded tumour blocks which had been histologically reviewed and demonstrated a high density of anaplastic nuclei. We obtained four such tumours, as well as a metastasis arising from one of these primary tumours. The PCR products from all samples displayed mobility shifts indicative of p53 gene mutations when analyzed by SSCP (Fig. 5). The primary tumour AN.-1 and its corresponding metastasis, MET.-1, harbour a guanine to adenine transition at codon 175 converting an arginine to a histidine. Both mutations are homozygous (Fig. 5). Tumour AN.-2 suffered a homozygous 8 nucleotide deletion in exon 5, resulting in a frameshift and early termination of translation. Tumour AN.-3 displayed a homozygous thymine to guanine transversion at codon 234 converting a tyrosine to an aspartic acid. Tumour AN.-4 harbours a cytosine to thymine transition at codon 196 predicted to cause early termination of translation.

To exclude the possibility that the p53 gene mutations we characterized were due to errors by the *Taq* polymerase arising during PCR amplification, we performed allele-specific amplification (ASA) for eight of the mutations present in the anaplastic tumours described above (Figs. 3-5). Oligonucleotide primers were synthesized in which the 3' bases specifically targetted the mutant alleles (see legend to Fig. 6). Thus, amplification reactions performed on DNA from an unafffected individual and a WT harbouring the appropriate mutation would be expected to specifically amplify only the mutated allele from the tumour. Oligonucleotides spanning the wild-type exon were performed in parallel as positive controls to asses the efficiency of the amplification reactions. When ASA was performed, product was observed only from tumour material (Fig. 6 *a-d*). Thus the *p53* mutations are indeed *in vivo* lesions present in these tumours. The finding of *p53* mutations in eight of 11 anaplastic WTs suggests that such lesions are involved in etiology to an unfavorable histological subtype.

The *MDM2* gene is not amplified in WTs.

The p53 gene product can be inactivated if sequestered by a p53-binding protein termed MDM2[23]. Amplification of *MDM2* is frequently found in human sarcomas[23], osteosarcomas[24], and 8-10% of human malignant gliomas without *p53* mutations[25], suggesting that overexpression of *MDM2* may represent a molecular mechanism by which malignant cells escape from p53-regulated growth control. We investigated the possibility that *MDM2* is amplified in WTs. Slot blots of genomic DNA from 91 WTs were hybridized to β-actin and *MDM2* probes (a gift of Drs. Oliner and Vogelstein (Johns Hopkins Oncology Center)). When standardized for variations in loading (by comparing to the β-actin values), the*MDM2* locus did not show any amplification (data not shown). Our results indicate that sequestration of functional p53 by amplified MDM2 in WTs is unlikely.

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Discussion

The treatment of WT is among the greatest successes of pediatric oncology. The employment of combined radio- and multidrug chemotherapy regimens following surgical resection has lead to an overall survival rate of >85% (for favorable histologies; Stages I and II)[20]. A special problem, however, is presented by the anaplastic subtype of WT which carries a significantly worse prognosis (~40-50% relapse-free survival rate) and hence is termed unfavorable histology[22]. Anaplasia is the most important indicator of poor prognosis and is defined by markedly enlarged and usually multipolar mitotic figures, a three-fold nuclear enlargement (compared with adjacent nuclei of the same cell type), and hyperchromasia of the enlarged nuclei. These cells have been suggested to be markers of increased genetic mutability and multiple mitotic errors[22]. The frequency of anaplasia among WT cases is ~5%[22].

We have observed *p53* mutations in eight of 11 anaplastic WTs analyzed. Our failure to detect *p53* mutations in anaplastic tumour A.I.#34 is likely to due the fact that this sample had a very low number of anaplastic cells confined to <10% of the microscopic fields evaluated. Our inability to detect mutations in samples P.G.#18 and B.U.#71 may be due to limitations of the PCR-SSCP assay - the detection rate of SSCP is not 100%[9], some of our primer pairs cross splice junctions and include exonic sequences, or other genetic lesions may be involved in promoting progression to anaplasia. We do not favor the latter explanation since we were able to identify *p53* mutations in all 4 samples containing a large proportion of anaplastic cells (Fig. 5). Although mutations were not defined in *p53* exons 2,3,4,8 or 9, we are confident that our oligonucleotides spanning these regions are informative for nucleotide changes in these exons since: i) we often detect polymorphisms in exons 2,3, and 4, ii) we have observed a rare polymorphism in exon 9 in some of our specimens, and iii) we have recently identified a *p53* mutation in exon 8 of an additional anaplastic WT (N. Bardeesy and J. Pelletier, unpublished observations). Thus, we are confident that mutations would be detected in these exons. Our data provides genetic evidence that *p53* mutations are associated with tumour progression in WTs, causing a more aggressive form of the disease. WTs without anaplasia, but with predominantly blastemal cell components, are also classified as unfavorable and have a poor prognosis[20,22]. Althought we failed to identify *p53* mutations in such samples, it is possible that only a small number of cells within the tumour bed have *p53* mutations and would thus escape detection. In general, *p53* mutations are rare in WTs since the majority of the samples analyzed in this study had no detectable alterations.

Most of the mutations we described occurred within conserved domains of the p53 gene (tumours T.J.#46, C.D.#79, AN.-1, AN.-2, and AN.-3). In tumour R.F.#57, the

mutation arose somatically, whereas in tumour T.J.#46 it is present in the germline of the affected individual (Figs. 3 and 4). In several of the malignancies (C.D.#79, AN.-1, AN.-2, and AN.-3), the mutations were present in the homozygous configuration. Expression of mutant p53 in anaplastic cells, with no endogenous p53 protein, may confer new or additional phenotypes[26]. Alternatively, loss of heterozygosity in the anaplastic tumours may suggest the presence of additional tumour suppressor loci on chromosome 17p necessary for tumour progression. Whether or not most anaplastic WTs with *p53* mutations demonstrate functional inactivation of the other p53 allele remains to be established. We cannot exclude the possiblity that specimens with apparently heterozygous mutations are contaminated with normal tissue. With the exception of tumours N.L.#4, C.D.#79, and AN.-2, all of the mutations we identified have been previously documented in other human malignancies[27].

Germline *p53* gene mutations are associated with the Li-Fraumeni cancer syndrome[28,29]. Recently, the occurence of WTs have been documented in several cancer-prone families[30], raising the possibility that *p53* gene mutations may be an initiating event for some WTs. We have described a similar family with two individuals suffering from WT (Fig. 4a). The WT from the proband harboured a germline mutation in the *p53* tumour suppressor gene (Fig. 4). The mutation is of maternal origin, whereas the second affected individual in this family is on the paternal side, therefore, the predisposition to WTs in this family is unlikely due to a *p53* gene mutation. The proband was born with hemihypertrophy, one of the stigmata of BWS and possibly an 11p15 lesion contributed to pathogenesis of this WT. Of note, no *WT1* mutation was detected in T.J.#46, ruling out inactivation of this gene as a potential mechanism leading to the two WTs on the paternal side (N. Bardeesy and J. Pelletier, data not shown). Our data would predict that WTs developing in individuals with germline *p53* mutations would have an increased likelihood of developing anaplastic features.

The p53 gene product is a mediator of an apoptotic pathway[17,18] activated by chemotherapeutic agents[19]. It is required for efficient activation of apoptosis by cytotoxic compounds and accordingly, the ability of tumour cells to activate this pathway in response to cellular damage may depend on the cellular status of *p53*. Tumour cells containing *p53* mutations may be expected to be resistant to anticancer agents. WTs provide a testing ground for this model since, diagnosed at an early stage and without anaplasia, this malignancy is readily treatable by multi-modal therapy. Before the advent of radio- and chemotherapy, surgical resection of WTs led only to a 20% cure rate[31]. With the introduction of postoperative radiotherapy, the cure rate increased to ~50%[32]. The addition of chemotherapy to the antitumour regimen, combined with more aggressive treatment of

the metastatic disease, survival rates currently are ~ 85%[20]. Of the 99 tumour specimens for which histopathology reports were available, we have found no evidence for *p53* mutations in non-anaplastic tumours. The failure to respond to therapy of some WTs may be due to the presence of a small number of *p53* mutant cells which escape detection and become selected for by the anticancer regimen. This issue will need to be addressed by analyzing *p53* in a series of histologically well defined sections containing a variety of cell types.

A large collection of WTs have been documented as having high expression levels of the p53 protein[33], and given our results, this likely represents wild-type protein. The stability of p53 protein is also increased in cells expressing E1A, resulting in elevated p53 levels[34]. Since normal cells express low levels of p53 without adversely affecting cell survival, p53 stabilization may be necessary for apoptosis. Thus, elevated levels of wild-type p53 protein in sporadic WTs may "sensitize" cells to the cytotoxic action of anticancer agents[19,34], thereby contributing to the effectiveness of chemotherapy in these tumours[20].

WT1 has been shown to interact with p53 protein and potentiate transactivation of the muscle creatine kinase promoter by p53 (ref. 35). This interaction is not essential to *WT1* function since mice homozygously mutated for *p53* have no developmental defects of the urogenital system[36], unlike what is observed in animals lacking *WT1* (ref. 37). We have analyzed tumours R.F.#57, N.L.#4, C.D.#79, and T.J.#46 for *WT1* mutations by PCR-SSCP and find no evidence for alterations (N. Bardeesy and J. Pelletier, unpublished observations). We do not know yet whether *WT1* and *p53* mutations are mutally exclusive. However, our data suggest that anaplastic WTs are part of a group of malignancies whose *p53* gene status may correlate with response to cytotoxic agents. No ultrastructural or immunocytochemical markers distinguish anaplastic from non-anaplastic WTs[22], and this assignment is based on experienced histological review of the diseased tissue. The detection of *p53* mutations in WTs may be useful in supporting histological diagnosis, providing a prognostic indicator for tumour progression, and an understanding of the biological basis of anaplasia.

Methodology

Isolation of genomic DNA. DNA prepared from 136 tumours were initially analyzed in this study. Five of these were from bilateral patients, contributing tumours T.J.#46 (with hemihypertrophy), M.T.#98 (with GU anomalies), N.L.#64, A.S.#35, and T.B.#14. One sample was from an individual with Denys-Drash syndrome (N.K.#97). Five samples were not WTs: a) J.K.#99 (a primitive neuroectodermal renal tumour), b) N.L.#65 (a nephroblastomatosis), c) K.A.#28 (a clear cell sarcoma), and d) N.H.#32 and J.L.#50 (rhabdoid tumours). The remaining samples were single WTs from unilateral sporadic patients. None of these remaining patients showed any stigmata of the WAGR or Beckwith-Wiedemann syndrome. With the exception of T.J.#46, the family history in all instances was negative for WTs. The wild-type DNA used as a negative control was from blood of an unaffected normal female. Constitutional DNA was prepared from blood obtained after informed consent from the patients, as previously described[9]. DNA was isolated from parrafin-embedded material of three anaplastic WTs, and a metastasis from one of the anaplastic WTs, essentially as described[38].

PCR and SSCP analysis. In order to detect possible point mutations, small deletions, or rearrangements in genomic DNA from tumours, sequences from the ten coding exons of p53 were amplified by the PCR and analyzed by SSCP. Ten pairs of oligonucleotides (presented in Fig. 2) were designed to cover the *p53* coding region: Exons 2 and 3, A (A1: 5'TGGATCCTCTTGCAGCAGCC3') and (A2:5'AACCCTTGTCCTTACCAGAA3'); Exon 4, B (B1:5'ATCTACAGTCCCCCTTGCCG3') and (B2:5'GCAACTGACCGTGCAAGTCA3'); Exon 5, C1* (5R:5'CCCCAGCTGCTCACCAT3') and (C1:5'TTCCTCTTCCTGCAGTACT3'); Exon 6, C2 (6U1:5'ACCATGAGCGCTGCTCAGAT3') and (C2:5'AGTTGCAAACCAGACCTCAG3'); Exon 7, D (D1:5'GTGTTGCCTCCTAGGTTGGC3') and (D2:5'CAAGTGGCTCCTGACCTGGA3'); Exons 8 and 9, E-1 (E1:5'CCTATCCTGAGTAGTGGTAA3') and (E2:5'CCAAGACTTAGTACCTGAAG3'); Exon 8, E-2 (8R:5'GTCCTGCTTGCTTACCTCG3') and (E1); Exon 9, E-3 (9F:5'TTGCCTCTTTCCTAGCACTG3') and (E2); Exon 10, F (F1:5'TGTTGCTGCAGATCCGTGGG3') and (F2:5'GAGGTCACTCACCTGGAGTG3'); Exon 11, G (G1:5'TCTCCTACAGCCACCTGAAG3') and (G2:5'CTGACGCACACCTATTGCAA3').Prior to the PCR, one oligonucleotide was radiolabeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (New England Nuclear). The PCR was performed using a DNA thermocycler (MJ Research) with ~50 ng of genomic DNA in a total volume of 20 ul. Thirty-five cycles of amplification were carried out; each

cycle consisted of 1 min at 94 °C, 1 min at the optimally determined annealing temperature for each oligonucleotide pair [Oligo pairs A, B, C2, D, E-1, E-2, E-3, F1, and G were annealed at 56 °C, whereas oligo pair C1* were annealed at 53 °C], and 1 min at 72 °C. After the PCRs, aliquots of 2 ul were mixed with 8 ul of sample buffer [95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF] and boiled for 5 min. Samples (2 ul) were loaded onto 8% polyacrylamide (50:1, acrylamide:bisacrylamide) gels, and electrophoresis was performed in TBE (90 mM Tris/90 mM boric acid/ 2.5 mM EDTA) buffer at 30 W in the cold room. The gel was dried on filter paper and exposed to XAR5 film at -70 °C for 12-24 hr with an intensifying screen.

PCR amplification products were subcloned into pKS II+ (Stratagene) and sequenced by the chain termination method[39] using Sequenase polymerase (U.S. Biochemical). No less than 6 clones were sequenced to ensure against *Taq* polymerase artifacts and to determine the sequence of both alleles.

Northern analysis. 10 µg was analysed on a 1.2% agarose/37% formaldehyde gel and blotted onto nitrocellulose (S&S) with 20X SSC (1X SSC: 150 mM NaCl, 15 mM sodium citrate). The blot was prehybridized in 50% formamide, 10% dextran sulfate, 5X Denhardt's solution, 0.5% SDS, 0.1% sodium pyrophosphate, and 100 µg ml^{-1} salmon sperm DNA at 42 °C and hybridized under the same conditions. The probe used for p53 detection was generated by PCR amplification using oligonucleotide pairs B1 and B2. The PCR product was gel purified, and radiolabelled by the random primer method[7] to achieve a specific activity of ~5 x 10^7 cpm µg^{-1}. After overnight hybridization, the blot was washed twice with 2X SSC/0.1% SDS at RT, twice with 2X SSC/0.1% SDS at 55 °C and once at 0.2X SSC/0.1% SDS at 55 °C. The blot was exposed to Kodak XAR film at -70 °C for 24 h with an intensifying screen.

Acknowledgments

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Figure Legends

Figure 1. Northern blot analysis of *p53* mRNA in seven sporadic, unilateral WTs. Total RNA was isolated from seven sporadic WTs and normal kidney as previously described[10]. The labels at the top of the autoradiograph refer to the RNA samples loaded on the gel: N.K., normal kidney; lanes 1 to 7, seven sporadic WTs. The size markers indicated on the left were obtained by co-migration of BRL RNA size ladders. The arrowhead indicates the position of migration of the *p53* transcript.

Figure 2. Schematic representation of the exons amplified and subjected to SSCP analysis from the *p53* gene. The regions of the p53 gene, corresponding to exons 2 to 11, targetted for mutational analysis are indicated by double-headed arrows. The coding regions of the exons are indicated by solid boxes with amino acid positions, while the noncoding regions are indicated by open boxes. The letter under each double-headed arrow corresponds to the primer pairs used to amplify that region. Five evolutionarily conserved regions are indicated by dotted boxes below the *p53* gene. The four "hot-spots" previously identified to be targets for mutational alteration are indicated by brackets above the *p53* gene[15,16].

Figure 3. *P53* gene mutations in sporadic WTs. *a*) PCR-SSCP analysis of exons 5, 7 and 10 of tumours C.D.#79, X.X.#27, R.F.#57 and N.L.#4, respectively (see Methodology). The arrowheads indicate the PCR products with altered mobility. The arrow indicates the position of the non-denatured PCR product. The lanes are: Non Den., nondenatured ; Water, (control containing no input DNA); Normal, DNA isolated from an unaffected individual; Blood, DNA isolated from peripheral lymphocytes of the patient; Tumour, tumour DNA. *b*) Summary of sequencing data obtained for sporadic Wilms' tumours displaying *p53* mutations. The sequence of the mutant alleles are summarized, as well as the predicted amino acid changes of the p53 polypeptide. N.D., not determined.
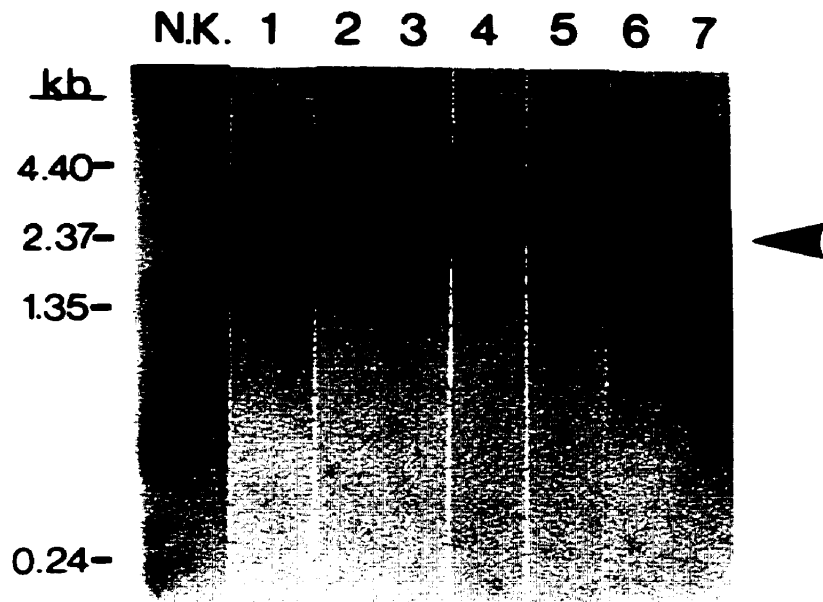
Figure 4. Pedigree and PCR-SSCP analysis of family T.J.#46. *a*) There are two cases of WTs in this family, a case of adrenal cancer in one of the siblings, and the mother has suffered from cervical cancer and a glioma. *b*) PCR-SSCP analysis of exon 7 from patient T.J.#46. The arrow indicates the PCR products with altered mobility. The open circle refer to the position of the normal exon 7 conformer. The lanes are: Nondenatured ; Normal, DNA isolated from an unaffected individual; Father, DNA isolated from peripheral lymphocytes of T.J.#46's father; T.J.#46, DNA isolated from peripheral lymphocytes of the patient; T.J.#46 [Tumour], DNA isolated from the patient's WT; Mother, DNA isolated
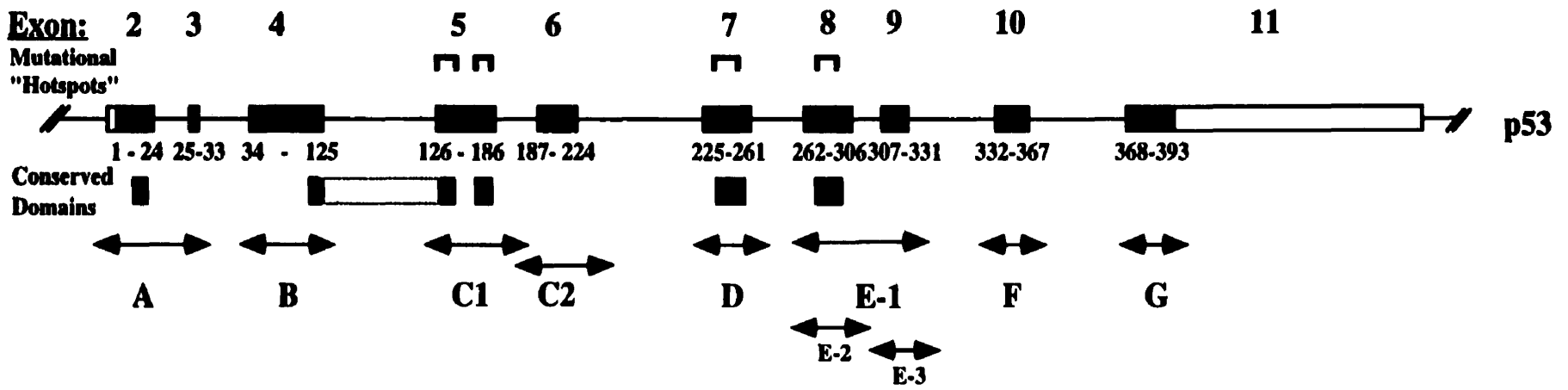
from peripheral lymphocytes of the patient's mother. The sequence of the mutant a p53 polypeptide.

Figure 5. Mutational analysis of the p53 gene in four anaplastic WTs and one metastatic WT. The arrowheads indicate the PCR product with altered mobility. The lanes are: Water, (control containing no input DNA); Normal, DNA isolated from an unaffected individual; AN., anaplastic; and MET., metastasis. The sequences of the mutant alleles are shown to the right, along with the predicted amino acid changes of the p53 polypeptide.

Figure 6. Allele-Specific Amplification (ASA) of the mutant WT1 alleles in anaplastic WTs. The mutations defined by cloning and sequencing for the eight sporadic and hereditary WTs in Figs. 3-5 were verified using allele-specific oligonucleotides targetted to the mutant alleles. Amplification reactions were performed as indicated in the Methodology with the following allele-specific primers targetting the indicated tumours: C.D.#79: 79s,^{5}'GTTTTGCCAACTGGCCAG^3'; AN.-1: 1as, ^{5}'GCTCATGGTGGGGGCAGT^3'; AN.-2: 2as, ^{5}'GGGGCAGCGCCTCACTC^3'; N.L.#4: L4as,^{5}'TGGCTCCTTCCCAGCCC^3'; R.F.#57: 57s, ^{5}'AGCGCTTCGAGATGTTCT^3'; AN.-4: 4as,^{5}'CTCCTCAGCATCTTATCT^3', T.J.#46: 46s, ^{5}' TTCCTGCATGGGCGGCG^3';AN.-3: 3s, ^{5}'ACTGTACCACCATCCACG^3'. The underlined nucleotides indicate the mutated base at each altered allele. The nucleotides highlighted in bold for oligo. 2as delineate the 2 bases which cross the deletion identified in tumour AN.-2. The oligonucleotides used as internal controls to span each exon are summarized in the Methodology. *a*) ASA of exon 5 from tumours C.D.#79, AN.-1, and AN.-2. *b*) ASA of exon 10 from tumours N.L.#4 and R.F.#57. *c*) ASA of exon 6 from tumour AN.-4. *d*) ASA of exon 7 from tumours T.J.#46 and AN.-3. The amplified products from the positive controls are on the left of each panel with the primer pair indicated below the panel. The arrowheads demarcate the ASA products from each tumour specimen analyzed and the allele-specific primer used is indicated below each panel.
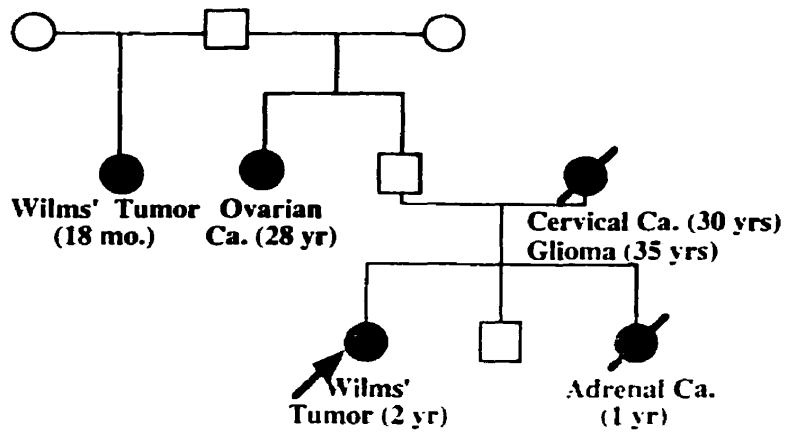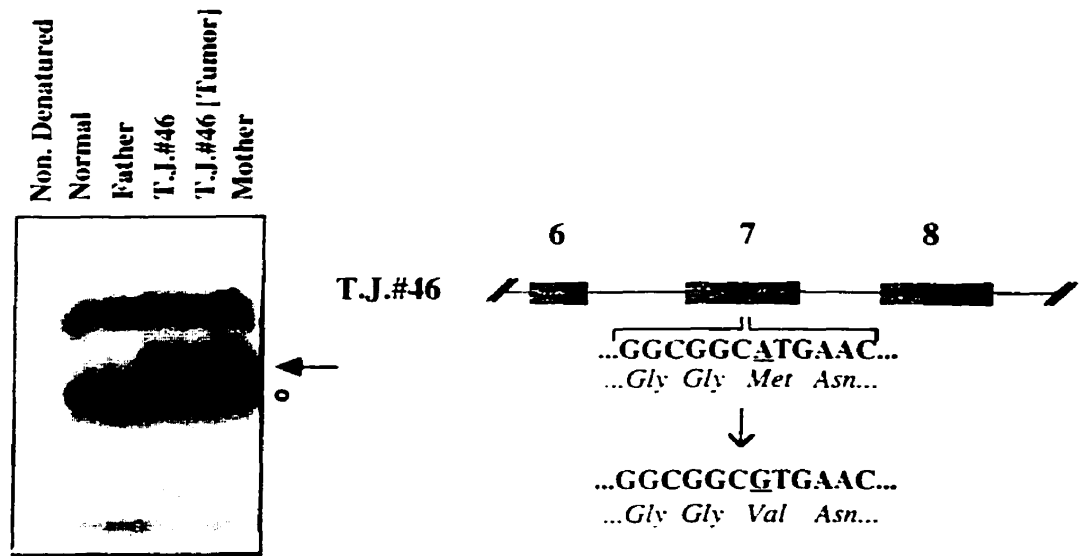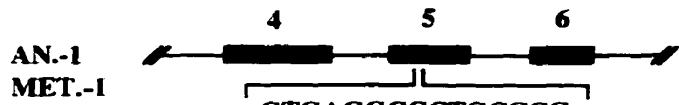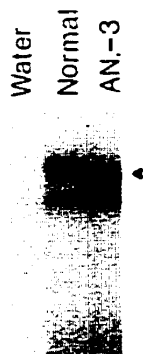
1

N.K. 1 2 3 4 5 6 7

kb

4.40-

2.37-

1.35-

0.24-

2

Exon: 2   3   4   5   6   7   8   9   10   11

Mutational "Hotspots"

p53

1 - 24 25-33 34 - 125   126 - 186 187- 224   225-261 262-306 307-331 332-367   368-393

Conserved Domains

A   B   C1   C2   D   E-1   F   G

E-2 E-3

3 A

Non Den. | Water | Normal | Blood | Tumor

Water | Normal | Tumor

Non Den. | Water | Normal | Tumor

Tumor | Normal

R.F.#57

C.D.#79

X.X.#27

N.L.#4
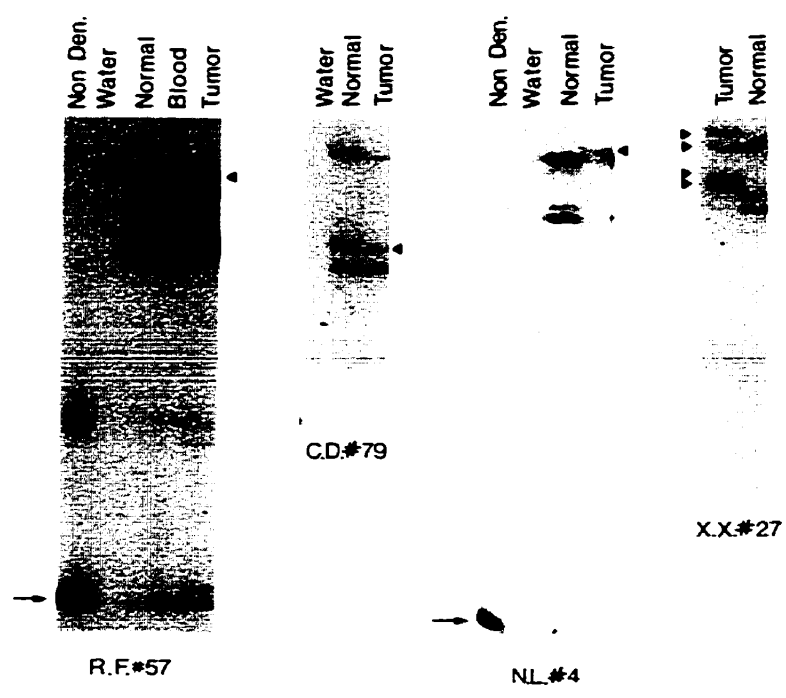
B

Tumor Code	Classification	SSCP Mobility Shift		p53 Mutation	
		Homozygous	Germline	Exon	Mutation
R.F.#57	Diffuse Anaplasia	No	No	Exon 10	...TTCCGAGAG... ..Phe Arg Glu... ↓ ...TTCTGAGAG... .. Phe ***
C.D.#79	Diffuse Anaplasia	Yes	N.D.	Exon 5	...GCCAAGACC... ...Ala Lys Thr... ↓ ...GCCAGGACC... ...Ala Arg Thr...
N.L.#4	Diffuse Anaplasia	No	N.D.	Exon 10	...GCCCAGGCT... ...Ala Gly Ala... ↓ ...GCCCGGGCT.. ...Ala Arg Ala...
X.X.#27	Unknown	Yes	N.D.	Exon 7	...AACCGGAGG... ...Asn Arg Arg ... ↓ ...AACCAGAGG... ...Asn Gln Arg...

4A.

Wilms' Tumor   Ovarian
(18 mo.)   Ca. (28 yr)

Cervical Ca. (30 yrs)
Glioma (35 yrs)

Wilms'
Tumor (2 yr)

Adrenal Ca.
(1 yr)

B.

Non. Denatured
Normal
Father
T.J.#46
T.J.#46 [Tumor]
Mother

T.J.#46

6   7   8

...GGCGGCATGAAC...
...Gly Gly Met Asn...

↓

...GGCGGCGTGAAC...
...Gly Gly Val Asn...

5

AN.-1
MET.-1

4   5   6
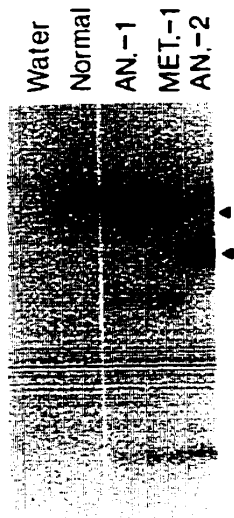
...GTGAGGCGCTGCCCC..
...Val Arg Arg Cys Pro...

↓

...GTGAGGCACTGCCCC..
...Val Arg His Cys Pro...

AN.-2

4   5   6

...CACATGACGGAGGTTGTGAGG...
...His Met Thr Glu Val Val Arg...

↓

14nt.

...CACATGAGTGAGG .... TGA...
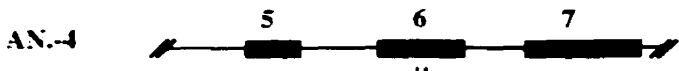...His Met Ser Glu .... ***

AN.-3

6   7   8
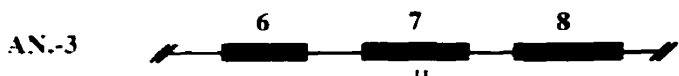
...ATCCACTACAACTAC...
...Ile His Tyr Asn Tyr...
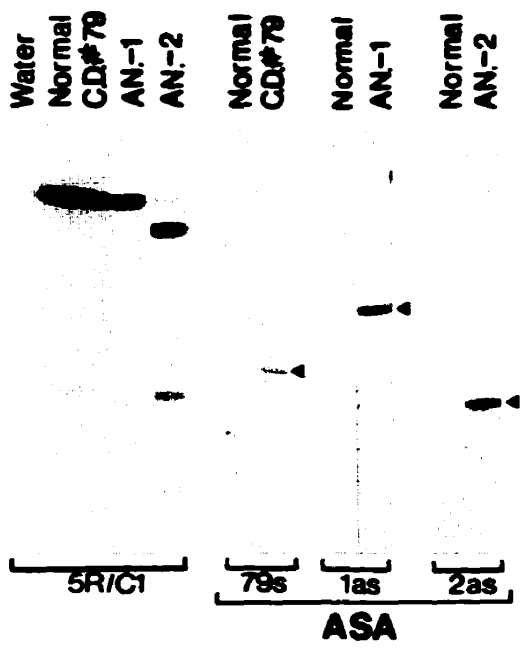
↓

...ATCCACGACAACTAC...
...Ile His Asp Asn Tyr...

AN.-4

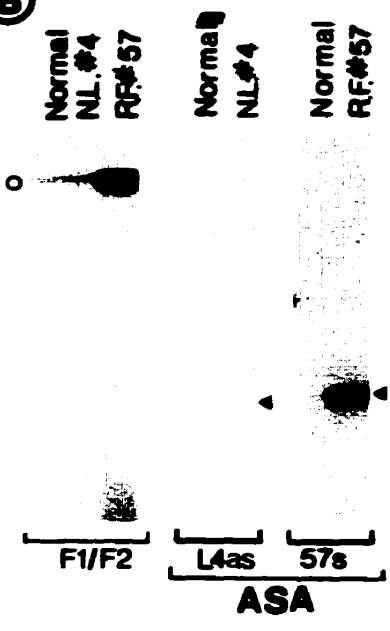5   6   7

...CTTATCCGAGTGGAA...
...Leu Ile Arg Val Glu...

↓

...CTTATCTGAGTGGAA...
...Leu Ile ***

6 Ⓐ

Ⓑ

Water
Normal CD#78
AN-1
AN-2

Normal CD#78
Normal AN-1
Normal AN-2

Normal NL #4
RF#57

Normal NL #4
Normal RF#57

5R/C1

79s   1as   2as

ASA

F1/F2   L4as   57s

ASA

6C

Water
AN.-4
Normal
AN.-4

6U1/C2 4as

ASA

D

Normal
T.J#46
AN.-3
Normal
T.J#46
Normal
AN.-3

D1/D2 46s 3s

ASA

CHAPTER 4

Following establishment of a strong association between anaplastic histology in Wilms' tumors with mutations in the *p53* gene in Chapter 4, we wanted to further address the role of such mutations in tumor progression. In particularly we wanted to determine at what step during tumorigenesis does *p53* become inactivated. Adult cancers undergo a multistep process in their malignant progression, involving an ordered series genetic lesions. Such a progression had not previously been documented in pediatric cancers which are generally considered to involve simple two-hit mutational events. We wanted to establish whether *p53* mutations are the rate limiting step in the onset of anaplasia or whether they are a secondary event. To answer this question, we required histologically defined sections of the same tumor containing well demarcated anaplastic and nonanaplastic tissue. If the mutations were confined to the anaplastic region it would strongly suggest that such a mutation would be a rate limiting step in the progression of the tumor and that there is clonal selection of cells with mutated *p53* genes. We also wished to test whether mutations in *p53* were associated with changes in the rate of apoptosis in Wilms' tumors. To address this we analysed the relative levels of apoptosis in adjacent anaplastic and nonanaplastic regions of Wilms' tumors.

Clonal Expansion and Attenuated Apoptosis in Wilms' Tumors are Associated with p53 Gene Mutations

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ABSTRACT

The p53 gene product is required for activation of an apoptotic pathway triggered by oncogenes and cytotoxic agents. Wilms' tumor (WT), a pediatric renal malignancy, provides a paradigm for evaluating genetic events involved in tumor progression. This malignancy is generally not associated with p53 mutations, and even in advanced disease states is quite responsive to current treatment regimens. The anaplastic histological variant of WT, however, is frequently associated with p53 gene mutations and shows poor prognosis. We analyzed seven WTs for which we had paired samples from non-anaplastic and anaplastic regions. P53 mutations were detected in six of these tumors, five of which demonstrated mutations restricted to anaplastic regions. Non-anaplastic cells of the sixth sample were heterozygous for a p53 mutation, whereas the anaplastic area of this tumor showed reduction to homozygosity. These results indicate that progression to anaplasia is associated with clonal expansion of cells which have acquired a p53 mutation. We demonstrated that tumor cells with p53 mutations show attenuated apoptosis, suggesting that such lesions may provide a selective advantage *in vivo* by decreasing cell death.

INTRODUCTION

The treatment of cancer with cytotoxic agents has significantly decreased morbidity and mortality associated with this disease. In a substantial proportion of patients, however, chemotherapy becomes ineffectual due to the acquisition of drug resistance, and relapse of the disease occurs. Mutations in the p53 gene have been noted: i) in association with a large number of malignancies arising from a variety of cell types, ii) as a late event in tumor progression, and iii) in advanced and relapsed disease (for a review, see ref. 1). The role of p53 in tumorigenesis is not well understood, although an apoptotic pathway induced by oncogenic activation or cytotoxic agents is directly dependent on the presence of active p53 protein (2-5). Thus, a function of the p53 gene product may be to sensitize damaged cells to apoptosis, thus acting to prevent the propagation of transforming mutations. Cells lacking functional p53 would be refractory to this process of elimination and predicted to be more aggressive in nature (2-5). These results, defined in primary cell cultures and transgenic mouse models, remain to be corroborated in human cancers.

The successful treatment of WTs with combined chemo- and radiotherapy is a major achievement of pediatric oncology. Before the advent of chemotherapy, the treatment of WTs by nephrectromy and postoperative irradiation yielded dismal survival rates: Stage I, 61%; Stage II, 47%; Stage III, 11%; and Stage IV, 0% (6). With current treatment protocols, the cure rate for WTs showing favorable histology is: Stages I and II, >90%; Stage III, >85%; and Stage IV, ~80% (7). The single most important indicator of poor prognosis for WTs is the presence of anaplasia within the tumor, as defined by three criteria: i)multipolar mitotic index, ii) a threefold nuclear enlargement (compared to adjacent nuclei of the same cell type), and iii) hyperchromasia of the enlarged nuclei (8). Before the implimentation of more aggressive treatment for anaplastic tumors, the recurrence rates were 90% for diffuse anaplasia versus 21% for WTs with favorable histology (6). Approximately 5% of WTs are anaplastic and the current cure rate for stage IV disease is only ~40-55% (7,8). In a recent study, we identified p53 gene mutations in a high percentage of anaplastic WTs (8 of 11 tumors), but failed to detect p53 gene mutations in a large number of WTs showing favorable histology (92 tumors) (9), suggesting that p53 lesions are linked with some of the aggressive features of anaplasia. Thus, WTs provide an excellent model for assessing the role of the p53 gene product in tumorigenesis.

In this report, we demonstrate that anaplastic cells arise from clonal progression of cells showing favorable histology, and that this progression is associated with p53 gene mutations. In addition, we have compared apoptosis of favorable and anaplastic paired tumour specimens. Our results support the notion that disrupting cell turnover plays an important role in tumor progression and suggests that p53 may be involved in this process in WTs.

MATERIALS AND METHODS

Tissues and DNAs. All tumor blocks were obtained through the National Wilms' tumor pathology center and have been extensively histologically reviewed by one of us (J.B.B.). Regions of focal anaplasia were demarcated for molecular genetic studies from tumor sections, and microdissection performed to isolate non-anaplastic and anaplastic regions. DNA was isolated from parrafin-embedded material as previously described (10), and titrated in control PCRs to determine the optimum DNA concentration with which to obtain the best signal-to-noise ratio.

Polymerase Chain Reaction (PCR) and Single Strand Conformational Polymorphism (SSCP) Analysis. Sequences from the ten coding exons of p53 were amplified by PCR and analyzed by SSCP as previously described (9). PCR products were generally analyzed on 8% polyacrylamide (50:1, acrylamide:bisacrylamide) gels, and electrophoresis was performed in TBE (90 mM Tris/90 mM boric acid/ 2.5 mM EDTA) buffer at 30 W in the cold room. When mutations within the p53 gene was not detected in a particular tumor specimen, the PCR samples were further anaylzed on 8% polyacrylamide gels (50:1) containing 5% glycerol and electrophoresed at room temperature. Gels were dried on filter paper and exposed to XAR5 film (Kodak) at room temperature for 12-24 hr.

When mutations were detected by SSCP, the appropriate tumor exon was re-amplified in 5 independent reactions, the products pooled, and cloned into pKS II+ (9). Sequencing was accomplished by the chain-termination method using double-stranded DNA template (11).

In Situ Detection of Apoptosis. The Terminal deoxynucleotide transferase - mediated dUTP-biotin Nick End Labeling (TUNEL) procedure (12) for *in situ* visualization of cells with fragmented DNA was performed as described in the *in situ* Apoptag kit (Oncor). Apoptosis was quantitated by determining the percentage of HRP-stained cells within a field of view at a magnification of 400x. A total of 10 randomly chosen fields were counted per slide assayed, and the counts were averaged to obtained the apoptotic index.

RESULTS AND DISCUSSION

In WTs, the frequency of anaplasia increases with stage of disease and patient age suggesting that genetic lesions associated with development of this histological subtype are a late event in progression (8). We have recently documented a high incidence of p53 mutations associated with anaplastic tumors, whereas we could not detect similar mutations in non-anaplastic WTs in our study (9). To address the relationship between p53 gene mutation and clonal expansion in anaplastic WTs, seven histologically defined WT sample pairs consisting of non-anaplastic and anaplastic regions were prepared from paraffin-embedded tumor blocks (Table 1). Specimens were carefully dissected to ensure optimal removal of contaminating tissue and DNA was isolated from anaplastic or non-anaplastic regions and analyzed as described in the Materials and Methods. Of the seven tumor pairs analyzed, six were found to harbor a p53 mutation (Table 1). Five of the tumors (7907, 8834, 8868, 6841, AN1) showed p53 mutations restricted to the anaplastic region (Table 1 and Fig. 1). Four of these (7907, 8834, 8868, AN1) had also lost the wild-type p53 allele indicating that most cells within the anaplastic region of these tumors were hemi- or homozygous for a mutant p53 allele (Table 1 and Fig. 1). Non-anaplastic cells from tumor #5191 were heterozygous for a mutant p53 allele, whereas anaplastic cells showed loss of the wild-type p53 allele (Table 1). All p53 lesions are missense mutations resulting in non-conservative amino acid changes. Identical mutations have been previously documented in other human cancers and are likely to inactivate the p53 gene product (13). These results are consistent with our previous studies indicating that a high percentage of anaplastic WTs harbor p53 gene mutations and suggest that progression to anaplasia is associated with clonal expansion of cells which acquire p53 mutations (9).

In light of results obtained with tumor 5191, indicating that p53 mutations can occur in non-anaplastic cells, albeit rarely, we addressed the possibility that a p53 gene mutation was present in one of the tumors characterized above in a fraction of non-anaplastic cells too small to be detectable by PCR-SSCP. This possibility has been previously noted during clonal expansion of p53 mutant cells in brain tumors (14). To address this issue, we used a sensitive colony hybridization assay which can identify a small number of cells harboring p53 mutations among a majority of cells with normal p53 genes. Tumor 7907 was chosen for further study since SSCP and sequence analysis demonstrated a homozygous p53 mutation at codon 179 restricted to the anaplastic region. Exon 5 of the p53 gene was amplified by the PCR from the anaplastic and non-anaplastic regions of this tumor and following cloning of the products into pKS II+, colony hybridization was performed with a specific oligonucleotide capable of recognizing the mutation defined in tumor 7907 (Fig. 2). No positive plaques were observed from clones

obtained with DNA isolated from the non-anaplastic region of tumor 7907 when hybridized with the mutant p53 allele-specific oligonucleotide. This probe detects the C to G transversion at amino acid 179 defined in the anaplastic portion of tumor 7907 (Fig. 1). The filters were then stripped and hybridized with a wild-type p53 oligonucleotide probe to detect all p53 containing clones (Fig. 2). There are a small number of cells with wild-type p53 alleles in the anaplastic region as indicated by the presence of clones which hybridized to the wild-type p53 oligonucleotide but not to the mutant p53 oligonucleotide (see crosses in Fig. 2). These likely represent contamination of the anaplastic region by a small number of non-anaplastic cells. Clearly no cells within the non-anaplastic region of tumor 7907 have an altered p53 gene indicating that p53 mutations are rare within non-anaplastic cell populations.

The growth rate of a tumor is defined by the balance of cell proliferation and cell death. Cell death may occur by the distinct processes of necrosis or apoptosis. Necrosis in a tumor usually occurs as a result of hypoxia affecting zones of contiguous cells whereas apoptotic cells are generally isolated and surrounded by viable neighbours (15). Distinguishing features of apoptosis include chromatin condensation, cytoplasmic shrinkage, and DNA fragmentation (15). Over-expression of wild-type p53 protein has been shown to sensitize transformed cells to apoptosis (16), whereas defects in the p53 gene are associated with attenuated apoptosis and chemo- and radio-resistance in a mouse sarcoma model (17). WTs have been shown to over-express the p53 gene product (ref. 18, 34 of 34 tumors analyzed), and accordingly, cells from non-anaplastic tumors may be expected to be sensitized to apoptosis. Tumors 7907 and 8834 provided us with the opportunity to determine if transformed cells harboring p53 mutations show decreased apoptosis compared to adjacent transformed cells with wild-type p53 alleles in a human cancer. The number of cells undergoing apoptosis in anaplastic (p53 mutant) versus non-anaplastic (wild-type p53 gene) regions from tumors 7907 and 8834 was directly compared. This was achieved by using a modification of the TUNEL procedure to visualize areas of increased DNA fragmentation *in situ* (12). The direct specific labeling of DNA breaks in nuclei demonstrated a higher apoptotic index in the non-anaplastic tumor regions [7.1 % (7907); 6.6% (8834)] relative to the anaplastic areas [<1.0% (7907): <1.0% (8834)] (Fig. 3). From the random distibution of dying cells in the non-anaplastic sections, it is apparent that apoptosis is not induced solely by over-expression of wild-type p53 in WTs and may require the accumulation of additional oncogenic insults to actuate the cell death program. Alternatively a certain threshold of p53 over-expression may be required, with the absolute levels of p53 differing among cells. The presence of a small number of apoptotic cells in the anaplastic region may be due to the presence of non-anaplastic cells

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activating the cell death program or triggering of a p53-independent pathway in anaplastic cells. A similar distribution of apoptotic cells in p53$^{+/+}$ or p53$^{-/-}$ tissue has also been noted by Seymonds et al. (5) in transgenic mice. These results suggest a link between wild-type p53 expression and apoptosis in human cancers, consistent with predictions from experiments in cell culture and murine model systems (4,5,16). Precisely how early in clonal evolution of WTs upregulation of p53 expression occurs remains to be defined.

Our results are consistent with the notion that p53 can directly limit tumor progression by enhancing apoptosis (15,16). Cells acquiring p53 mutations would have a selective advantage - being more refractory to apoptosis in response to physiological situations that would normally counter tumor growth, or cytotoxic stimuli encountered during chemotherapy. This is in keeping with the more aggressive nature of anaplastic cells since relapse-free survival is inversely related to the number of anaplastic cells observed per microscopic field analyzed histologically (8). A substantial number of anaplastic WTs are successfully treated in the clinical setting (7,8) possibly reflecting confinement of the anaplastic cells within the tumor bed which can be successfully excised during surgical intervention. Additionally, more aggressive treatment of this histological variant (7) may in turn activate a p53 independent apoptotic pathway. Such a mechanism has been demonstrated by Lowe et al. (4) where high concentrations of chemotherapeutic agents were capable of inducing apoptosis in p53 deficient cells.

Non-anaplastic cells from tumor 5191 are heterozygous for a mutant p53 allele. The anaplastic portion of this tumor is homozygous for the same p53 mutation, indicating that it has arisen by clonal progression from non-anaplastic cells. This tumor provides genetic evidence for the clonal evolution of anaplasia from more favorable histological variants. Two previous reports have also documented p53 lesions in non-anaplastic WTs (19,20). Such lesions are rare (refs. 9,19,20; 2 samples of 143 analyzed), and when characterized, have been found to be heterozygous (20). The high correlation between p53 mutations and the anaplastic phenotype (ref. 9 and this report) suggests that p53 mutations are necessary, but not sufficient, for progression to anaplasia with additional events being required to achieve commitment to the anaplastic phenotype. This may include loss of the normal p53 allele since most of our anaplastic samples show a complete absence of wild-type p53 allele (9, this report).

The presence of p53 mutations in anaplastic tumors correlates well with the genetic features of anaplasia and biological properties of the p53 protein. Anaplastic tumors are characterized by chromosomal instability with hyperdiploid content (>70 chromosomes) and numerous complex translocations while non-anaplastic WTs lack such abnormalities (21). A similar correlation has been noted between colorectal cancers with alterations in p53

(detected by immunocytochemical stabilization) and aneuploid clonal divergence (22). Our data are consistent with Nowell's model for acquired mutations during tumor progression (23), and suggest that p53 loss can lead to attenuated apoptosis in a human cancer. The generally favorable prognostic outcome of non-anaplastic WTs may be due to the presence of wild-type p53 protein (9,18), which mediates initiation of apoptosis in response to appropriate chemotherapeutic agents (4).

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TABLE 1 P53 Gene Mutations in Paired Wilms' Tumor Samples with
Anaplastic and Favorable Histology

Sample	Histology	Codon	Sequence Change	Amino Acid Change	Mutation Present in Non-Anaplastic Tumour Cells?
5191	Focal Ana.	248	CGG to CAG	Arg to Gly	Yes[1]
7907	Focal Ana.	179	CAT to GAT	His to Asp	No
8834	Focal Ana.	175	CGC to CAC	Arg to His	No
8465	Focal Ana.	-	None Detected	-	-
6481	Diffuse Ana.	266	GGA to GTA	Gly to Val	No
8868	Diffuse Ana.	156	CGC to CCC	Arg to Leu	No
AN.-1[2]	Diffuse Ana.	175	CGC to CAC	Arg to His	No

All specimens have been histologically reviewed by one of us (J.B.B.) and focal regions of anaplasia clearly demarcated for molecular genetic studies. DNA was isolated from tumors and analyzed as described in the Materials and Methods.

[1]DNA from non-anaplastic cells of this sample demonstrated a heterozygous mobility shift when analyzed by PCR-SSCP, whereas DNA from anaplastic cells showed loss of the wild-type p53 allele. Normal tissue was not available from this child to determine if the mutation is germline.

[2] The p53 mutation in the anaplastic portion of this sample has been previously defined (9). We recently obtained a non-anaplastic portion of this tumor and analyzed it for p53 mutations as described in the Materials and Methods.

FIGURE LEGENDS

FIG. 1. Micrographs and PCR-SSCP analysis of the p53 gene in tumors 7907 (A) and 8834 (A) having two distinct cellular patterns: non-anaplastic (upper panel) and anaplastic (lower panel). The PCR-SSCP result of each analysis is shown to the right of the micrographs. In A, DNA for the amplification reaction was from the following sources: lane 1, no input DNA; lane 2, non-anaplastic tumor bed; lane 3, anaplastic tumor bed; lane 4, blood of an unaffected individual. In B, DNA for the amplification reaction was from the following sources: lane 1, no input DNA; lane 2, non-anaplastic tumor bed; lane 3, anaplastic tumor bed. The non-anaplastic micrographs were taken at an original magnification of 100x, whereas the anaplastic ones were at 200x.

FIG. 2. P53 gene status in anaplastic and non-anaplastic cells from tumor 7907. Following amplification of p53 exon 5 from tumor 7907 with oligonucleotides 90 (^{5}'TCACTTGTGCCCTGACTTTCAACT^3') and 5R (^{5}'CCCCAGCTGCTCACCAT^3'), DNA was gel purified and subcloned in pKS II+. Bacterial colonies were replica plated onto nitrocellulose filters and processed for colony hybridization with oligonucleotides as previously described (23). The mutant oligonucleotide 7907ASO (^{5}'CCCCCAC$\underline{G}$ATGAGCG^3') (where the underlined G detects the mismatch) and wild-type oligonucleotide C1 (5'TTCCTCTTCCTGCAGTACT3') were used to screen colonies containing the mutant p53 allele or containing a p53 gene insert, respectively. Filters in (A) were probed with oligo 7907ASO, whereas those in (B) were hybridized with C1. The source of the PCR product is indicated below the filter. *Arrowheads* and open triangles position the colonies for reference points and *crosses* beside the *arrowhead* or open triangle identify some of the colonies hybridizing to C1 but not 7907ASO, indicate the presence of a wild-type p53 allele within the anaplastic tumor region. Note that the two upper filters are inverted with respect to each other.
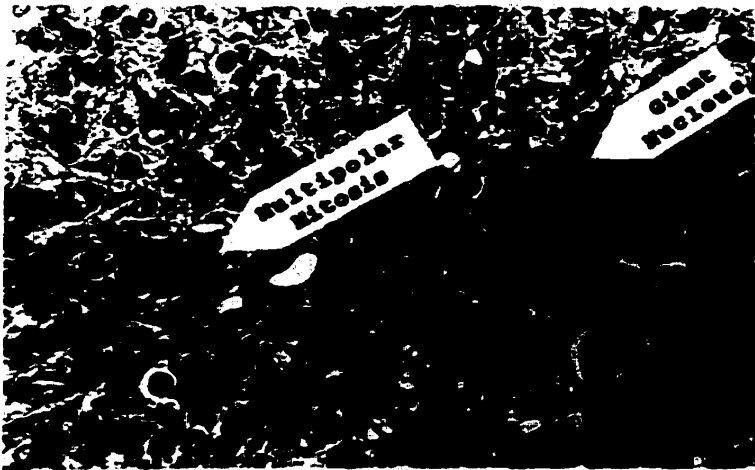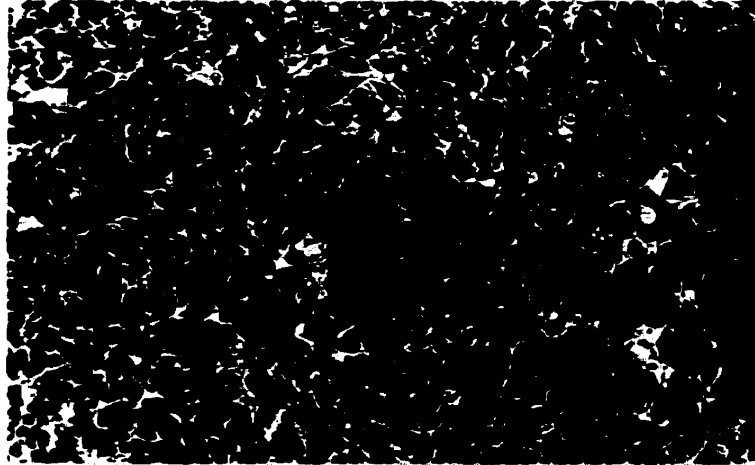
FIG. 3. Anaplastic cells show decreased apoptosis relative to non-anaplastic transformed cells from the same tumor. Sections from tumors 7909 and 8834 were subjected to the modified terminal deoxynucleotide transferase-mediated dUTP-biotin nick end labelling procedure. Micrographs of fields from the same section containing non-anaplastic cells (upper panels) or anaplastic cells (lower panels) are shown. Small arrows denote some of the cells showing nuclear staining. A peculiarity noted with this assay was background staining within the cytoplasm of the large, anaplastic cells, whereas very few cells showed

cytoplasmic staining in the non-anaplastic regions.Original magnification of all micrographs are at 500x.

1 A.

...CCCCAC**C**ATGAGCGC...
...Pro His His Glu Arg...

↓

...CCCCAC**G**ATGAGCGC...
...Pro His Asp Glu Arg...

1B

...GTGAGGC**G**CTGCCCCCAC...
...*Val Arg Arg Cys Pro His*...

...GTGAGGC**A**CTGCCCCCAC...
... *Val Arg His Cys Pro His*...

2
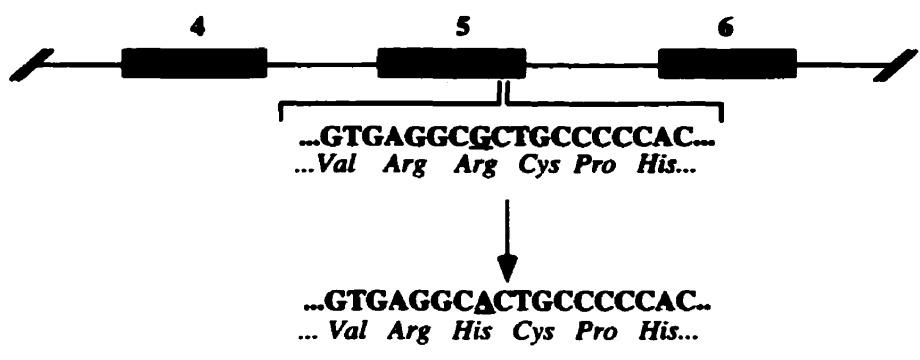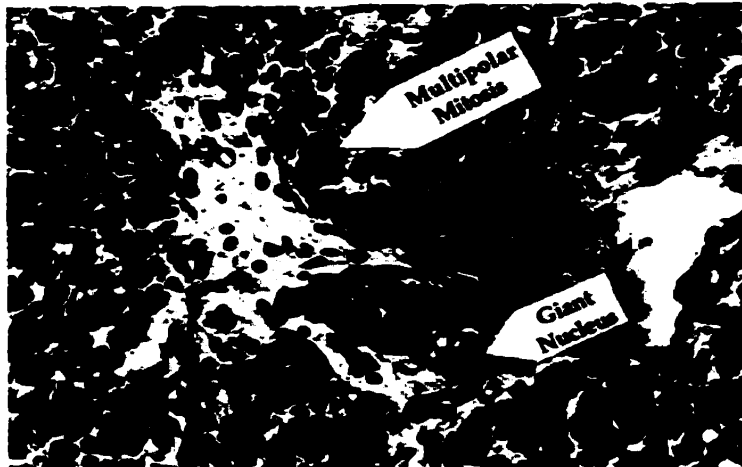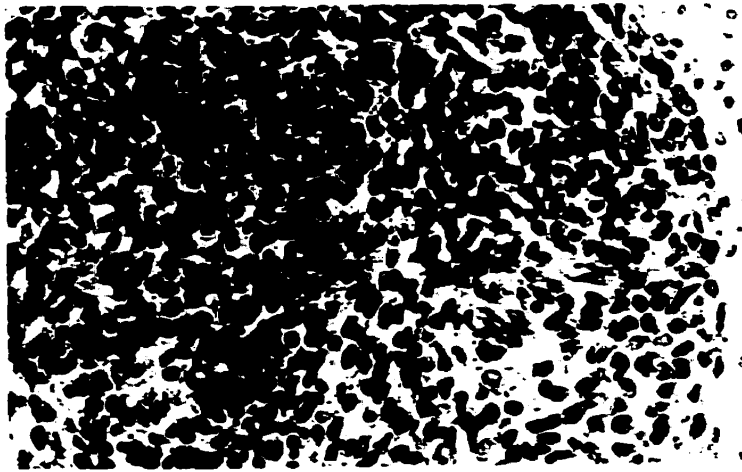
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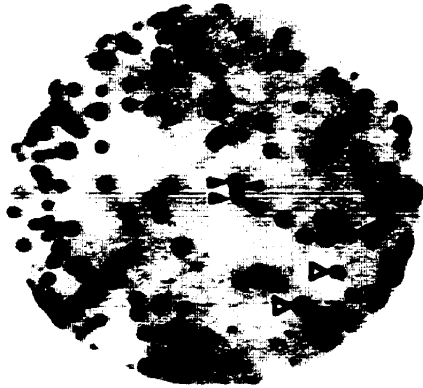
Oligo 7907ASO
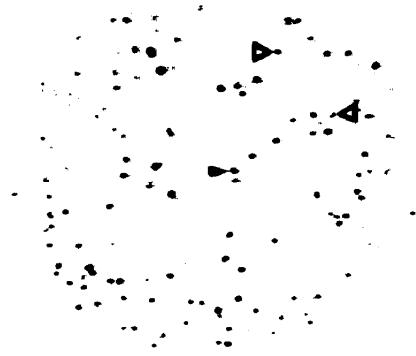
Anaplastic

Anaplastic

Non-Anaplastic

Non-Anaplastic

3

7907-Non-anaplastic

8834-Non-anaplastic
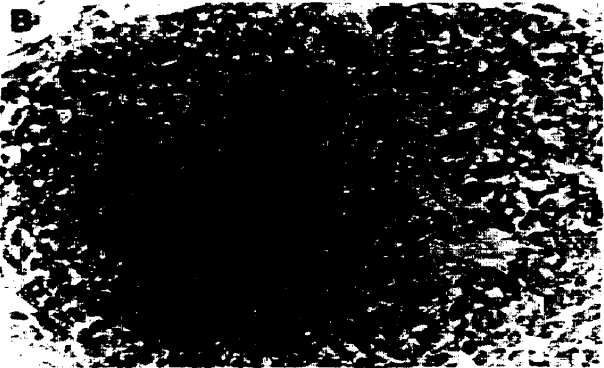
7907-Anaplastic
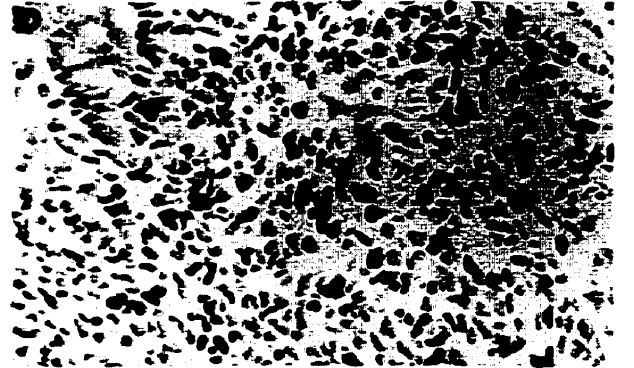
8834-Anaplastic

CHAPTER 5

Having completed the series of genetic studies on Wilms' tumors described in previous chapters, we next undertook to employ some of this genetic data which we obtained to help study some of the functional properties of the WT1 protein. In particular we were studying the effects of the mutations which had been identified on the transcriptional properties of WT1. During this time reports appeared suggesting that WT1 may have a role not just as a DNA binding protein but also in RNA metabolism. Imm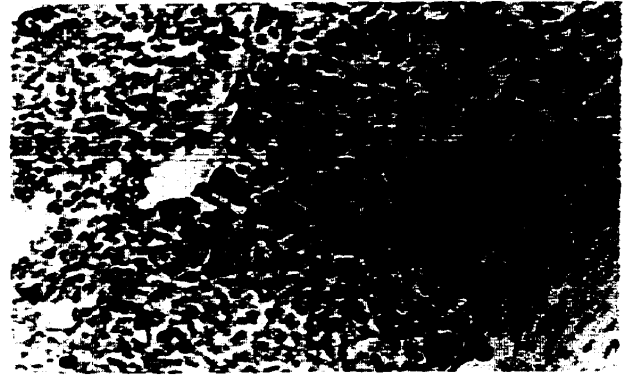unofluorescence data showed that certain WT1 isoforms were distributed in nuclear compartments where splicing factors were localized, and additionally it was possible to immunoprecipitate WT1 with antibodies to these splicing factors. It was also reported that the subnuclear localization could be RNAse sensitive. Another group demonstrated that in gel shift assays that the WT1 zinc fingers could bind to sequences in the insulin-like growth factor II mRNA, a gene previously demonstrated to be regulated by WT1. Also, computer modelling predicted that the amino terminus of WT1 has structural homology to the RNA recognition motif of splicing factor U1A. Based on these observations we wished to analyse the possible role for WT1 in RNA metabolism. We wished to address whether WT1 can bind to specific RNA targets by using an RNA selection method.

Overlapping RNA and DNA Binding Domains of the *wt1* Tumor Suppressor Gene Product

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(WT1/ RNA Binding/ SELEX/)

Running Title: RNA Binding by WT1

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ABSTRACT

The Wilms' tumor suppressor gene, *wt1*, is mutated in a subset of patients with Wilms' tumor and has a critical role in urogenital development. *wt1* encodes a zinc finger transcription factor which regulates the expression of several genes involved in cellular proliferation and differentiation. Although a number of studies have characterized the DNA binding properties of the WT1 protein, recent evidence has suggested that WT1 may also have a role in RNA metabolism. We have used an RNA selection method to identify WT1-binding ligands from a random RNA pool. Three groups of RNA ligands specifically recognized by WT1 were identified. Mutational analysis pinpointed ribonucleotide sequences critical for binding. The analysis of truncated WT1 proteins demonstrated that three of four zinc fingers were necessary for RNA-protein interaction. The naturally occurring WT1 isoforms with the insertion of lysine, threonine and serine between zinc fingers three and four were unable to bind the selected RNAs. The selected RNA ligands competed with the cognate WT1 DNA binding site for complex formation with WT1. Our findings suggest potential cellular RNA target sequences for WT1 and provide tools for studying the structural and functional properties of this tumor suppressor protein.

INTRODUCTION

Wilms' tumor (WT) is a malignancy which affects 1 in 10 000 children, usually before the age of five years (1). It is thought to arise when multipotential cells of the metanephric blastema fail to differentiate and remain locked in a state of continual proliferation. WT has long been considered an excellent model for studying the relationship of cancer to development. The tumors derive from mesenchymal stem cells which would normally differentiate into epithelial components of the nephron. These tumors are remarkable in attempting to recapitulate the different stages of nephron development, albeit abnormally. A tumor suppressor gene, *wt1*, implicated in predisposition to WT, has been identified by positional cloning at chromosome 11p13 (2, 3). *Wt1* has been extensively characterized and is mutated in 10-15% of sporadic WTs (4, 5) and in some hereditary WT cases (6). Genetic evidence suggests that *wt1* mutations are involved in the initiation of this disease (7). *Wt1* is also thought to play a functional role in the inductive processes of urogenital development (reviewed in ref. 8). Consistent with this hypothesis is the observation that mice in which the *wt1* gene is homozygously deleted fail to develop kidneys and gonads (9). As well, germline *wt1* lesions in humans are associated with predisposition to WTs and aberrant differentiation of the urogenital system (6, 10).

The *wt1* gene encodes a protein having many characteristics of a transcription factor, including a glutamine/proline-rich amino-terminus, nuclear localization, and four Cys_2-His_2 zinc finger motifs (reviewed in ref. 8). The three carboxy-terminal-most zinc fingers share 64% identity to the three zinc fingers of the early growth response gene-1 (EGR-1). The mRNA contains two alternative sites of translation initiation (11), two alternatively spliced exons (12, 13), and undergoes RNA editing (14), thus potentially encoding 16 different protein isoforms with predicted molecular masses of 52-65 kDa. The function of the alternative translation initiation event, the RNA editing, and the first alternative splicing event (exon V), have not been well defined, although exon V can repress transcription when fused to a heterologous DNA binding domain (15). Alternative splicing of exon IX inserts or removes three amino acids (+/-KTS) between zinc fingers III and IV and changes the DNA binding specificity of WT1 (16). The WT1(-KTS) isoforms can bind to two DNA motifs: i) a GC rich motif, $5'GG^G/\gamma GTGGGC^G/C3'$, similar to the EGR-1 binding site (16); and ii) a $(^5TCC^{3'})_n$ containing sequence (17). The DNA binding properties of the WT1(+KTS) isoforms are not well understood, since no high affinity, specific binding site has been elucidated for these splice variants. A number of genes involved in growth regulation and cellular differentiation contain WT1 binding sites

within their promoters and their expression can be modulated by WT1 in transfection assays (reviewed in ref. 8). The *wt1* gene product mediates both transcriptional repression and activation, depending on the architecture of the promoter under study and the cell lines in which the assays are performed (18).

Recently, Larsson *et al.* (19) demonstrated that different WT1 isoforms localize to distinct compartments of the nucleus: with -KTS isoforms displaying a distribution that parallels that of classical transcription factors such as Sp1 and TFIIB; whereas +KTS isoforms are preferentially associated with interchromatin granules and coiled bodies. These results were reproduced and extended by Englert *et al.* (20). Caricasole et al. (21) have shown that the subnuclear localization of WT1(+KTS) is RNAse, but not DNAse, sensitive. It was also demonstrated that WT1, but not EGR-1, can bind Igf-II exonic RNA sequences and that this binding is mediated by the zinc finger domain (21). In addition, Kennedy *et al.* (22) used protein structural modeling techniques to identify a region in the amino terminal region of WT1 which is predicted to have structural homology to the RNA recognition motif of splicing factor U1A. These results suggest that WT1 may regulate gene expression by both transcriptional and post-transcriptional mechanisms.

In this study we have identified RNA ligands to WT1 as an initial step in determining a possible role for this protein in RNA metabolism. These ligands were capable of binding to the WT1(-KTS) isoform indicating that this spliced variant is also capable of interacting with RNA in a specific fashion. These RNA ligands may also be useful tools for structural and functional studies of this protein.

MATERIALS AND METHODS

Plasmid Construction.

Preparation of plasmid DNA, restriction enzyme digestion, agarose gel electrophoresis of DNA, DNA ligation, and bacterial transformations were carried out using standard methods (23). Subclones of DNA PCR amplifications were sequenced by the chain termination method (24) using double-stranded DNA templates to ensure the absence of unwanted secondary mutations.

To generate pET15B/WT(+/-), the 1.5 kbp Sau3A1 fragment from the full length mouse WT1 cDNA (25) was cloned into the Bam H1 site of pET15B (Novagen). The sequences coding for the carboxy terminus (codons 297-449) were replaced by a synthetic gene fragment generated by overlap extension PCR (16) in order to obtain favorable codon usage for expression in bacteria (see Fig. 1A). To obtain pET15/WTZF[(I-IV)-KTS] and pET15/WTZF[(I-IV)+KTS], the synthetic gene fragment (codons 297-449) with or without the alternatively spliced codons, respectively, were cloned into the Bam H1 site of pET15B. pET15B/WTZF[(I-III)-KTS] codes for amino acids 297-422 and was obtained by digesting pET15B/WTZF[(I-IV)-KTS] with Xmn I and Blp I to excise the 3'portion of the gene, followed by Klenow repair to create blunt ends and religation of the vector. The resulting plasmid has sequences derived from pET15B at the 3' end coding for an extra 22 amino acids. pET15B/WTZF[(II-IV)-KTS] codes for amino acids 342-449. pET15B/WT(+/-)^{394}R/W contains the most common WT1 zinc finger Denys-Drash syndrome mutation (10) in the context of the full length cDNA. pET15B/WT(Del) is missing a cytosine residue at codon 368 which results in premature termination of translation at codon 370 (Fig.1). The expression vector for generating the EGR-1 zinc finger domain was derived by cloning the 469 bp Fsp I fragment (encoding the zinc finger region) of the mouse EGR-1 cDNA (26) into pET15B, which had been linearized with Xho I and converted to blunt ends with Klenow polymerase.

Protein Purifications.

For the expression of recombinant proteins, pET15 expression vectors were introduced into the BL21 (DE3) pLysS strain of *Escherichia coli* and proteins were induced using the recommended conditions (Novagen). Proteins were purified using nickel-chelate affinity chromatography (Qiagen) under native conditions as recommended by the manufacturer. Eluted proteins were dialyzed against a buffer containing 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; pH 7.5), 70 mM KCl, 12 % glycerol, 0.05% NP-40, 100 μM ZnSO$_4$, and 0.5 mM DTT. The purity and integrity of

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the fusion proteins was assessed by Coomassie blue staining of SDS-polyacrylamide gels (for example, see Fig. 1B).

Identification of RNA binding Sites by Systematic Evolution of Ligands by Exponential Enrichment (SELEX).

For generation of a random DNA template, PCR amplification was performed using 5 µg of N20 (^{5}TGGGCACTATTTATATCAAC(N)$_{20}$AATGTGTCGTTGGTGGCCC^3), an oligonucleotide consisting of 20 degenerate nucleotides flanked by T7 and Rev primer binding sites, and 10 µg each of T7 (^{5}CGCGGATCCTAATACGACTCACTATAGGGGCCACCAACGACATT^3) and Rev (^{5}CCCGACACCCGCGGATCCATGGGCACTATTTATATCAAC^3) oligonucleotides as described (27). Half of the amplification product was transcribed with T7 RNA Polymerase (NEB) in the presence of α-^{32}P-CTP (3000 Ci/mmol) to yield about 5 µg of radiolabelled random RNA sequences. The RNA was gel purified on an 8% sequencing gel, eluted into 0.5 M ammonium acetate, and ethanol precipitated. For selection, the RNA was resuspended in SELEX buffer (20 mM HEPES [pH 7.5], 70 mM KCl, 2 mM MgCl$_2$, 12.5% glycerol, 0.5 mM DTT, 50 µM ZnSO$_4$, 0.05% NP-40), heated to 65 °C, and slowly cooled to room temperature. The RNA was added to a binding reaction with 10 µg of WTZF[(I-IV)-KTS] fusion protein in 100 µl of SELEX buffer. Binding was allowed to proceed for 30 min at 4 °C and the mixture was then filtered through a nitrocellulose filter (Millipore HAWP 02500) which had been prewashed with 50 mM Tris-HCl (pH 7.5). Filters were rinsed with 50 mM Tris-HCl (pH 7.5) and the RNA was eluted and precipitated as described (28). The selected RNA was reverse transcribed at 42°C for 1 hour using 100 ng of Rev oligonucleotide with Superscript II Reverse Transcriptase (Gibco BRL) as recommended by the supplier. Subsequent cycles of PCR and *in vitro* transcription were performed as described (27). The amount of WTZF[(I-IV)-KTS] protein used for RNA selection was reduced by approximately one half every second cycle. The final round of selection involved incubating 1 µg of protein and 1 µg of RNA in SELEX buffer plus 5 µg of calf liver tRNA on ice for 30 minutes. The RNA-protein mixture was electrophoresed on a 4% polyacrylamide/ 0.5X TBE gel at 140 V at 4°C. The wet gel was exposed to Kodak X-Omat film, and the RNA-protein complex was excised from the gel by alignment with the developed film. The RNA in the complex was eluted from the gel

and precipitated as described above. Following reverse transcription and PCR, the SELEX products were digested with Bam HI, converted to blunt ends with Klenow polymerase, and cloned into pUC18 linearized with Sma I. The SELEX clones were analyzed by sequencing miniprep DNA. To generate RNA for EMSA, PCR products amplified directly from the bacterial colonies were used to generate templates for RNA synthesis.

Electrophoretic Mobility Shift Assay (EMSA).

EMSAs were performed with 50 ng of recombinant protein unless otherwise indicated. The binding reactions were performed in a total of 15 μl in binding buffer (20 mM HEPES-KOH [pH 7.5], 50 mM KCl, 5 mM $MgCl_2$, 10 μM $ZnSO_4$, 0.67 mM DTT, 12% glycerol, 10 μg BSA, 1 μg poly(dI)-poly(dC), 1μg calf liver tRNA). Binding reactions were pre-incubated for 15 min at 25 °C in the absence of RNA probe. Radiolabelled RNA (20 fmol) was heated to 65 °C, slowly cooled to room temperature, then added to the binding solution. Incubation of the RNA-protein mixture was for 20 min at 25 °C. For supershift experiments, 200 ng of antibody was subsequently added and incubation was continued for a further 15 min at 25 °C. For competition experiments, competitor RNA was labelled with ^{3}H-CTP during the *in vitro* transcription reactions, thus allowing for accurate quantitation of RNA amounts. When competition experiments were performed, ^{3}H-labeled RNA or unlabelled DNA oligonucleotide was pre-incubated with the recombinant protein for 15 min before the addition of the probe. Protein-RNA complexes were resolved on 0.5X TBE-4% polyacrylamide gels electrophoresed at 140V at 4 °C. Dried gels were exposed overnight to X-Omat film (Kodak) at -70°C overnight with an intensifying screen. PhospoImager scanning (Fuji) was used to quantitate the efficiency of complex formation.

RESULTS

Several members of the Cys_2- His_2 zinc finger family are known to interact with both DNA and RNA (see Discussion). There is also suggestive evidence that the WT1 (+KTS) isoforms may be involved in RNA metabolism (see Introduction). *In vitro* studies have also shown that the WT1 (-KTS) isoforms can bind to sequences in exon 2 of the Igf-2 mRNA (21). We have examined the RNA binding properties of WT1 by using an affinity elution-based RNA selection to identify specific WT1-binding RNA ligands (29). We employed Systematic Evolution of Ligands by Exponential Enrichment (SELEX) with a recombinant WT1 protein encoding zinc fingers I-IV and a $(His)_6$ tag at the amino terminus WTZF[(I-IV)-KTS]. In order to maximize expression levels of recombinant WT1 protein, we generated a synthetic gene fragment harboring favorable codon bias for expression in *E. Coli* (Fig. 1A). The bacterially expressed protein was purified using nickel-chelate chromatography and assessed to be >90% homogenous by SDS-PAGE (see Fig. 1B, lane 7). RNA was transcribed from a pool of PCR-amplified oligonucleotides composed of 20-nucleotide random-mers with an estimated complexity of 10^{14} molecules. RNA binding to WT1 (-KTS) was enriched using ten rounds of nitrocellulose filter binding/ reverse transcription-PCR amplification, followed by one round of selection by electrophoretic mobility shift assay (EMSA). EMSA analysis comparing the RNA pools at the second and eighth cycle of SELEX revealed a clear enrichment for WT1-binding RNA sequences (Fig. 2A, compare lane 4 to 2). Following cloning of the SELEX products, individual clones were analyzed by EMSA. All clones analyzed showed specific binding when incubated with 50 ng of protein (Fig. 2B and data not shown), whereas RNA generated from a clone of the random pool of unselected PCR products showed no complex formation (data not shown). Sequence analysis of 31 clones revealed that they could be classified into 3 distinct families, based on nucleotide conservation at specific residues (Fig. 2C).

The largest group with eighteen clones, family A, is characterized by high sequence conservation within the first nine nucleotide positions with identity at five of these positions (Fig. 2C). Family B contains three members and is characterized by the core motif: ^{5}GAAUG^3. Family C contains 10 members and is characterized by the core consensus: ^{5}CCC(A/G)^3 (Fig. 2C). Computer analysis using the Zuker RNA folding algorithm (30) failed to predict conserved stable secondary structures for these RNAs (N.B., unpublished data). To characterize the WT1-RNA interaction, we quantitated the binding of RNA species #22 to WTZF[(I-IV)-KTS]. In an EMSA, WTZF[(I-IV)-KTS] demonstrated saturable binding to RNA #22 (K_D of ~ .7 μM) (Fig. 2D). This dissociation

constant is slightly higher than that reported for bacterially produced recombinant WTZF[(I-IV)-KTS] to DNA target sites (K_D of ~ 0.14 µM) (31).

To establish the specificity of the WT1/RNA interaction a number of competition experiments were performed. Competition with RNA ligands from the three families indicated that all were capable of significantly reducing complex formation of RNA ligand #22, when present at a 50-fold molar excess (Fig. 3A, compare lanes 3 - 5 to lane 2). An unrelated RNA sequence did not compete for WT1 RNA binding when present at 50-fold molar excess (compare lane 6 to 2). Complex formation by probes from the other two RNA families (i.e. - RNA ligand #38 and #20) were also inhibited by these same specific competitor RNA ligands (data not shown). To determine if DNA and RNA binding to WT1 were mutually exclusive, we incubated a radiolabelled double stranded (ds) DNA oligonucleotide probe containing the cognate WT1 binding site with WTZF[(I-IV)(-KTS] in the presence of 50-fold molar excess of a member from each RNA ligand family. The RNA ligands significantly reduced WT1-DNA complex formation (Fig. 3B, compare lanes 3 - 5 to lane 2). A random RNA sequence did not compete for WT1 binding to DNA when present at 50-fold molar excess (N.B., data not shown). Additionally, WT1/RNA complexes can be inhibited by pre-incubation of WT1 with DNA oligonucleotides containing a WT1 DNA binding site (Fig. 3C, compare lane 5 to 2), whereas an ETS binding site, which is not recognized by WT1, shows no effect on WT1-RNA complex formation (compare lane 6 to 2). We conclude that WT1 zinc fingers can bind to DNA and RNA, but binding to both is mutually exclusive.

Supershift experiments of the protein-RNA complexes revealed a slow migrating complex in the presence of C19, an anti-WT1 antibody directed against the C-terminal domain of the protein (Fig. 3C, compare lane 7 to 2 and lane 11 to 10). The specificity of this complex was demonstrated by the use of a control antibody, WT180, which recognizes an N-terminal epitope of full length WT1 protein, absent in WTZF[(I-IV)-KTS]. WT180 was unable to supershift the WTZF[(I-IV)-KTS]-RNA complex (compare lane 8 to 2). The supershifted complex was specific for the WTZF[(I-IV)-KTS]-RNA complex since incubation of the C19 antibody did not show cross-reactivity with the RNA (compare lane 12 to 11). The observed WT1-RNA interactions are not particular to the bacterially expressed protein since in vitro synthesis of WT1 in rabbit reticulocyte lysates produced protein capable of specific WT1-RNA complex formation (data not shown).

To probe the structural requirements for WT1/RNA interaction, the binding capacity of a series of WT1 proteins was studied using RNA ligand #22. SDS-PAGE analysis of these purified, recombinant proteins is shown in Fig. 1B. The presence of the alternatively

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spliced amino acids, KTS, between zinc fingers three and four greatly reduced RNA binding (Fig. 4, compare lane 3 to 2). This protein shows a similar reduced ability to bind to EGR-1 DNA recognition sites (10). Deletion of the first zinc finger produced a recombinant protein still capable of forming protein/RNA complexes (compare lane 4 to 2), whereas removal of zinc finger IV significantly reduced RNA recognition (compare lane 5 to 2). Full length recombinant WT1(+/-) was also able to form specific complexes with this RNA (lane 6). An arginine to tryptophane mutation at codon 394 of WT1 represents the most commonly detected mutation in Denys-Drash Syndrome, a wt1-associated developmental disorder. This protein is unable to bind the cognate WT1 DNA binding site (10). This mutation, in the context of the full length recombinant WT1(+/-) protein showed a 10-fold reduction in RNA binding (compare lane 7 to 6). This effect, although strong, is less dramatic than the complete loss of DNA binding observed for this mutant. A WT1 mutant containing an intact amino terminus but lacking part of zinc finger II and all of zinc fingers III and IV cannot bind RNA efficiently (compare lane 8 to 2). We have found that bacterially purified EGR-1 can also bind to RNA #22 (compare lane 9 to 2) suggesting that, like the EGR-1 DNA recognition motif, RNA #22 might be recognized by a number of proteins with related zinc fingers. Likewise, we have found that the other two families of ligands have similar protein structural requirements for binding to WT1 (N.B., data not shown). Taken together these data demonstrate that residues within WT1 zinc fingers II-IV are sufficient and necessary for RNA binding and that the DNA and RNA sites are overlapping.

To identify primary nucleotide sequence requirements for binding, mutagenesis was performed using clones #22 and #38. A series of RNA probes with mutations of the highly conserved nucleotides as well as flanking sequences was employed in EMSA (Table 1). All mutations tested in the context of RNA #22 abolished recognition by WT1, indicating that the RNA recognition site extends over the region which characterizes this family. With respect to RNA #38, all mutant products, with the exception of that derived from M2 abolished RNA recognition. The mutation harbored by M2 showed weak but detectable binding to WT1. These results establish specific sequence requirements for WT1 recognition of the RNA ligands.

We performed BLAST searches using the SELEX ligands to identify naturally occurring RNAs which could potentially be in vivo targets for WT1(-KTS). For query sequences in the searches we employed the first 11 nucleotides of the family A ligands since these showed the largest regions of homology. Table 2 lists a number of genes identified from data bases which match RNA ligands and which may have relevance to

WT1 function. It remains to be established whether WT1 can bind to any of these potential targets *in vivo*.

DISCUSSION

Zinc finger domains, found in a large family of proteins, were originally characterized for their ability to bind DNA in a sequence-specific manner. The Cys_2-His_2 subfamily of zinc finger proteins has been extensively studied. This small, independently folding motif has regularly spaced cysteine and histidine residues which ligand one zinc ion (32). X-ray crystallographic and NMR studies have shown that these domains are composed of a B-hairpin followed by an alpha helix (33, 34). Structural studies of the zinc finger domains of Zif268 bound to a cognate DNA binding site have demonstrated that sequence-specific binding is acquired through direct nucleotide contact by three amino acids in the alpha helical region (34). These studies have lead to proposals of a general code for sequence-specific recognition of DNA by zinc fingers (35 - 37). However, it is now clear that rather than simply being DNA binding modules, these domains may behave in a more complex, multifunctional manner. The *Xenopus laevis* TFIIIA protein contains nine zinc fingers of the Cys_2-His_2 class which mediate specific binding to both the 5S rRNA gene (38) and 5S ribosomal RNA (39), implicating a role in both gene transcription and RNA metabolism. The human and mouse MOK2 proteins have ten and seven Cys_2-His_2 zinc fingers, respectively, and show a similar ability to bind specifically to both DNA and RNA (40). In addition to nucleic acid binding, Cys_2-His_2 zinc fingers can mediate protein-protein interactions. The zinc fingers of GATA-1 specifically interact with the Cys_2-His_2 zinc fingers of both Sp1 and EKLF (erythroid Krüppel-like factor), (41). The Sp1 zinc fingers also interact with the amino terminal DNA binding domain of RelA(p65) (42) and the Cys_2-His_2 zinc fingers of YY1 (43). Each of these interactions leads to cooperative DNA binding. The zinc finger can also harbor a nuclear localization signal (44). Another interesting feature of some Cys_2-His_2 zinc fingers proteins is the ability to bind to DNA-RNA hybrids with affinities comparable or higher than for DNA duplexes (45). Although the significance of this interaction is unknown, it is a further indication of the multifunctional nature of zinc finger domains.

The functional diversity of Cys_2-His_2 zinc fingers is well exemplified by WT1. The WT1(-KTS) isoform recognizes DNA specifically, regulating transcription through binding

to the EGR1 consensus binding site. However, the WT1 zinc finger domains have been found to mediate a number of other functions. The par-4 transcriptional repressor binds to this domain and modulates the transcriptional properties of WT1 (46). Similarly, WT1 and the p53 tumor suppressor protein influence each others transcriptional properties through an interaction mapping to the first two zinc fingers of WT1 (47). The WT1 zinc finger region also contains nuclear localization signals (20, 48). The zinc finger region of WT1 has also been reported to mediate binding to sequences in exon 2 of the Igf-2 mRNA (21). This observation together with data showing association of WT1(+KTS) with components of the RNA splicing machinery (19) has lead to suggestions that WT1 may be involved in RNA metabolism and may indeed be an RNA-binding protein.

In this study, we used the SELEX methodology to identify three unrelated families of RNA ligands which show specific binding to WT1(-KTS) with affinities comparable to that seen for binding of this protein to DNA [see Fig. 1D and ref. 31]. The structural requirements of WT1 are similar for binding to the different families of RNA ligands and they also share features in common with WT1(-KTS) recognition of DNA. The zinc fingers critical for binding are the same, that is, the first zinc finger is not required while zinc fingers II-IV are indispensable. Additionally, the WT1(+KTS) isoform and the WT1(+/-) [394]R-W mutant show reduced ability for binding to both DNA and RNA. These data, along with the observation that DNA and RNA can compete with each other for binding to WT1(-KTS), demonstrate that they likely interact with the same or overlapping regions. Due to the similarity of structural features involved in RNA and DNA binding, we addressed whether WT1 can bind to the SELEX sequences when they are present in a double stranded DNA oligonucleotide, rather than as single strand RNA sequences. No binding to WT1 was observed when representative SELEX clones were used as DNA probes in gel shift assays (data not shown).

Inspection of ligands from family A reveals conserved features in addition to the consensus sequence. All clones have i) the consensus sequence present at the same position relative to the 5' end, and ii) except for clones #25 and #44, stretches of at least three cytosines in the 3' region (Fig. 2C). These features suggest that recognition of family A ligands by WT1 may involve additional sequence information than simply the nine nucleotide consensus sequence. Alternatively, we cannot exclude the possibility that these ligands evolved from a common ancestor. The consensus sequence elements in clones from families B and C vary in their relative positions (Fig. 2C) suggesting independent evolution of the individual clones and attesting to a strong selection for the consensus sequence in the competitive binding of RNA to WT1(-KTS). Comparison of the sequences of the different families of ligands does not reveal any obvious common elements, thus, we cannot

currently define general parameters for the recognition of RNA by WT1(-KTS). It is not unusual for an RNA SELEX procedure to yield sets of unrelated high affinity ligands (28, 49). Although computer modeling did not demonstrate stable secondary structures for the RNA ligands we cannot rule out that such structures may be a component of WT1 binding to these ligands. It should be noted that TFIIIA binds DNA in a primary sequence specific manner (50, 51) but binds RNA based on conserved secondary/tertiary structure, independent of the primary RNA sequence (52).

As mentioned above, it has been shown that the WT1 zinc fingers can bind to sequences in exon 2 of the Igf-2 RNA (20). The putative site of interaction contains the same sequence as that of the cognate WT1 DNA binding site (the EGR1 site). DNA binding by WT1, however, involves recognition of the guanine-rich DNA strand (16), whereas, the observed RNA binding is to the complementary cytosine-rich strand of the Igf-2 mRNA (21). We have used an RNA probe from a circumscribed region of Igf-2 spanning the EGR1 site postulated to interact with WT1 proteins but failed to detect a specific RNA-protein complex (N.B., data not shown). Thus the parameters of the WT1-Igf-2 mRNA interaction remain unclear at this time. The data on the RNA ligands we present here, in contrast, employs competition and mutational studies to describe highly specific binding. Fifty-fold excess of specific competitor is able to eliminate the interaction while nonspecific competitor has no effect and single nucleotide substitutions completely eliminate binding.

It is possible to use SELEX products to generate ligands with higher affinity for WT1. The RNA pool from the last cycle can be subjected to selection under conditions of high stringency such as increased salt or detergent concentration. Alternatively, a SELEX clone could be mutagenized randomly. The resulting RNA pool could them be used in further rounds of selection for WT1 binding. Such a method has been successfully employed to obtain very high affinity ligands for HIV-1 Rev (53).

The RNA ligands we have identified represent potential reagents to disrupt certain aspects of WT1 function for studies of this protein in tissue culture cells. Since the ligands can compete with DNA as targets for WT1 binding they may be able to inhibit the ability of WT1 to regulate its downstream targets while leaving other functions of WT1 intact. In addition, they represent isoform-specific reagents since they would not be expected to hinder WT1(+KTS) function.

In desmoplastic small round cell tumor (DSRCT), a malignant abdominal neoplasm, a chromosomal translocation leads to the generation of a chimeric protein fusing the amino terminal region of the Ewing Sarcoma protein (EWS) to zinc fingers II-IV of WT1 (54). One of the resulting proteins, EWS-WT1(-KTS), is capable of acting as a

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dominant oncoprotein (55). EWS-WT1(-KTS) is a strong transactivator of gene expression through the behavior of the EWS moiety as a transcriptional activation domain (55, 56). Since the RNA ligands we have identified interact with zinc fingers II-IV of WT1 they likely also bind to EWS-WT1. The identification of RNA ligands which inhibit EWS-WT1 DNA binding may provide clues for the design of drugs which mimic the activity of these ligands and could potentially interfere with the activity of this oncoprotein.

It remains unclear whether WT1 isoforms are involved in post-transcriptional RNA metabolism *in vivo*. Given that our study does not address the biological significance of WT1/RNA interaction, we recommend its cautious interpretation. WT1 was initially thought to be exclusively localized to the nucleus, although recently a cytoplasmic role for WT1 has been postulated based on immunohistochemical data showing that cells treated with protein kinase A activators demonstrate cytoplasmic as well as nuclear staining (57). One therefore cannot rule out a possible role of WT1 in mRNA translation. The immunohistochemical data of Larsson *et al.* (19), showing the WT1(+KTS) isoforms colocalize with splicing factors, suggest an involvement of this isoform with nuclear RNA metabolism, possibly splicing. Structural modeling techniques (22) have suggested that a portion of the amino terminal region of WT1, spanning amino acids 20-110, may have an RNA recognition motif (RRM) based on predicted three dimensional structural (but not amino acid sequence) homology. The protein we used for the SELEX procedure lacked this region. Gel shift analysis using full length WT1 bound to the RNA with similar affinity as the zinc finger region alone (see fig. 3). Further SELEX experiments will be useful for identifying possible ligands to this putative RRM of WT1.

Interestingly, it has recently been demonstrated that p53, like WT1 also a tumor suppressor protein and transcription factor, is in a ribonucleotide complex (58) and is associated with cytoplasmic ribosomes (59) suggesting a role in RNA processing. Other classes of DNA binding domains may also possess bifunctional DNA and RNA binding capabilities, and thus be involved in transcriptional as well as post-transcriptional processes. ETS-like DNA binding domain of Spi-1/PU.1 binds to DNA in a sequence-specific manner and also exhibits binding to RNA homopolymers (60). The authors demonstrate that Spi-1/PU.1 is able to inhibit RNA splicing *in vitro*. It seems likely that similar bifunctionality will be identified for a number of other proteins originally presumed to be exclusively transcription factors. Our identification of RNA ligands for WT1(-KTS) with a complementary SELEX study of the WT1(+KTS) isofoms should provide useful materials for the study of a possible role of this protein in RNA metabolism.

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Table 1 Mutational Analysis of RNA Ligands

	RNA sequence	binding		RNA sequence	binding
#22	G<u>AU</u>**AUGG**<u>U</u>GACCACCCCGGC	+	#38	A<u>UCAC</u>**C**C<u>A</u>CCCC<u>C</u>GAGCUGGC	+
M1	**C**AUAUGGUGACCACCCCGGC	−	M1	A**G**CACCCACCCCGAGCUGGC	−
M2	GA**C**AUGGUGACCACCCCGGC	−	M2	AUC**C**CCCACCCCGAGCUGGC	+/−
M3	GAU**C**UGGUGACCACCCCGGC	−	M3	AUCA**G**CCACCCCGAGCUGGC	−
M4	GAUA**C**GGUGACCACCCCGGC	−	M4	AUCAC**G**CACCCCGAGCUGGC	−
M5	GAUAU**C**GUGACCACCCCGGC	−	M5	AUCACC**G**ACCCCGAGCUGGC	−
M6	GAUAUG**C**UGACCACCCCGGC	−	M6	AUCACCC**C**CCCCGAGCUGGC	−

Summary of nucleotide requirements for WT1 recognition of RNA ligands. Table shows RNA sequences used in EMSA with WTZF[(I-IV)-KTS]. Clones #22 and #38 were chosen as representatives for RNA families A and C, respectively. Nucleotides conserved between all members of the family are in bold. Other strongly conserved positions are underlined. Altered positions in each mutant RNA are indicated in bold. The RNA sequences are flanked by sequences derived from the T7 and Rev oligonucleotides (see Materials and Methods). The (+) and (−) symbols indicate whether or not the individual RNA probes formed complexes with WT1 protein. Weak binding (<10% of input RNA species present in complex) is indicated by (+/−).

Table 2. Genes Containing Consensus Sequences from Family A SELEX ligands

Ligand #11/#50	**GAUAUGGAUGG**		
Integrin α6 (Human)	AAGAC**GAUAUGGAUGG**GGGAG	#493	(coding)
AP-2 β (Human)	CUGGA**GAUAUGGAUGG**AUAGA	#8238	(intron)
Ligand #24	**GAUAUGGUGCG**		
B-raf oncogene (Human)	AGGGG**GAUAUGGUGCG**UUUCC	#977	(coding)
Ligand #12	**GAAAUGGAUGG**		
p107 (Human)	AUGAA**GAAAUGGAUGG**ACAUG	#401	(coding)
p107 (Mouse)	AUGAA**GAAAUGGAUGG**ACAUG	#396	(coding)
PDGFα Receptor (Mouse)	GTTTG**GAAAUGGAUGG**ACAAG	#3707	(3'UTR)
Ligand #46	**GCUAUGGUGAA**		
CHED (cdc2-related protein kinase)	GCCUC**GCUAUGGUGAA**ACCAA	#1111	(coding)

Blast searches were performed using the first 11 nucleotides from each of the ligands from family A. The sequences used for searching are displayed in bold type. Selected genes with sequences matches are displayed. The numbers to the right of the sequences refer to the relative position of the nucleotides as entered in the GenBank and EMBL data bases. The region of the gene where the sequence is located is indicated in brackets at the far right. Accession numbers: Integrin α 6 (emb-X59512), AP-2 β (emb-Y09912), human p107 (gb-L14812), mouse p107 (gb-U27177), PDGFα-R (gb-M84607), B-raf (gb-M21001), CHED (gb-M80629)

FIGURE LEGENDS

Figure 1. (A) Sequence of the synthetic gene encoding the WT1 zinc finger region. Numbers to the right refer to the amino acid residues in the context of the full length murine protein as (25). We note the reporting of a murine WT1 sequence coding for QL at amino acids 448-9 instead of HV (61). Underlined nucleotides correspond to a naturally occurring splice variant (+KTS) and are present in pET15B/WT(+KTS) but not in pET15B/WT(-KTS). The arginine residue at position 394 (**CGT**) is changed to a tryptophan in the construct pET15B/WT(+/-)^{394}R/W. The cytosine nucleotide in bold is missing in the truncated construct WT(del) resulting in a frameshift and early termination of translation. (B) Analysis of purified recombinant WT1 proteins. Nickel-chelate affinity purified, recombinant proteins (2 μg) were resolved on a 12.5% SDS-PAGE gel and stained with Coomassie blue. WTZF[(I-III)-KTS] migrates slightly slower than expected due to the presence of an extra 22 amino acids contributed by pET15B vector sequences. The sizes of the molecular mass standards (New England Biolabs) are to the left of the figure.

Figure 2. Selection of WT1 RNA ligands from a random RNA library. (A) WT1-bound sequences enriched in cycles two and eight were transcribed *in vitro* into RNA and used in EMSAs with WTZF[(I-IV)-KTS] protein. Protein-RNA complexes are indicated by an arrow and free probe by an arrowhead. The presence or absence of WTZF[(I-IV)-KTS] is indicated above the panel and the cycle number indicated below the panel. (B) EMSA used to screen clones obtained from the eleventh round of SELEX. Inserts from individual clones were amplified by the PCR and transcribed *in vitro* using oligonucleotides employed in SELEX. Following gel purification, RNA species were used in EMSA with 50 ng of recombinant WTZF[(I-IV)-KTS] and resolved on an 8% polyacrylamide gel. The RNA species used in the EMSA is indicated above the panel. (C) Nucleotide sequences of WT1 selected RNAs. Sequences of 31 PCR clones encoding RNA ligands cloned after SELEX are shown. Conserved identical nucleotides are shown in bold and nucleotide positions showing strong sequence conservation are underlined. Sequences derived from the nonrandom region of the RNA ligand (primer binding sites) are in lowercase. A consensus sequence is shown below each class of RNA ligands. (D) WT1 binding to an RNA selection ligand (#22). Increasing amounts of recombinant WTZF[(I-IV)-KTS] protein (0.26 μM to 8.32 μM concentration) was mixed with a constant amount of RNA ligand (1.3 nM). The amount of bound and free probe was quantitated after separation by gel electrophoresis using Phosphorimage analysis. The result of one representative experiment for RNA ligand #22 is shown.

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Figure 3. Specificity of WT1/RNA interaction. (A) Competition of WT1/RNA complexes by RNA ligands. Fifty-fold molar excess of unlabelled RNA was incubated with 50 ng of WTZF[(I-IV)-KTS] and radiolabelled RNA #22 probe. Complexes were resolved on 4% polyacrylamide gels, dried, and exposed to X-ray (X-OMAT) film at -70°C overnight. Reactions containing recombinant WTZF[(I-IV)-KTS] protein and competitor RNA are indicated above the panel. R6 is an RNA species generated from a clone obtained from a random unselected oligonucleotide pool. (B) Competition of WT1/DNA complexes by RNA ligands. Fifty-fold molar excess of unlabelled RNA was incubated with 50 ng of WTZF[(I-IV)-KTS] and radiolabelled EGR-1 DNA binding site oligonucleotide as indicated above each lane. The position of the WT1-DNA complexes and free probe are indicated on the left. Binding reactions were electrophoresed on a 4% nondenaturing polyacrylamide gel and visualised by autoradiography by exposing the X-Omat film (Kodak) at -70°C overnight with an intensifying screen. (C) Competition of WT1/RNA complexes with RNA and DNA, and supershift of WT1 RNA complexes. Fifty-fold molar excess of unlabelled RNA (lanes 3 and 4) or DNA (lanes 5 and 6) oligonucleotide was incubated with WTZF[(I-IV)-KTS] and radiolabelled RNA#41 probe. Lanes 1 and 9 contain the probe alone. EGR1 (5'-TCGAGCGGGGGCGGGCGT-3') and ETS (5'-GATCCAGGAAGTGA-3') oligonucleotides are specific and nonspecific DNA competitors, respectively. WT1 supershift assays (lanes 7, 8, 11,12) were performed by preincubating 200 ng of antibody prior to the addition of probe. Lane 12 contains C19 antibody and RNA probe in the absence of WT1 protein. Binding reactions were electrophoresed on a 4% nondenaturing polyacrylamide gel and visualised by autoradiography. The region of the gel containing WT1 supershifted complexes is delineated to the right. The position of complexes are indicated to the left.

Figure 4. WT1 structural requirements for RNA binding. A series of WT1 deletion and missense mutants were incubated with radiolabelled RNA#22 probes. Protein-RNA complexes were resolved on 4% polyacrylamide gels, dried and exposed to X-ray (X-OMAT) film at -70°C overnight. The nature of the recombinant protein used is indicated above each lane. The position of complexes are indicated to the left.

```
     R   G   I   Q   D   V   R   R   V   S   G   V   A   P   T    309
    CGA GGC ATT CAG GAT GTG CGT CGT GTG AGC GGC GTG GCG CCG ACC

     L   V   R   S   A   S   E   T   S   E   K   R   P   F   M    324
    CTG GTG CGT AGC GCA TCC GAA ACC AGC GAA AAA CGT CCG TTC ATG

     C   A   Y   P   G   C   N   K   R   Y   F   K   L   S   H    339
    TGC GCA TAC CCG GGT TGC AAC AAA CGT TAC TTC AAA CTG TCC CAC

     L   Q   M   H   S   R   K   H   T   G   E   K   P   Y   Q    354
    CTG CAG ATG CAC TCC CGT AAA CAC ACC GGT GAA AAA CCG TAC CAG

     C   D   F   K   D   C   E   R   R   F   S   R   S   D   Q    369
    TGC GAC TTC AAA GAC TGC GAA CGT CGT TTC TCC CGT TCC GAC CAG

     L   K   R   H   Q   R   R   H   T   G   V   K   P   F   Q    384
    CTG AAA CGT CAC CAG CGT CGT CAC ACC GGT GTT AAA CCG TTC CAG

     C   K   T   C   Q   R   K   F   S   R   S   D   H   L   K    399
    TGC AAA ACC TGC CAG CGT AAA TTC TCC CGT TCC GAC CAC CTG AAA

     T   H   T   R   T   H   T   G   K   T   S   E   K   P   F    414
    ACC CAC ACC CGT ACC CAC ACC GGT AAA ACC TCC GAA AAA CCG TTC

     S   C   R   W   H   S   C   Q   K   K   F   A   R   S   D    429
    AGC TGC CGT TGG CAC AGC TGC CAG AAA AAA TTC GCA CGT TCC GAC

     E   L   V   R   H   H   N   M   H   Q   R   N   M   T   K    444
    GAA CTG GTT CGT CAC CAC AAC ATG CAC CAG CGT AAC ATG ACC AAA

     L   H   V   A   L   .                                        449
    CTG CAC GTT GCA CTG TGA
```

1B

Lane labels (top, vertical):
- WT1(Del)
- WT1(+/-)394R/W
- WT1(+/-)
- WTZF[(I-III)-KTS]
- WTZF[(I-IV)-KTS]
- WTZF[(I-IV)+KTS]
- WTZF[(I-IV)-KTS]

kDa

175.0
83.0
62.0
47.5
32.5
25.0
16.5

1 2 3 4 5 6 7 8

2A

ZF(I-IV):   −   +   −   +

2B

WTZF(I-IV): - + - + - + - + - + - + - + - +

RNA #:   16   17   20   21   22   38   40   41

Complex →

Free
Probe

2c

FAMILY A

7	GAUAUGGUGCCUAGCCCCGG
8	GAUAUGGUAGCGCCCGCACG
11	GAUAUGGAUGGCCCGGUCCCG
12	GAAAUGGAUGGCUCUCCCCG
16	GUAAUGGUCAAACCCCCCCC
17	GCUAUGGUUGCGCCCUUACG
21	GAUAUGGUGGCUGUAUACCC
22	GAUAUGGUGACCACCCCGGC
24	GAUAUGGUGCGAGCCCCGGG
25	GAUAUGGAUGGCCCUGGCUG
26	GAUAUGGUGUGAUGCCCCCC
30	GCUAUGGUGCCUUCCCCCCU
31	GAUAUGGUGACCUCCAGCGC
36	GAUAUGGUGAAGCACGCCCC
44	GAUAUGGUGGCGACUGCUGG
46	GCUAUGGUGAAAUGCCCCGG
48	GCUAUGGAGUGCCCCGGACC
50	GAUAUGGAUGGCCCGGUCCCG

CONSENSUS: GAUAUGGAG
                         U

FAMILY B

4	CCUUAGAAUGAUGCGACAUG
20	GACGAAUGCGUAAAUUGCUAG
23	GACGAAUGCGACGCUGGGCC

CONSENSUS: GAAUG

FAMILY C

5	AUACACCCGAAUCCGGAGCU
6	AUACUCCCACAAGAUGGUGA
9	auuCACCCACGACAUUUGCUAGG
38	AUCACCCACCCCGAGCUGGC
39	AUACGCCCAAAUGCUGGCGC
40	auuGACCCAGGGCCGUCACGUGG
41	auuCUCCCAACUGACUGACCUGGCGC
43	AUUACCCACGCGCAUGAUAGG
45	auuCUCCCGAACAAACACCCGGU
51	GGCGAACCCAUAAGUGCUUG

CONSENSUS: AUUCACCCAxxxG
                        A U   G   C

2D

% RNA Bound (y-axis): 0, 25, 50, 75, 100, 125

Protein Concentration
log [μM]
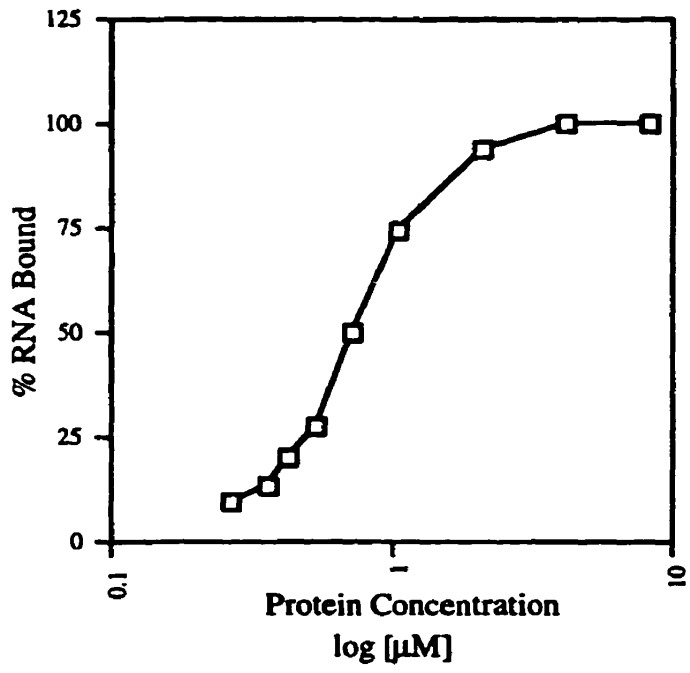
3A

Competitor: - - #38 #20 #22 R6
WTFZ(I-IV): - + + + + +

Complex →

Free
Probe →

1   2   3   4   5   6
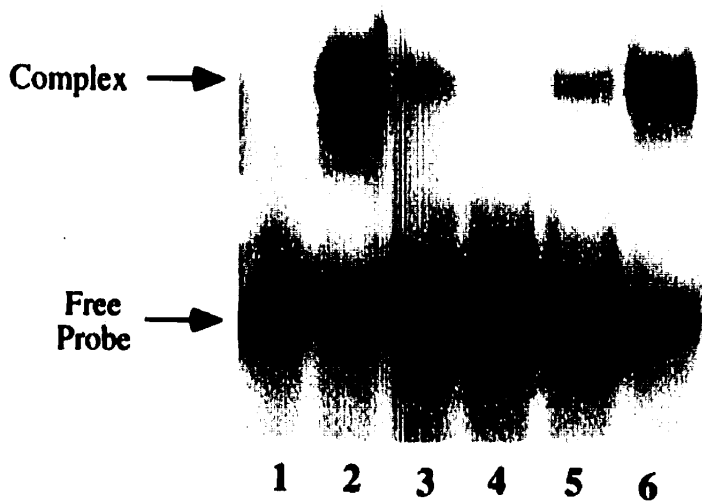
3B

Competitor: - - #20 #22 #38
WTFZ(I-IV): - + + + +

Complex →

Free
Probe →

1   2   3   4   5

3c

4

Protein: WTZF[(I-IV)-KTS] WTZF[(I-IV)+KTS] WTZF[(II-IV)-KTS] WTZF[(I-III)-KTS] WT1(+/-) WT1(+/-)β394R/W WT1(Del) EGR-1

1 2 3 4 5 6 7 8 9
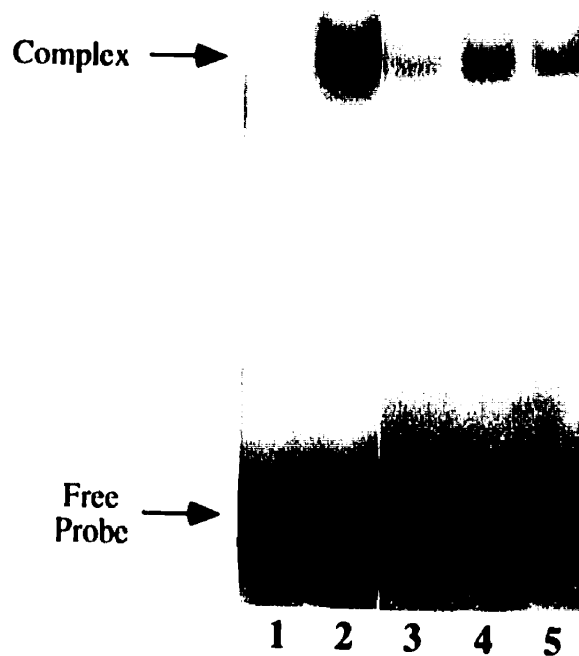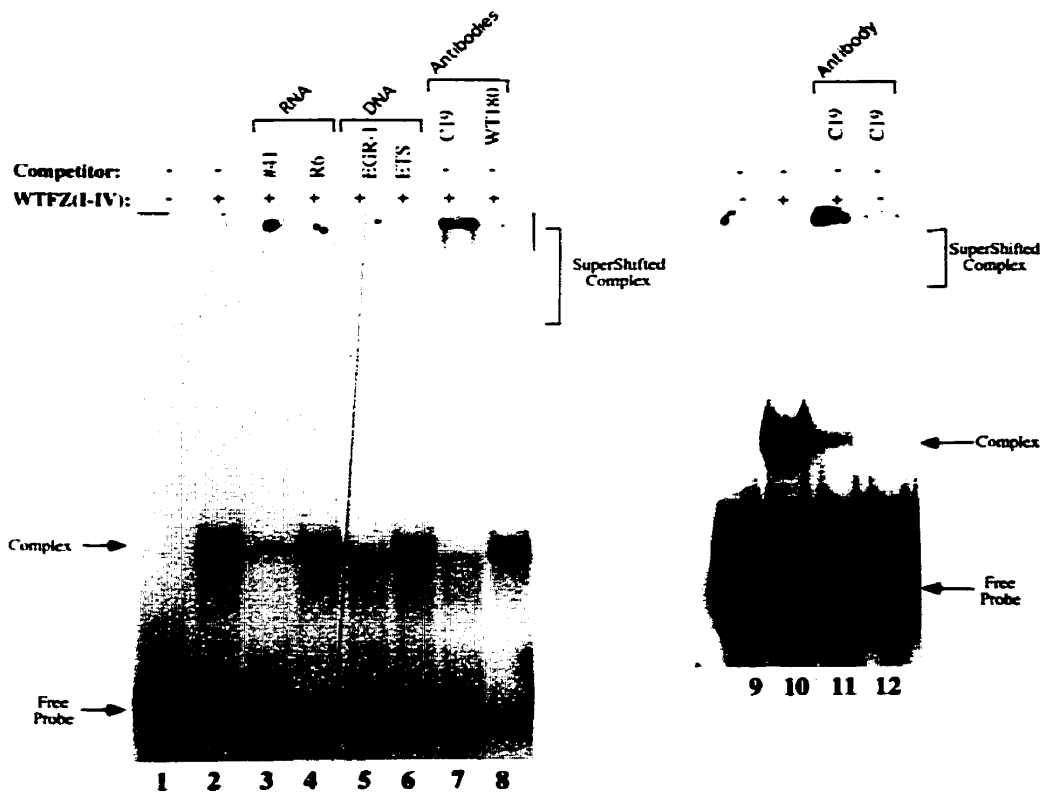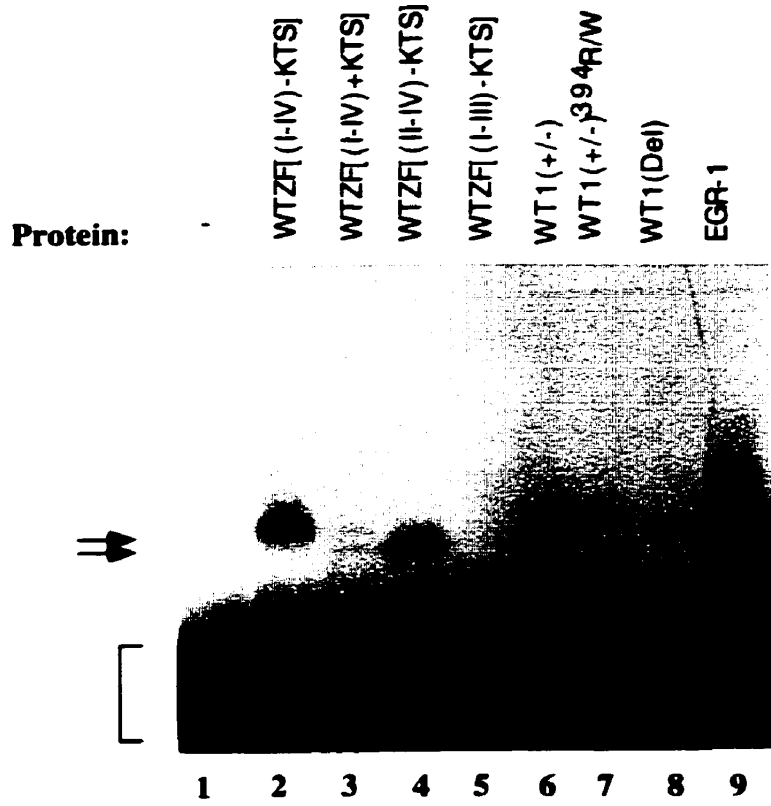
DISCUSSION

This section will evaluate how the data presented in the results section of this thesis accords with the general body of molecular genetic studies of Wilms' tumor. In addition, a number of questions will be addressed in a speculative attempt at resolving problems in elucidating the genetic basis for this disease.

The Role of WT1 Mutations in Development of Wilms' tumor

Wilms' tumors show heterogeneous clinical presentation with considerable variation in histology and age of onset. As we have seen, Wilms' tumors also displays genetic heterogeneity with several loci implicated in tumorigenesis. By compilation of genetic analyses of Wilms' tumors we can attempt to correlate genetic changes with specific clinicopathologic patterns. Such an analysis may allow resolution of Wilms' tumors into subtypes dependent on genetic etiology and may permit rationalization of phenotypic differences between them. More importantly, it may eventually permit the design of specific treatment regimens according to which genetic lesion is involved.

Wilms' tumor is thought to arise from malignant transformation of renal stem cells (metanephric blastema). The tumors generally show triphasic histology consisting of blastemal, epithelial and stromal cells, although some tumors show a predominance of a single cell type (Gonzalez-Crussi, 1984). Wilms' tumor cells may undergo varying degrees of differentiation which mimic normal nephrogenesis. In some cases this aberrant differentiation produces heterologous elements such as skeletal muscle and bone. Precursor lesions to Wilms' tumors have been identified. These structures, known as nephrogenic rests (NR), are abnormal foci of persistent embryonal cells often found in kidneys with Wilms' tumor. These features suggest that Wilms' tumor development results from defects in kidney differentiation and that it is a multistep process. There are two distinct categories of NR, classified according to their position in the renal lobe. Intralobar NR (ILNR) are dispersed randomly throughout the lobe while perilobar NR (PNLR) are restricted to the periphery and are demarcated from the normal renal tissue (Beckwith et al., 1990). ILNR are found in most patients with DDS or WAGR syndrome. They are also associated with an earlier age of presentation of Wilms' tumor which show stromal predominant histology and often contain heterologous elements such as skeletal muscle. PNLR, in contrast, are associated with Wilms' tumors in patients with BWS and hemihypertrophy. These tumors show later onset, usually consist of blastemal or epithelial cells, and do not contain heterologous elements. These observations suggest that the histological subtypes of Wilms'

tumors are associated with mutations in different genes and that these genes regulate different stages of kidney differentiation. Tumors associated with ILNR mimic the entire spectrum of nephrogenesis while those associated with PLNR only mimic later stages of this process, thus it is likely that genes involved in ILNR control early phases of differentiation while genes causing PLNR function later in renal development.

These epidemiological observations implicate the *WT1* gene in the development of ILNR and the BWS gene(s) in PNLR-related tumorigenesis. These predictions are well supported by recent molecular data. WT1 is expressed in blastemal and epithelial cells but not stromal cells in Wilms' tumors (Pritchard-Jones & Fleming, 1991; Miwa *et al.*, 1992a). This expression pattern is analogous to what is observed in normal cells. In addition *WT1* mutations are correlated with stromal-predominant histology (Schumacher *et al.*, 1997). In a series of 21 stromal-predominant tumors, 62% had *WT1* mutations, whereas only about 15% of Wilms' tumors overall harbor WT1 mutations. In a similar fashion, *WT1* expression levels are generally lower in tumors with heterologous differentiation than in those lacking heterologous elements (Gerald *et al.*, 1992). Correspondingly, ectopic myogenesis in Wilms' tumors has recently been shown to be associated with *WT1* mutations (Miyagawa *et al.*, 1998). This association is supported by a cursory review of the literature in which *WT1* mutations have been described revealing a number of cases displaying myogenesis (*e.g.* Glaser *et al.*, 1989; Park *et al.*, 1993; Kaplinsky *et al.*, 1996). Taken together, these observations indicate in the absence of WT1, nephrogenic cells are developmentally arrested at the primitive stromal stage and are restricted in proceeding to form blastemal or epithelial cells. These aberrant cells, lacking functional WT1, may attempt to follow other pathways of differentiation and thus produce heterologous elements such as skeletal muscle. It is noteworthy that WT1 expression can suppress muscle differentiation *in vitro* (Miyagawa *et al.*, 1998). Epithelial/blastemal tumors show a low frequency of *WT1* mutations and are likely involve different genes which act later in kidney differentiation.

Although the discussion above shows that classification of Wilms' tumors is possible through the association of particular phenotypic features with specific genetic lesions, these associations are not absolute. It is necessary to account for tumors which do not fit with this classification system. For example, tumors with *WT1* mutations frequently display other histological types instead of stromal-predominance and conversely, a significant proportion of stromal-predominant Wilms' tumors do not harbor WT1 mutations. Addressing the first point, the different histological types which are associated with *WT1* mutations may arise due to differences in the specific timing of the *WT1* mutation, to mutations in other genes or to stochastic factors. It is unlikely that the type of

WT1 mutation affects these histological differences as similar mutations can be associated with different histological types (*e.g.* see Glaser *et al.*, 1989; Park *et al.*, 1993; Schumacher *et al.*, 1997) The observation that some stromal-predominant Wilms' tumors do not harbor *WT1* mutations may indicate that a gene operating in a similar pathway to *WT1* is mutated in these tumors and hence, with respect to tumorigenesis, mutations in such a gene show degeneracy with *WT1* mutations.

In general, the classification of histologically distinct Wilms' tumors into specific molecular subsets appears to be valid. There also appears to be association of specific molecular lesions with other clinical features of Wilms' tumor. Familial Wilms' tumor is associated with at least three different loci (see Introduction). Families in which tumorigenesis segregates with the chromosome 17 *FWT1* locus have a median age of diagnosis 5.5 years (Rahman *et al.*, unpublished) in contrast to an age of less than two years in patients with inherited *WT1* mutations result in an age of diagnosis of less than two years (Green *et al.*, 1993). The median age of diagnosis is less than one year in families unlinked either locus.

There are now some clues available to interpret data on *WT1* genetics with respect to the clinicopathology of Wilms' tumor. An important question may presently be addressed: can *WT1* be considered a 'classical' tumor suppressor gene, *i.e.* is it likely that the homozygous inactivation of *WT1* is the rate limiting step in the development of Wilms' tumor? A number of challenges to this two-hit model for *WT1* in tumorigenesis must be resolved. These challenges are derived from a number of paradoxical observations.

1) **Only a small proportion of Wilms' tumors have *WT1* mutations.** Wilms' tumor represents a group of distinct pediatric renal neoplasms and not a single disease and thus an unselected set of such tumors will include a considerable proportion in which *WT1* mutations are not relevant. The subdivision of Wilms' tumors into appropriate histological types implicates *WT1* mutations at a high frequency in a specific group of such tumors. Hence, this apparent paradox arises from difficulties in classifying distinct but related tumor types.

2) **The proportion of familial Wilms' tumor cases is much lower than predicted by Knudson's two-hit model.** The low number of familial Wilms' tumor cases associated with *WT1* mutations is easily accounted for. Patients with germline *WT1* mutations likely have reduced reproductive fitness due to genitourinary abnormalities associated with these mutations and additionally, until recently the survival of Wilms' tumor patients was low. These two factors significantly reduce the transmission of *WT1* mutations.

3) **A high proportion of the** *WT1* **mutations in Wilms' tumors are constitutional.** The incidence of two somatic WT1 lesions in Wilms' tumors is uncommon in comparison with that of a constitutional mutation associated with loss of the wild type *WT1* allele. The relative rarity of somatic mutations may indicate that inactivation of WT is tumorigenic only in early stages of kidney development as was suggested above. In such a case, the target cell population and temporal window for such mutations would be limited. These factors would likely reduce the probability of two independent mutations, and hence increase the relative proportion of constitutional mutations.

4) **Frequent absence of LOH at chromosome 11p13 in Wilms' tumors of patients with constitutional deletions at 11p13.** This observation may appear to call into question whether *WT1* undergoes two-hit inactivation in Wilms' tumorigenesis. It is now clear that most of these patients have intragenic *WT1* mutations in the second allele and thus the gene indeed shows homozygous inactivation (Baird *et al.*, 1992; Gessler *et al.*, 1993; Huff *et al.*, 1995; Schumacher *et al.*, 1997). The rarity of LOH associated with large deletions may result from constraints imposed by homozygous loss of adjacent genes essential for cell viability (Glaser *et al.*, 1989).

Hence, these apparent paradoxes which are suggestive of a more complex genetic mechanism can be resolved by more careful analysis of the biological context of Wilms' tumorigenesis. We can conclude, therefore, that *WT1* probably conforms to two-hit models for its role in Wilms' tumor development. Hence, tumorigenesis associated with *WT1* mutation is unlikely to involve a complex multistep pathway. The data supporting these assertions are that a) inheritance of a mutated *WT1* allele is associated with the development of Wilms' tumor at high penetrance, b) distinct histological subtypes of Wilms' tumor show a high frequency of *WT1* mutations, and c) most *WT1* mutations are associated with loss of the wild type allele.

Epigenetic changes resulting in biallelic paternal expression of *IGF2* and maternal LOH at chromosome 11p15 are the most common molecular changes observed in Wilms' tumor (Reeve, 1996). Both of these lesions are expected to result in increased IGF2 levels. The relation of these alterations to *WT1* mutations is unclear since no molecular studies have analysed this association. The positioning of *WT1* and *IGF2* on the same chromosomal segment (*i.e.* chromosome 11p) may result in a cooperation between these loci. LOH at chromosome 11p occurs frequently in conjunction with *WT1* mutations (see Introduction) and in most cases this event affects both the 11p13 and 11p15 regions. If maternal alleles are lost in concurrence with duplication of the paternal chromosome segment, this event may contribute to tumorigenesis by 'unmasking' a tumorigenic mutation in one *WT1* allele and simultaneously causing biallelic expression of *IGF2*.

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Consistently, the majority of LOH at 11p involves loss of maternal chromosome (Coppes *et al.*, 1992b), however, no studies have extensively documented the parental origin of LOH in association with *WT1* mutations. Future studies should address the coincidence biallelic *IGF2* expression and *WT1* mutations in Wilms' tumors to resolve the relationship of these loci in tumorigenesis.

Bilateral tumors arise in individuals with constitutional predisposing mutations or with mutations that occur early in development. The association of germline mutations of a particular gene with a high frequency of bilateral tumors suggests that this gene is conforming to two-hit kinetics since subsequent tumorigenic mutations must be occurring with a high incidence in these patients (Knudson, 1971). A very low incidence of bilateral tumors in the context of a germline mutation, in contrast, may imply that tumorigenesis associated with this gene involves complex genetics, requiring mutations at a number of other loci. The observations that *WT1* germline mutations are disproportionately common (in comparison to *de novo* somatic mutations) and that they are highly penetrant predict that an increased proportion of bilateral Wilms' tumors are associated with *WT1* mutations. This is supported by studies showing *WT1* mutations in nine of 17 bilateral tumors (Nordenskjold *et al.*, 1995; Schumacher *et al.*, 1997). The penetrance of Wilms' tumor appears to be low in patients with predisposing mutations associated with the Beckwith-Weidemann syndrome (BWS) (Sotelo-Avila *et al.*, 1980), Simpson-Golabi-Behmel syndrome (SGBS) (Hughes-Benzie *et al.*, 1996; Lindsay *et al.*, 1997) and some familial Wilms' tumor pedigrees (Grundy *et al.*, 1988; Schwartz *et al.*, 1991). Additionally, tumors associated with these conditions are unilateral. These observations are suggestive of an increased genetic complexity of tumorigenicity in these non-*WT1*-associated diseases.

In summary, while it is likely that homozygous mutation of *WT1* itself is not sufficient to induce Wilms' tumors, this appears to be the rate-limiting step in a histologically and clinically distinct subset of Wilms' tumors. Subsequent mutations likely occur at high probability and/or affect few genes. Wilms' tumors associated with BWS, SGBS, and a subset of familial cases, in contrast, may deviate from two-hit models of tumorigenesis.

Molecular Genetic Analysis of Denys-Drash Syndrome

Wilms' tumor is associated genitourinary anomalies and germline WT1 mutations in two discrete syndromes. The genitourinary malformations in the WAGR syndrome are relatively mild and include undescended testes and hypospadias in the male. Gross chromosomal deletions are often observed cytogenetically, and these span the *WT1* locus

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on chromosome 11p13. Fine scale characterization of the extent of WAGR deletions has demonstrated that deletion of the *WT1* gene is responsible for the Wilms' tumor and genitourinary defects in this disease. This association is confirmed by the detection of constitutional intragenic *WT1* mutations in some patients with these abnormalities (Pelletier, 1991b; Nordenskjold *et al.*, 1995). These intragenic mutations either alter the translational reading frame or produce nonsense codons and thus are predicted to result in severely truncated polypeptides. Given that deletions of *WT1* and intragenic *WT1* mutations produce similar effects on tumorigenesis and genitourinary development, it is likely that the pathogenic mechanisms of these different mutations are identical and result from reduced expression of the WT1 protein.

The Denys-Drash syndrome (DDS) involves the disease triad of Wilms' tumor, severe genitourinary defects and nephrotic syndrome resulting in renal failure. The association of DDS with mutations in the *WT1* has been clearly established (Pelletier et al., 1991c). In contrast to the deletions found in WAGR syndrome, the vast majority of DDS patients have missense point mutations in the zinc finger region of WT1. Thus a more severe phenotype is associated with heterozygous amino acid changes in the WT1 zinc fingers compared with reduction in WT1 dosage. This effect is generally attributed to a dominant negative activity of WT1 proteins whose DNA binding capability has been disrupted (Coppes *et al.*, 1993c). In the Appendix to this thesis (Bardeesy *et al.*, 1994b) and in Bruening et al. (1992) we continued investigations of the the role of *WT1* mutations in DDS and addressed the genetics of a less severe variant referred to as incomplete DDS. The concept of incomplete DDS was introduced by clinicians to account for patients with only partial manifestation of the DDS triad of anomalies (Manivel et al., 1987; Schmitt et al., 1995). In our analysis of patients diagnosed with incomplete DDS we identified constitutional heterozygous point mutations in *WT1*. In contrast to the missense mutations described for fully penetrant DDS, these mutations created premature stop codons predicted to cause production of truncated WT1 proteins. In addition, we identified mutations at the alternative splice donor site of intron 9 in two patients diagnosed with DDS, although neither developed Wilms' tumor. Investigation of the functional consequences of these intronic mutations in an *in vitro* system indicated that they cause disrupted splicing of the +KTS isoforms from the mutated *WT1* allele (Bruening *et al.*, 1992).

To summarize, the following observations were made for each disorder:

1. DDS patients have missense point mutations in zinc finger region or mutations which truncate the protein in the zinc finger region (*i.e.* toward the carboxy terminus). A small

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number of DDS cases have intronic mutations interfere with alternative splicing of exon 9, preventing production of the +KTS isoforms.

2. Incomplete DDS patients have mutations leading to insertion of a premature stop codon before the zinc fingers (*i.e.* more toward the amino terminus).

3. WAGR patients have either large deletions spanning *WT1*, smaller intragenic deletions, or mutations producing premature stop codons.

In attempts to synthesize this data to interpret genotype-correlations, we made the following suggestions. The less severe disorder WAGR is produced by a decreased gene dosage of *WT1*. Such patients retain partial WT1 function by retaining one wild type allele. DDS patients develop more severe symptoms because the mutated allele codes for a protein which is able to disrupt functioning of the wild type allele. It is unlikely that DDS arises due to WT1 binding to new DNA targets in a gain of function manner since several different mutations in the zinc finger regions give identical phenotypes including mutations in the zinc chelating residues responsible for the structural integrity of these domains. In addition, the identification of patients with intronic mutations causing alteration of the ratio of alternatively spliced transcripts and, thus, not resulting in production of novel proteins, indicated that a gain-of-function mechanism was not involved. To account for the phenotypic differences between incomplete DDS and WAGR, both of which can be caused by mutations which result in premature stop codons, we suggested that different mutations may result in mRNA or protein with different biological properties, such as altered mRNA or protein stability. For example, the missense mutations in WAGR patients produce the same phenotype as deletions of the entire gene and so do not result in interference of wild type protein function. Incomplete DDS, with an intermediate phenotype between WAGR and DDS, was suggested to be due to a production of molecules which could partially disrupt WT1 function. The dominant-negative models for WT1 behaviour are supported by observations that WT1 can multimerize and that mutated WT1 can antagonize the transcriptional function of wild-type WT1 (Reddy et al., 1995; Moffett et al., 1995). This dominant negative activity requires the nuclear targeting of WT1 mediated by signals in the zinc finger region (Bruening et al., 1996). *In vitro* studies of the mutated WT1 alleles from WAGR and incomplete DDS patients indicated that neither are targeted to the nucleus nor inhibit the transcriptional properties of WT1. It is possible that the distinct phenotypes brought upon by the mutation are due to interference with other functions not detected *in vitro*, and that these properties are not manifested by the WAGR mutants due to decreased mRNA or protein stability. Conversely the distinction between the phenotypes may not be due to the biochemical differences resulting from these mutations. Instead, phenotypic

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differences may be caused by genetic differences at other loci in the genome of affected individuals. The number of patients analysed to date is too small to draw conclusions on whether specific germline mutations in the 5' portion of *WT1* result in specific phenotypic differences.

The classification of rare dysmorphic syndromes is often complicated due to extensive clinical overlap between disorders and variably penetrant phenotypes (for example see: Moorthy *et al.*, 1987; Massart *et al.*, 1995; Schmitt *et al.*, 1995). It is not uncommon for a rare disorder to be misdiagnosed in favor of a clinically overlapping, but distinct disease. Since these syndromes occur at very low frequency, such inappropriate diagnoses can create confusion when molecular studies are conducted. The Frasier syndrome (FS), provides an example of the difficulties associated with clinically overlapping disorders (Moorthy *et al.*, 1987). This rare disease is defined by male pseudo-hermaphroditism and progressive renal glomerulopathy and thus is comparable to DDS (Frasier *et al.*, 1964). Patients with FS are characterized by normal female external genitalia, streak gonads and an XY karyotype. Patients have glomerular dysfunction resulting in childhood proteinuria and develop focal and segmental glomerular sclerosis resulting in end-stage renal failure in adolescence of early adulthood. Patients are predisposed to gonadoblastoma but, significantly, do not develop Wilms' tumor. The pathology of DDS is related but distinct, involving ambiguous or normal female genitalia, dysgenic or streak gonads, and diffuse mesangial sclerosis. More than 90% of DDS patients develop Wilms' tumor. Additionally, DDS results in end-stage renal failure at a much younger age and with more rapid progression than FS. In a retrospective analysis, Moorthy *et al* (1987) have suggested that a number of cases DDS should be reclassified as FS.

It has recently been reported that donor splice site mutations between exons 9 and 10 of *WT1* are associated with FS in eight patients (Barbaux *et al.*, 1997; Kikucki *et al.*, 1998). The mutations all disrupt alternative splicing from one allele of *WT1* resulting in diminution of the level of WT1+KTS isoforms. We and others had previously identified similar mutations in three patients who had been diagnosed clinically as having DDS (Bruening et al., 1992; König et al., 1993; Bardeesy et al., 1994b). Review of the clinical features of these patients suggests that they more suitably display the criteria for FS. Most notably, none of the patients developed Wilms' tumor (analysis of kidneys removed from one patient with renal failure showed no tumor). All of the patients have an XY karyotype with normal female external genitalia. Other features consistent with differential diagnosis as FS included renal failure in adolescence (*i.e.* later onset than DDS). In summary, then, we favor the re-evaluation of these cases as FS.

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It is apparent that germline intronic *WT1* mutations mutations affecting alternative splicing of exon 9 do not predispose patients to the development of Wilms' tumors. Thus, the deregulation of the ratio of -KTS to +KTS splice forms is malformative in genitourinary developmental processes but appears not to be oncogenic. It is tempting to speculate that the -KTS isoform of WT1, which is capable of DNA binding, is responsible for the tumor suppressor activity. These observations, together with evidence that germline deletions of *WT1* are highly predisposing to malignancy but produce relative mild malformations, suggests that WT1 may play distinct functions in either process. Consideration of the oncogenic fusion protein resulting from translocation of the *EWS* and *WT1* genes in desmoplastic small round cell tumors (DSRCT) supports the notion of an exclusive role of the [-KTS] isoform in tumorigenesis. In NIH3T3 cell transfection assays, only the EWS-WT1[-KTS] isoform is capable of inducing transformation. Overall, it is apparent that specific mutations in *WT1* are clearly associated with discrete phenotypes.

The involvement of *WT1* in syndromes which display progressive renal failure has led some investigators to postulate that this gene may have a postnatal role in tissue homeostasis (Hastie, 1994; van Heyningen, 1997) in addition to its function in embryonic development. While this suggestion is plausible, an alternative explanation can be advanced to account for this progressive nephropathy, *i.e.* a subtle defect in nephrogenesis causing mild structural abnormalities could be manifested only following cumulative damage brought on through physiological processes. In such a case the defect would appear to involve a postnatal function, although in fact original disturbance occurred during embryonic development. It is not currently possible to resolve which pathogenic mechanism is responsible although the persistent expression of WT1 in certain cells may support the former hypothesis.

Role of *p53* in the Pathogenesis of Wilms' tumor

Genetic models for tumorigenesis maintain that tumors are clonal, *i.e.* they arise from a single abnormal cell (Nowell, 1976; Fearon & Vogelstein, 1990). Opposing models propose that aberrant differentiation or field effects are responsible for neoplastic changes and predict that tumors may be polyclonal (Rubin, 1994). In the genetic models, progression of the cancer to a more highly malignant form is thought to result from the acquired genetic variability of the tumor cell population. A cell which acquires mutations giving it a selective growth advantage (due to increased proliferation or decreased cell death) can give rise to a clonal subpopulation of cells with increasingly aggressive characteristics. The clonal nature of many cancers has been confirmed by associating

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specific acquired genetic changes with cancer progression (*e.g.* Fearon *et al.*, 1987; Sidransky *et al.*, 1992; Furnari *et al.*, 1995). The detection of such a change in all or nearly all the cells of a tumor suggests that this change is responsible for conferring the growth advantage allowing it to outgrow other cells in the population. Confirmation that this association is not merely coincidental is demonstrated if such a change occurs frequently in other tumors of the same type. It is now clear that many cancers follow multistep pathways for tumor progression in which specific genetic changes are associated with particular stages of tumor development. In the well characterized example of colorectal tumorigenesis, loss of the *APC* tumor suppressor gene and activating mutations in the *K-ras* oncogene are observed in early stages while *p53* mutations only appear after the tumor has shown many histological features of neoplastic change (Fearon & Vogelstein, 1990). From these observations it is presumed that some lesions are involved in the initiation of tumorigenesis while others accumulate in cells which are already transformed, conferring to these cells specific growth advantages as well as the the properties of invasion and metastasis.

The progression of most cancers is associated with numerous chromosomal abnormalities and other mutations. This increased accumulation of mutations is higher than can be accounted for by the mutation rate in normal somatic cells (Loeb, 1996). Genetic instability of cancer cells must then reflect a mutator phenotype which produces a greatly increased mutation rate and these cells are likely to have a defect in pathways for DNA damage surveillance or DNA repair. Such a defect would increase the likelihood of the acquisition of new favorable mutations and thus a gene involved in this process would be expected to be a central regulator of tumorigenic progression. Tumor progression is often associated with resistance to treatment regimens, a phenomenon which may be due to genomic plasticity which readily permits the acquisition of mutations, of which some are favorable to drug resistance, or it may be due to defects in a gene which causes cell death in response to cytotoxic stimuli (Fisher, 1994).

The progression of Wilms' tumor follows the pattern of tumor progression discussed above. The tumor may display stages showing increasing histological abnormalities and genomic instability. In Chapters 3 and 4 of this thesis (Bardeesy *et al.*, 1994a; 1995a) we present an extensive analysis of the *p53* gene in Wilms' tumors. We observed the complete absence of *p53* mutations in tumors with favorable (*i.e.* non-anaplastic) histology. *p53* mutations and loss of the wild type allele is restricted to regions of anaplastic histology demonstrating an association of Wilms' tumor progression with clonal expansion of cells with acquired *p53* mutations. This histological subtype of Wilms' tumor is characterized by dramatic genetic instability and is associated with poor patient

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prognosis due to enhanced resistance to multimodal (*i.e.* chemo- and radiotherapeutic) treatment (Zuppan *et al.*, 1988; Beckwith, 1997). This is in sharp contrast to Wilms' tumors with favorable histology which show few chromosomal abnormalities and are highly responsive to treatment. In stage IV tumors (*i.e.* those showing metastasis) the survival rate is 7% for diffuse anaplastic histology versus 84% for non-anaplastic tumors (Beckwith *et al.*, 1997).

The histological definition of anaplasia in Wilms' tumors involves the simultaneous presence of a) markedly enlarged and usually multipolar mitotic figures, b) three-fold nuclear enlargement compared with nuclei of the same cell type, c) hyperchromasia (dark staining) of the enlarged nuclei. The anaplastic phenotype correlates well with *in vitro* observations of cells lacking p53. In mouse fibroblasts, p53 controls a mitotic spindle checkpoint necessary for preventing abnormalities in mitotic poles and maintenance of diploidy (Cross *et al.*, 1995). The spindle defects associated with p53 deficiency are reminiscent of the abnormal mitotic figures in anaplastic cells. The correlation of p53 mutations with anaplastic Wilms' tumors is strong evidence that p53 is involved in regulating the genetic stability of tumor cells *in vivo*. The process by which p53 maintains genetic stability involves the induction of G1 or apoptosis in damaged cells (reviewed in Levine, 1997). This mechanism prevents the propagation of potentially harmful mutations and has been observed *in vivo* in a mouse tumor model system (Symonds *et al.*, 1994). In this system, tumor growth and progression was associated with the loss of wild type p53 and a corresponding decrease in the level of apoptosis suggesting that p53-dependent apoptosis serves as a critical regulator of tumorigenesis. We detected an increased rate of apoptosis in non-anaplastic regions (containing wild type *p53*) compared with adjacent anaplastic regions (which harbor *p53* mutations) from the same suggesting that the ability of wild type p53 to sensitize cells to apoptosis is relevant to human tumorigenesis *in vivo* (Chapter 4; Bardeesy *et al.*, 1995a).

The conventional model to explain the mechanism of action of anti-cancer treatments proposed that these treatments selectively target rapidly dividing cells (reviewed in Fisher, 1994). A number of modes of resistance to treatment have been identified and these included the amplification or increased expression of genes encoding proteins which limit drug accumulation, mediate drug detoxification, or are the target of drug action. These mechanisms, however, cannot account for the drug resistance of a large proportion of malignancies. Recent studies have show that many anti-cancer agents function by triggering apoptosis in malignant cells (Dive & Hickman, 1991). For a wide range of agents this process is p53-dependent, and hence, transformed cells lacking functional p53 are greatly desensitized these treatments (Lowe *et al.*, 1993b; 1994). Untransformed cells do not

undergo apoptosis in response to these treatments, regardless of the p53 status, an observation which is consistent with the specificity of such agents for cancer cells *in vivo*. Since p53 mutations are widely observed in human cancers, it is possible that defects in p53-dependent apoptosis may be a common mechanism of treatment resistance in cancer.

Given the association of *p53* mutations with anaplastic Wilms' tumors, it appears likely that such mutations cause the poor prognosis associated with this tumor variant. Correspondingly, the favorable treatment outcome of most Wilms' tumors is consistent with the absence of *p53* mutations in such tumors. The hypothesis that the abrogation of a p53-dependent apoptotic pathway is responsible for the treatment failure of anaplastic Wilms' tumors concurs with the histopathological determination that anaplasia is a marker of resistance to therapy but not of increased aggressiveness (Beckwith, 1997). The property of chemo- and radiotherapeutic resistance in the absence of increased aggressiveness implies that a direct role for defects in a central regulator (*i.e.* p53), and not general genetic instability, is responsible for the morbidity of anaplastic Wilms' tumor.

There have been a number of studies by other investigators which have addressed the involvement of p53 in the pathogenesis of Wilms' tumors using immunohistochemical detection and mutational analysis. One immunohistochemical study found generalized overexpression of p53 in a large group of Wilms' tumors (Lemoine *et al.*, 1992), while a second study (Lahoti *et al.*, 1996) showed an increased level of p53 expression in recurrent and metastatic tumors. Mutational analysis corroborated our observations of the rarity of p53 mutations in tumors of normal histology. Malkin *et al.* (1994), found *p53* mutations in two of 23 Wilms' tumor specimens, one in an anaplastic tumor and the other in a metastatic tumor. The series included two other samples with anaplastic histology but which lacked *p53* mutations. Waber *et al.* (1993) found no *p53* mutations in a set of 38 Wilms' tumors, consisting of 37 tumors with favorable histology and one tumor with unfavorable histology of an unspecified type (this could include anaplastic Wilms' tumor or other variants). Kusafuka *et al.* (1997) failed to observe *p53* mutations in a set of 13 Wilms' tumors of undefined histology. Lahoti *et al.* (1996) identified *p53* mutations in four of 24 Wilms' tumors. Two of these had been reported previously in by Malkin *et al.* (1994) and the others consisted of an anaplastic tumor and a non-anaplastic tumor which metastasized (the mutation was present in the primary tumor). The finding of apparent *p53* mutations in two tumors without anaplasia may appear to challenge our conclusions tightly linking such mutations to the anaplastic phenotype. Closer scrutiny, however, reveals that some of this data is suspect. The nucleotide alteration, designated to be a 'mutation', in the nonanaplastic tumor which metastasized was an insertion of a guanine nucleotide in the middle region of intron five (396 base pairs away from the end of exon 5). This nucleotide

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variant has no effect on the coding sequence of p53 and is unlikely to affect splicing given its position within the intron. It is not indicated whether the alteration is heterozygous or homozygous, nor is it reported what proportion of the cells harbored this variant allele. Additionally, the authors do not address whether the nucleotide variant occurred *de novo* in the tumor or was present constitutionally. Finally this intronic mutation was restricted to the primary tumor; the metastatic recurrence was negative for *p53* mutations, hence in this tumor, the *p53* alteration was unlikely to be relevant to tumorigenesis. It is highly probable that this variant is a neutral polymorphism, lacking functional consequences, and thus should not be considered a mutation.

Some investigators have questioned the extent of the association between anaplastic Wilms' tumor and *p53* mutations because of their inability to detect such mutations in a some anaplastic tumors (Lahoti *et al.*, 1996). This is likely due to the large size and heterogeneous nature of Wilms' tumors in which molecular analysis of a single tumor area is often not representative of the entire tumor. The studies of other groups exclusively employed uncharacterized portions of the frozen tumor instead of histologically defined tumor sections for their mutational analysis. Since anaplasia is commonly confined to restricted regions within tumors, there is considerable likelihood of low proportions of anaplastic cells within a given tumor fragment. For example, Lahoti *et al.* (1996) note a case in which anaplasia was only present in two of 19 tumor sections. Mutational analysis of an undefined portion of this tumor would be very unlikely to detect any mutations which are restricted to anaplastic cells. In our own molecular studies using histologically defined sections we observed *p53* mutations in 9 of 10 anaplastic tumors, whereas in undefined sections from tumors designated as anaplastic we detected mutations in only 4 of 7 tumors. The disparity in detection rates between our group and others, therefore, is likely due to our more stringent method of analysis. In conclusion, homozygous *p53* mutations are highly specific markers for anaplasia in Wilms' tumor. The phenotype of anaplasia correlates well with characterized *in vitro* consequences of p53 dysfunction. The implication of *p53* mutations in the etiology of anaplasia provides a basis for informed design of therapeutic approaches to this prognostically unfavorable malignancy.

The occasional detection of *p53* mutations in non-anaplastic tumors may be due to the requirement for homozygous *p53* mutations in the development of anaplastic histology. We observed that all the mutations in specimens which contained exclusively anaplastic cells were homozygous (Chapter 5; Bardeesy *et al.*, 1995a). *p53* mutations were detected in non-anaplastic regions in two tumors which also contained anaplastic histology. In both cases the mutations were heterozygous in the nonanaplastic regions. In one case, T.J.#46 (Chapter 4; Bardeesy *et al.*, 1994a), the mutation was constitutional and the tumor

contained a small, focal region of anaplasia. DNA prepared from the frozen tumor revealed a heterozygous mutation. Unfortunately no material was available from the anaplastic nodule to allow assessment of whether the wild type allele had been lost in the progression to anaplasia. In the other case, #5191 (Chapter 5; Bardeesy *et al.*, 1995a), the mutation was heterozygous in the nonanaplastic region and homozygous in the adjacent region of anaplasia. It is evident that the progression to anaplasia is associated with the homozygous inactivation of *p53* and thus it is likely that the genetic etiology of anaplasia in Wilms' tumor requires loss of wild type *p53* alleles. There is likely to be a high probability that Wilms' tumor cells with heterozygous mutant *p53* will lose the wild-type allele.

The type and intragenic distribution of *p53* mutations is variable between types of malignancies (Greenblatt *et al.*, 1994). Osteosarcomas generally display gene rearrangements while most carcinomas harbor missense mutations which affect residues in the DNA binding domain. Toxic exposure is associated with specific mutational spectra in tumors of particular tissues. Examination of the *p53* in Wilms' tumors reveals 13 of 17 tumors with missense mutations, of which all but one alter amino acids in the DNA binding domain. Four of these mutations affected residues which are mutational 'hot spots' accounting for 40% of all missense mutations in *p53*. Structural studies have shown that these residues are critical for DNA binding, either having direct contacts with the DNA bases or are responsible for the structural integrity of the binding domain (Cho *et al.*, 1994). Three of the *p53* lesions associated with Wilms' tumors were nonsense mutations and one was a deletion. The distribution of *p53* mutations in Wilms' tumor is similar to what is observed for cancers overall. There is no indication of an environmental etiology in these mutations, and, conversely, the considerable number of C to T transitions of CpG dinucleotides is indicative of a spontaneous origin of these lesions.

Despite a wide body of *in vitro* data demonstrating a central role for p53 in cellular transformation and responsiveness to cancer therapeutic agents, there appears to be specificity of p53 function with respect to cancer type. The rate, timing, and prognostic significance of p53 mutations varies considerably between different malignancies (Sidransky & Hollstein, 1996), thus there does not appear to be a universally valid model for the necessity and consequences of p53 dysfunction in malignancy. Genetic analysis of some, but not all, cancer types clearly implicate p53 as a 'guardian of the genome', *i.e.* mutations and phenotypes correlate in a way consistent with *in vitro* experiments. In comparison with adult cancers which show a mutation rate of more than 50% (Greenblatt *et al.*, 1994), the incidence of *p53* mutations in most types of pediatric solid tumor is very low. For example, mutations are detected is less than 5% of Wilms' tumors (Waber *et al.*, 1993; Bardeesy *et al.*, 1994a; Malkin *et al.*, 1994), less than 2% of neuroblastomas (Vogan

et al., 1993; Imamura *et al.*, 1993; Kusafuka *et al.*, 1997), and rarely in hepatoblastomas (Kennedy *et al.*, 1994; Kusafuka *et al.*, 1997). The conspicuously different mutation frequencies suggest there are fundamental differences between the pathology of adult versus pediatric tumors. The cell types in pediatric cancers resemble undifferentiated cells seen in normal development (Gonzalez-Crussi, 1984). Speculatively, the primitive, embryonal cells from which pediatric tumors are derived may have different means of regulated the cell cycle than the more mature cells which are precursors to adult cancers. The responsiveness of these cells to *p53* expression may thus be altered, obviating the requirement of *p53* loss for malignant progression. The rarity of *p53* mutations in pediatric cancers supports the hypothesis that wild type p53 expression is an important in chemotherapeutic responsiveness since these tumors are generally very responsive to chemotherapy (Beckwith, 1997).

The variable involvement of *p53* may be partially accounted for by the activities of the recently discovered *p53*-homologue, designated *p73* (Kaghad *et al.*, 1997). This gene maps to chromosome 1q36 and shows LOH in neuroblastoma as well as a number of other cancers. p73 shares many biochemical properties with p53 including similar DNA binding specificity and involvement in cell cycle arrest. p73 also appears capable of association with p53 which could potentially result in reciprocal modulation of these two proteins. These properties strongly indicate that *p73* is a tumor suppressor gene. Specificity of expression and functional interplay of these proteins may modulate the involvement of *p53* in human cancers.

Our analysis of Wilms' tumors conclusively shows an association of *p53* mutations with the progression to anaplasia. In nearly all cases the mutations were late events since they were absent in regions of favorable histology which represent earlier stages of tumorigenesis. It remains unclear, however, whether *p53* mutations can cause predisposition to Wilms' tumor, *i.e.* whether such mutations can be involved in the *initiation* as opposed to the *progression* of Wilms' tumors. The Li-Fraumeni hereditary cancer syndrome is associated with the inheritance of a mutated *p53* allele (Malkin *et al.*, 1990). An increased incidence of Wilms' tumors associated with the Li-Fraumeni syndrome would suggest a role for *p53* mutations in the initiation of this malignancy. Kleihues *et al.* (1997) reported three Wilms' tumors in an analysis of 475 tumors in 91 families with *p53* germline mutations. Hartley *et al.* (1993) noted Li-Fraumeni syndrome in the families of two of 176 patients with Wilms' tumor. Both of these studies are suggestive of an increased incidence of Wilms' tumor in patients with the Li-Fraumeni syndrome. Since the number of cases is small, however, it is not currently possible to establish the statistical significance of this association and it remains possible that the link is merely

coincidental. We have identified a patient with a germline *p53* mutation and anaplastic Wilms' tumor arising in the context of Li-Fraumeni syndrome and familial Wilms' tumor (T.J. #46). The Li-Fraumeni syndrome was inherited maternally whereas as the other case of Wilms' tumor was on the paternal side of the family and hence the *p53* mutation did not segregate with Wilms' tumor in this family. We analysed the *p53* in a set of 40 patients with familial Wilms' tumor and found no germline mutations in these patients (data not shown). The absence of inherited *p53* mutations in patients with familial Wilms' tumor is consistent with our observations that p53 mutations are a late event in Wilms' tumor progression. Patient T.J. #46 was born with hemihypertrophy (asymmetric growth), a factor associated with predisposition to Wilms' tumor, albeit with low penetrance (Green *et al.*, 1993); it is possible that a coincident *p53* germline mutation increases the penetrance of Wilms' tumor in patients with hemihypertrophy *i.e.* perhaps *p53* mutations can initiate Wilms' tumorigenesis in cooperation with other lesions.

The genetic interplay of the *WT1* and *p53* loci in Wilms' tumorigenesis should be addressed. Two studies have described the association of the WT1 and p53 proteins (Maheswaran *et al.*, 1993; 1995). It is important to consider how this putative interaction relates to the well characterized genetic involvement of these genes in the pathogenesis of Wilms' tumor. The identification of this interaction emerged from experiments in which the genes were overexpressed in tissue culture cells and the corresponding proteins coimmunoprecipitated (Maheswaran *et al.*, 1993). Additionally the endogenous proteins from Wilms' tumors could be coimmunoprecipitated, albeit under conditions of low stringency. Functionally, it appears that coexpression of the two proteins results in modulation of their respective transcriptional properties. Licht (1996) has noted that this modulatory effect may be difficult to interpret since p53 is known to behave pleiotropic effects on transcription. There have been no reports of a demonstration of the interaction *in vitro* and the authors have stated that attempts to identify such an interaction in a yeast two-hybrid system have failed (Maheswaran *et al.*, 1995). The coexpression of these proteins results in the stabilization of p53 protein levels, an activity mapping to the zinc finger region of WT1. Finally, it is demonstrated that WT1 can either inhibit p53-dependent apoptosis or activate p53-independent apoptosis (Maheswaran *et al.*, 1995; Englert *et al.*, 1995). The authors attribute this variable behaviour to relative levels of WT1 expression. Consideration of the phenotypes of mice with homozygous null mutations for the p53 and WT1 genes may help in the interpretation of the significance of the biochemical interaction between the WT1 and p53 proteins. WT1-null mice display embryonic lethality and are characterized by complete absence of genitourinary system development. In contrast, *p53*-null mice are viable and undergo normal embryonic development. It appears then, that

normal functioning of WT1 does not require the presence of p53. Such a behaviour may be accounted for redundancy of *p53* (*e.g.* a role for the homologous gene, *p73*) or may indicate that the interaction has limited biological significance.

It is currently unknown whether *WT1* and *p53* mutations ever coincide in a Wilms' tumor. We have only analysed the status of the WT1 gene in two tumors in which we have identified *p53* mutations, and in neither did we detect *WT1* mutations. LOH at chromosome 11p occurs at lower frequency in association with anaplasia than with Wilms' tumors in general (Grundy *et al.*, 1996), which may suggest a negative correlation between anaplasia and *WT1* mutations. Anaplasia, however, does arise in some patients with *WT1* mutations (*e.g.* see Kaplinsky *et al.*, 1996). The decreased incidence of anaplasia in association with *WT1* mutations may reflect differences in the developmental stage of cells from which these tumors originated.

An intriguing problem which relates to the previous discussion is the absence of Wilms' tumors in the mouse. Humans with germline heterozygous or hemizygous *WT1* mutations (such as WAGR and DDS patients) develop such tumors with high penetrance while mice hemizygous for *WT1* (*i.e.* those with germline targeting of *WT1* or the Dickie small eye mouse mutant) do not show such a predisposition (Glaser *et al.*, 1990; Kriedberg *et al.*, 1993). Mice homozygous for *WT1* mutations cannot be evaluated for tumor development because of embryonic lethality. It appears that embryonic kidney tumors are exceedingly rare in the mouse, and there is no well characterized murine malignancy analogous to Wilms' tumor (reviewed in Pelletier *et al.*, 1991d). It has been speculated that the discrepancy between mice and humans may be due to differences in the size of the two organisms and in the time period of embryonic development. The small size and shortened growth period in the mouse result in a reduced target cell population and limited time window for additional random mutational events to occur (*e.g.* such as loss of the wild type *WT1* allele). Alternatively there may be more generalized variations in the physiology of these animals. Another possibility is that the role of *WT1* in tumorigenesis may involve cooperation with genes located at chromosome 11p15.5 in human (see above). Since the equivalent regions are not syntenic in the mouse a single LOH event is insufficient to alter both loci, thus reducing the probability of tumor development. Moreover, imprinting is poorly conserved between species (Jaenisch, 1997), thus, if imprinted genes cooperate with *WT1* in tumorigenesis, variations in imprinting may cause the phenotypic differences.

The lack of concurrence of phenotypes between homologous mutants in the mouse and human has been observed frequently. It is not unusual genes between these two species to share nearly identical sequences and yet for corresponding mutants to result in very difficult biological consequences (reviewed in Jacks, 1996). A number of human

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tumor suppressor genes display this behaviour. For example, heterozygous germline mutations in the *RB1* and *BRCA1* genes predispose humans, but not mice, to retinal and breast cancers respectively. It has been demonstrated that homozygous mutations of these genes in the mouse results in widespread cell death due to apoptosis. This apoptosis is dependant on the status of the *p53* gene such that mice lacking functional p53 have a rescue from apoptosis and tumor formation ensues (reviewed in Jacks, 1996). It appears that in the mouse, therefore, that a homozygous-null configuration of these genes, in the presence of wild type p53, is sensed as an abnormal growth state, triggering removal of these cells through apoptosis. An analogous process may be involved the case of the mouse with targeted disruption of *Wt1*. Indeed, WT1-null homozygotes are characterized by widespread apoptosis in precursor cells to the kidney. It tempting to speculate that this apoptosis may involve p53. An interesting experiment which is immediately feasible is the crossing of *p53* and *Wt1* mutant mice in order to generate the genotype *Wt1(+/-)/p53(-/-)*. By analogy to the case for *Rb1*, such animals may show predisposition to a murine Wilms' tumor. In addition it would of interest to evaluate the histology of such tumors, should they occur, to determine whether anaplasia was present.

Analysis of RNA-binding Properties of WT1

Zinc finger domains have conventionally been considered to be DNA binding modules, however, it is now apparent that they may function in a considerable diversity of processes. In the case of WT1, there is evidence to suggest that this protein may be involved in RNA metabolism. Original observations noted that, in transfected cells, the +KTS isoforms of WT1 colocalize with RNA splicing factors while the -KTS isoforms colocalize with transcription factors in the nucleus (Larsson *et al.*, 1995), thus implicating the +KTS isoforms in RNA binding. However, it was also demonstrated that interfering of the DNA binding ability of WT1-KTS causes altered subnuclear localization resulting in a similar distribution to that of splicing factors (Larsson *et al.*, 1995; Englert *et al.*, 1995b), thus both WT1 isoforms can be directed to regions of RNA processing. A cellular mechanism for the regulation of WT1-DNA binding may exist since cAMP-dependent protein kinase (PKA) phosphorylation serine residues in the zinc finger of WT1 causes decreased WT1-DNA binding *in vitro* and within the cell (Ye *et al.*, 1996; Sakamoto *et al.*, 1997) (see the Introduction for more complete discussion of these data). The phosphorylation of WT1-KTS, preventing DNA binding, may shunt this isoform into pathways for RNA metabolism and thus both the -KTS and +KTS isoforms of WT1 zinc

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fingers are potentially involved in RNA metabolism. *In vitro* RNA binding studies have supported this notion (Caricasole *et al.*, 1996; Ye *et al.*, 1996).

It has recently been demonstrated that the WT1 protein can be phosphorylated by protein kinase A (PKA) both *in vitro* and in transfected cells (Ye *et al.*, 1996; Sakamoto *et al.*, 1997). The sites of phosphorylation have been mapped to Ser-365 and Ser-393 which are in zinc fingers 2 and 3, respectively. Phosphorylation inhibits the DNA binding and transcriptional repression properties of WT1. A potential function for this this disruption of WT1 DNA binding may be to divert this protein to activity in RNA metabolism. It has been demonstrated in immunofluorescence experiments, that in cells treated with DNAse, the subnuclear localization of WT changes from nuclear domains overlapping with transcription factors to regions where splicing factors are localized (Larsson *et al.*, 1995; Englert *et al.*, 1995b; Caricasole *et al.*, 1996). It has not yet been demonstrated how phosphorylation of WT1 effects its subnuclear localization although upregulation of PKA activity by forskolin treatment causes increased cytoplasmic retention of WT1 (Ye *et al.*, 1996), an effect which may be a consequence of the proximity of the nuclear localization signal to the sites of phosphorylation. The authors, unfortunately, do not address the relative phosphorylation of cytoplasmic versus nuclear WT1 and hence change in protein localization may not be directly the result of PKA-mediated phosphorylation of WT1.

Chapter 6 of this thesis (Bardeesy et al., 1998), reported the characterization of potential RNA ligands to the -KTS isoform of WT1 using an RNA selection method. All of the RNA ligands shared WT1 structural requirements for efficient binding, *i.e.* zinc fingers 2 to 4 were critical. In addition all could competitively inhibit binding of WT1 to its DNA recognition sequence and thus the sites for RNA and DNA binding likely overlapped. Although specific binding was identified, it remains to be established whether these ligands are relevant to WT1 function *in vivo* since RNA molecules can form a plenitude of complex structures and, thus, the iterative selection procedure is able to generate ligands which are not relevant to the authentic function of a protein (*e.g.* see Jellinek *et al.*, 1993; Binkley *et al.*, 1995).The identification of ligands which show specific, high affinity binding to WT1, however, should facilitate the development of functional assays for a potential role of WT1 in RNA metabolism.

Although it is highly speculative, we can comment on a possible relationship between the interaction of p53 and WT1 and the ability of WT1 to bind to RNA. As was discussed above, there is evidence for a physical interaction between the p53 and WT1 proteins involving the zinc finger domain of WT1 (Maheswaran *et al.*, 1993; Maheswaran, *et al.*, 1995). The interaction, however, cannot be demonstrated *in vitro* or in a yeast two-hybrid system, and thus it may involve another component mediating the interaction. Since

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in the cell, a proportion of the p53 molecules are associated with RNA it is conceivable that WT1 and p53 interact as a ternary complex mediated by mutual binding to RNA. The requirement for such a complex would account for the inability to reconstitute the interaction of these proteins in the systems discussed above. This hypothesis can be tested experimentally by determining whether the WT1-p53 association in cell extracts is sensitive to RNAse treatment.

A further indication of the biochemical versatility of the WT1 zinc fingers is the ability of this domain to bind to heteroduplex DNA (Elser *et al.*, 1997). In the cell, heteroduplex DNA occurs during transcription due to 'bubble formation' in which double-stranded DNA is melted allowing recognition of the template strand by RNA polymerase II. WT1 is able to bind to heteroduplex DNA of a random sequence *in vitro* and point mutations in the zinc fingers reduces binding. Kid-1, also a transcriptional repressor protein with C_2H_2 zinc fingers, can also bind to DNA heteroduplexes. The authors suggest that these structures may be natural targets for some transcriptional repressors. Another type of nucleic acid molecule which may be relevant to WT1 function is the RNA-DNA hybrid. The related C_2H_2 zinc finger protein, Sp1, can bind to a duplex of complementary RNA and DNA consisting of the sequence of the Sp1 cognate DNA binding site, with similar affinity to that for DNA duplexes (Yi & Berg, 1995). A similar behaviour was also observed for the synthetic C_2H_2 zinc finger protein which, in fact, showed stronger binding for DNA-RNA hybrids than for DNA-DNA duplexes. The authors note that DNA-RNA hybrids are observed *in vivo* during gene transcription and thus may also be targets for transcriptional regulatory proteins. Taken together, these observation are hints that the WT1 zinc fingers may be highly versatile. Given that WT1 is capable of self-association, there is an intriguing possibility that WT1 may regulate gene transcription at several levels and that different WT1-nucleic acid complexes may be brought together by this self association. Little is known about the mechanisms of WT1-mediated transcriptional repression. The development of an *in vitro* transcription assay for WT1 activity may be highly informative in assessing possible biochemical role of WT1 in the functions discussed above.

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APPENDIX

This appendix describes the mutational status of *WT1* in a variant of the Wilms tumor-associated development disorder, Denys-Drash syndrome (DDS). Patients with full manifestation of the disease (the triad of Wilms' tumor, severe nephropathy, and intersex disorders) were known to harbor constitutional *WT1* mutations (Pelletier et al., 1991). Variants, sometimes referred to as incomplete DDS, lacking one of the three characteristic abnormalities have been noted in the literature (Manivel *et al.*, 1987). It was not clear whether incomplete DDS was truly a distinct clinical entity. We were interested in the molecular etiology of this variant with respect to the genetics of *WT1*. We wished to establish whether the pathology of these patients is attributable to lesions in *WT1*, and if so how do such lesions compare with the alterations which cause the complete form of the disorder. Through the detection of a spectrum of mutations associated with different phenotypes, it was hoped that we could obtain clues as to the origins of the relatively reduced severity of this disorder and obtain insights into the function of the WT1 protein and its domains.

WT1 Mutations Associated with Incomplete Denys–Drash Syndrome Define a Domain Predicted to Behave in a Dominant-Negative Fashion

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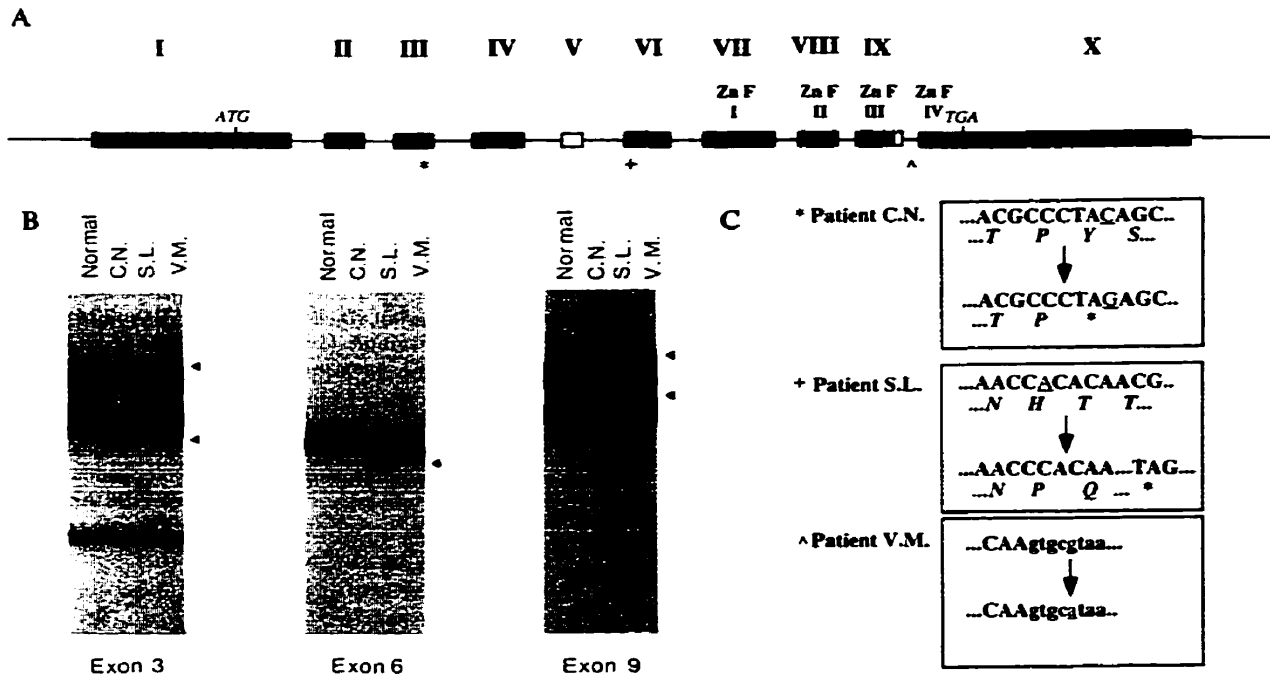
Denys–Drash syndrome (DDS) is a complex congenital disorder consisting of intersex disorders (XY pseudohermaphroditism or XY female), nephrotic syndrome, and Wilms tumor (WT) (1, 2). The 11p13 WT suppressor gene, WT1, encodes a transcription factor containing four zinc fingers of the Cys_2–His_2 variety (3, 4). Two of the 10 WT1 coding exons are alternatively spliced to produce 4 mRNA species (5). WT1 mRNA and protein are predominantly expressed in components of the urogenital system, spleen, and mesothelial cells (6, 7). Mice in which the WT1 gene is homozygously deleted fail to activate development of the urogenital system, indicating a key role for WT1 in the formation of this system (8). Consistent with this, germline missense mutations within the WT1 DNA binding domain are the most common lesions defined in individuals with DDS, providing genetic evidence of a role for WT1 in pathogenesis of WTs and development of the urogenital system (9–11). An incomplete form of DDS occurs in individuals suffering from renal nephropathy who either do not develop WT or exhibit normal sexual differentiation

(12). It is not clear that incomplete and complete DDS share a common molecular etiology.

To address this issue, we analyzed three individuals with incomplete DDS for possible germline WT1 mutations. The clinical features of these individuals have been described (13). Briefly, patient C.N. developed bilateral WTs and was diagnosed with nephrotic syndrome at the age of 11 months. He also suffers from bilateral cryptorchidism. Since this individual does not suffer from the severe intersex disorders associated with DDS, he is considered to have incomplete DDS. Patient S.L. is an XX female diagnosed with WT at the age of 2 years, developed nephrotic syndrome at 15 years, and has a normal genital system. Patient V.M. is an XY pseudohermaphrodite who developed nephrotic syndrome at the age of 2.5 years. This individual is currently 14 years of age and has not developed a WT. The renal lesions of all three affected individuals are characteristic of DDS (K. Schmitt, unpublished observations). Following isolation of genomic DNA from blood of the affected individuals, each WT1 exon (Fig. 1A) was amplified by the polymerase chain reaction (PCR) (9). The products were screened for possible mutations by single-strand conformational polymorphism (SSCP) (9). Mobility shifts in exon 3 (patient C.N.), exon 6 (patient S.L.), and intron 9 (patient V.M.) indicated that all three individuals had WT1 mutations in the heterozygous configuration (Fig. 1B). Cloning and sequence analysis of these PCR products revealed a [663]C to G transversion converting [95]Tyr to a stop codon in patient C.N., a deletion of [827]A in patient S.L., and a G to A transition at the +5 position of intron 9 for patient V.M. (Fig. 1C).

These results provide evidence that incomplete and complete DDS have a common molecular basis. The mutations described for patients C.N. and S.L. are predicted to result in early termination of translation, producing truncated polypeptides lacking a DNA binding domain. The majority of DDS lesions are missense mutations occurring within WT1 zinc finger III converting an arginine to a tryptophan residue (9). Constitutional mutations within the WT1 gene have been described for individuals with WTs, cryptorchidism, and hypospadias, but not suffering from the renal nephropathy typical of DDS (13, 14). These lesions are thought to be functionally equivalent to the loss of one WT1 allele, since the phenotypes they produce are also observed in individuals with WAGR syndrome (an association of Wilms tumor, aniridia, genitourinary anomalies, and mental retardation) associated with constitutional deletions of 11p13. These results suggest that WT1 haploinsufficiency during gonadal development interferes with proper development of the male genital system. However, the DDS mutations have more severe effects on development of the urogenital system (i.e., renal nephropathy and XY pseudohermaphroditism) compared to those observed in the context of WAGR syndrome, and thus have been proposed to behave in a dominant-negative fashion. As such, the mutation described in patient C.N. defines the smallest WT1 domain (a polypeptide encoded by exons 1, 2, and 3) predicted to behave in such a fashion. The splicing mutation defined for patient V.M. has been previously reported and experimentally shown to affect alternative splice site selection (10). The net result is a skewing of the ratio of WT1 isoforms, consistent with a dominant-negative mode of behavior for DDS mutations (10).

A

I   II III   IV   V   VI   VII   VIII IX   X

ZnF   ZnF ZnF ZnF
I   II   III IV TGA

ATG

B

Exon 3   Exon 6   Exon 9

C

* Patient C.N.

...ACGCCCTACAGC...
...T   P   Y   S...

↓

...ACGCCCTAGAGC...
...T   P   *

+ Patient S.L.

...AACCACACAACG...
...N   H   T   T...

↓

...AACCCACAA...TAG...
...N   P   Q —   *

^ Patient V.M.

...CAAgtgcgtaa...

↓

...CAAgtgcataa...

FIG. 1. PCR/SSCP analysis of the WT1 gene in three individuals with incomplete DDS. (A) Schematic diagram of the WT1 gene. The stippled boxes represent the WT1 zinc fingers, and the open boxes are the two alternatively spliced exons. The ATG and TGA are indicated. Each exon is numbered. The asterisk, cross, and caret symbols are the mutated areas defined in this study for patients C.N., S.L., and V.M., respectively. (B) PCR-SSCP analysis of the WT1 gene. Twelve pairs of oligonucleotides designed to cover the WT1 coding region were used in PCR/SSCP assays as previously described (9). The patient code is denoted above each lane. The WT1 exon amplified and being analyzed is indicated below each panel. Aberrant SSCP mobility shifts are indicated with arrowheads. (C) Nucleotide sequence of PCR products displaying SSCP mobility shifts. The nucleotide sequence of exon showing a different mobility than control DNA was determined as previously described (9, 10). The predicted WT1 amino acid sequence is shown in italics below the nucleotide sequence. Mutated nucleotides are indicated by an underline. Exon and intronic sequences are represented by upper- and lowercase lettering, respectively.

Individuals with germline WT1 mutations do not necessarily develop the full spectrum of DDS features. The incomplete penetrance of WT in some DDS individuals (e.g., patient V.M.) probably reflects the requirement of additional mutational events to progress to malignancy. In a hereditary setting, the penetrance of WT has been reported to be ~40–60% (15). Variability in expressivity of WT1 germline mutations on genital system development may be due to differences in genetic background or individual mosaicism. What determines the presence or absence of nephrotic syndrome in individuals with WT1 germline mutations? One possibility is that various WT1 nonsense or frameshift mutations produce macromolecules with different biochemical properties. We have previously described two males with frameshift mutations in WT1 exons 4 and 6, predicted to produce truncated polypeptides very similar to those described herein for patients C.N. and S.L. (13). These individuals do not suffer from nephrotic syndrome and may produce unstable mRNA or protein species unable to behave in a dominant-negative fashion. Understanding the phenotypic differences for these mutations will require biochemical characterization of the altered WT1 proteins and mRNAs. The novel mutations described in this report define unique reagents with which to experimentally address these issues.

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