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Wilms' tumour - a case of disrupted development

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SUMMARY

Wilms' tumour is a paediatric kidney malignancy that arises through aberrant differentiation of nephric stem cells. We are studying the role of one Wilms' tumour predisposition gene, WT1. This is a tumour suppressor gene whose function is required for normal development of the genitourinary system. WT1 encodes a putative transcriptional repressor of the zinc finger family. Here we discuss how one of the normal functions of WT1 may be to suppress myogenesis during kidney development. Furthermore, we describe how we are proposing to use YAC (yeast artificial chromosome) transgenesis to analyse WT1 regulation and function in mice. We also discuss the evolution of the WT1 gene amongst different vertebrate classes and how this may provide insights into genitourinary evolution.

Key words: Wilms' tumour, WTI, transcriptional repression, tumour suppression, zinc finger

INTRODUCTION

Wilms' tumour (nephroblastoma) has long been considered a wonderful example of the way in which tumours can arise through abnormal development (van Heyningen and Hastie, 1992; Haber and Housman, 1992; Hastie, 1993). These tumours arise from mesenchymal stem cells, which usually differentiate into the epithelial components of the nephron. Normally during kidney development the mesenchyme condenses around the ureteric buds and in response to inductive signals from the bud differentiates into epithelial cells, which go on to form the proximal and distal tubules and the glomerulus of the nephron (Fig. 1). The tumours appear to arise from aberrant rests of stem cells, which persist into early childhood. Normally such rests are transient structures that would disappear by 36 weeks of gestation.

The majority of tumours consist of a combination of blastemal stem cells, stromal cells and epithelial structures that often look like tubules and incomplete glomeruli of the nephron. However, a minority of tumours contain ectopic tissues of mesodermal origin including skeletal muscle, bone and cartilage. We are keen to explore the molecular basis for the production of these ectopic tissues in the tumour.

By studying these tumours we hope to gain insight into normal kidney development. Conversely we will not understand tumorigenesis without an appreciation of the mechanisms controlling normal nephrogenesis.

Unlike the situation for the other well known paediatric tumour, retinoblastoma, there appear to be several different Wilms' tumour predisposition genes (van Heyningen and Hastie, 1992; Haber and Housman, 1992; Hastie, 1993). We are studying one of these, the WT1 gene, which is located on chromosome 11p13 (Call et al., 1990; Gessler et al., 1990). The WT1 gene is a tumour suppressor gene that encodes a protein with four zinc fingers of the Kruppel type. The last three zinc fingers of WT1 share strong homology with those found in the known transcription factors SP1, EGR1 (krox24) and EGR2 (krox20). Unlike these other zinc finger proteins four different isoforms of WT1 are produced through two alternative splices, one leading to the insertion of 17 amino acids upstream of the zinc fingers, the other inserting three amino acids (KTS) between zinc fingers 3 and 4 (Haber et al., 1991). It has been shown that WT1 proteins bind to G-rich target DNA sequences, three nucleotides to each finger (Rauscher et al., 1990; Bickmore et al., 1992). Also the products that differ by the insertion of the KTS have different DNA binding site preferences and may therefore bind to different target genes (Drummond et al., 1994).

The WT1 gene is expressed in a limited set of tissues during development, chief among those being the kidney, the gonads, the spleen and the mesothelium (Pritchard-Jones et al., 1990; Armstrong et al., 1992). All these tissues are of mesodermal origin and all experience a mesenchymal to epithelial transition when WT1 is being expressed. We have proposed that WT1 plays a role in switching cells from a mesenchymal to an epithelial fate. In the kidney expression of WT1 is very low in the uninduced mesenchyme but then increases dramatically during the induction process within the condensed blastema (Fig. 1). Expression then becomes restricted to the posterior part of the newly formed epithelium and is eventually limited to the epithelial layer of the glomerulus. Genetic evidence suggests that WT1 may have different functions at three different stages of nephrogenesis (Fig. 1).

Children who inherit heterozygous deletions of the WT1 gene often develop kidney tumours during the first five years of life but may also suffer from mild abnormalities of the genital system. The tumours arise when the second copy of the gene is also mutated, consistent with Knudson's two-hit hypothesis for tumour suppressor genes. However, children who inherit heterozygous mutations (usually missense mutations) restricted to the zinc finger region of the protein can develop very much more extreme anomalies including severe glomerular nephropa-

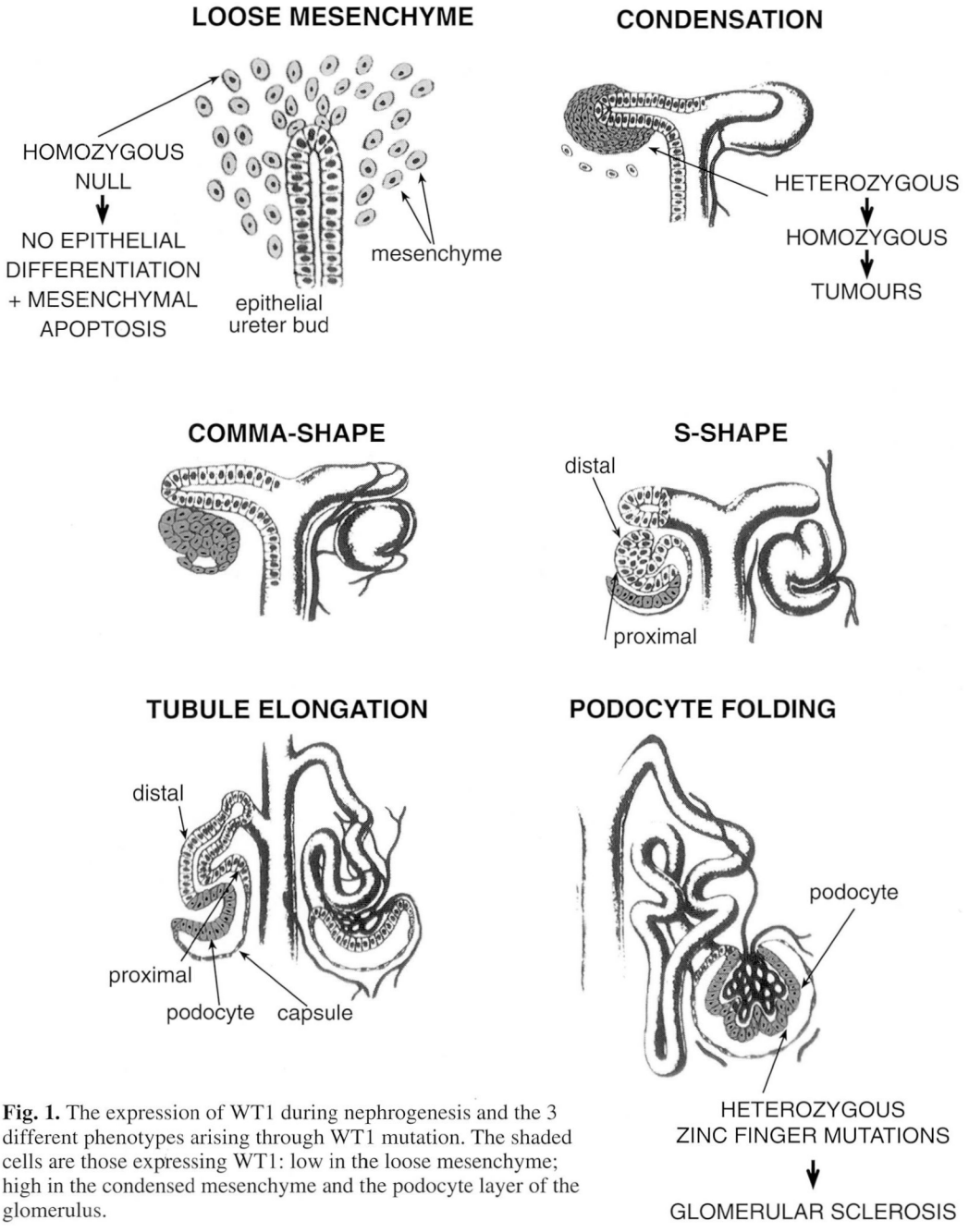

Fig. 1. The expression of WT1 during nephrogenesis and the 3 different phenotypes arising through WT1 mutation. The shaded cells are those expressing WT1: low in the loose mesenchyme; high in the condensed mesenchyme and the podocyte layer of the glomerulus.

thy, gonadal dysgenesis and Wilms' tumour (Pelletier et al., 1991; Bruening et al., 1992; Baird et al., 1992; Little et al., 1993). Hence it has been proposed that these mutant proteins are acting in a dominant negative fashion; perhaps the mutant form partially inactivates the wild-type protein encoded by the normal allele by forming a heterodimer. Children with the zinc finger mutations and severe genitourinary abnormalities have the Denys-Drash syndrome (DDS).

Proof that the WT1 gene is essential for the development of the genitourinary system has come from the analysis of homozygous null mice (Kreidberg et al., 1993). WT1 null mice completely lack gonads and kidneys and the mesothelium is abnormal. In these mice the mesenchyme of the kidney fails to differentiate into epithelium and is subject to apoptosis.

It is now apparent that mutations in the WT1 gene are only found in 10-15% of Wilms' tumours. So far there have been no clues to suggest that tumours arising through WT1 mutation have different pathological features from those arising in the absence of WT1 mutation. If such differences could be defined they could provide significant insights into WT1 function.

WT1 always behaves in transient transfection assays as a repressor of gene expression (Madden et al., 1991). Hence we can envisage that the normal function of WT1 is to shut off the expression of genes required to maintain kidney stem cells in cycle. When WT1 function is lost stem cells keep in cycle and fail to differentiate. Compelling candidate target genes for regulation by WT1 include the insulin-like growth factor 2 gene (IGF2) and the insulin-like growth factor 1 and 2 receptor

(IGF1/2 receptor) (Drummond et al., 1992; Werner et al., 1993). Both of these genes can be expressed at high levels in Wilms' tumours and are normally expressed at maximum level in undifferentiated kidney mesenchyme. Furthermore it has been shown that WT1 can bind to the promoters of these genes and repress reporter expression from these promoters in transient transfection systems. However, there is no proof as yet to support the idea that these are true physiological targets of WT1 during development.

Our experiments are now directed towards understanding the function of the WT1 gene during development: can WT1 exert a mesenchyme-epithelial transition when expressed ectopically? What are the physiological target genes regulated by WT1 during the development of these mesodermal tissues? Also as the genitourinary system is one that has evolved during vertebrate development we are characterising the WT1 gene from all the major vertebrate classes, with the hope that this might provide insights into the function of WT1 as well as into the evolution of the genitourinary system.

ACTIVATION OF THE MYOGENIC PROGRAMME IN TUMOURS WITH WT1 MUTATION

We were provided with a clue concerning the pathology of tumours involving WT1 mutation when the two nephroblastomas of a child with DDS were examined. As is often the case with inherited WT1 mutations this child had bilateral Wilms' tumours, one on the left kidney the other on the right kidney. Whereas one of the tumours had typical pathology, that is a mixture of epithelial structures, blastemal cells and stromal cells, the other tumour had very large regions that appeared to consist entirely of skeletal muscle. Immunohistochemical markers confirmed that skeletal muscle proteins such as myosin were being expressed in this tumour.

Skeletal muscle is known to develop through the action of the so-called myogenic genes, a group of transcription factors with the helix-loop-helix (HLH) domain, which include myoD, myogenin, Myf5 and Myf6. Recently we have examined whether these genes are expressed in Wilms' tumours and whether such expression is a common feature of tumours arising through WT1 mutation.

We have found that tumours arising through WT1 mutations have high levels of expression of these myogenic genes but that tumours not involving WT1 mutations do not express detectable levels. We have not been able to detect expression of these genes in normal developing kidney thus this appears to be true ectopic activity of myogenic genes.

This finding raises two interesting possibilities. First, that kidney stem cells are capable of differentiating into alternative mesodermal structures, including kidney, bone and cartilage but are normally repressed from doing this. Secondly, that when WT1 function is lost the strict direction of differentiation into epithelial cells is also lost and the stem cells are now capable of differentiating in various directions.

This also begs the question of whether WT1 can directly repress the differentiation of mesenchymal stem cells into muscle and other structures. To test this we have introduced constructs expressing the different spliced forms of WT1 into cultured C3H 10T1/2 cells, mesenchymal cells that can be induced to differentiate into muscle, cartilage and adipose

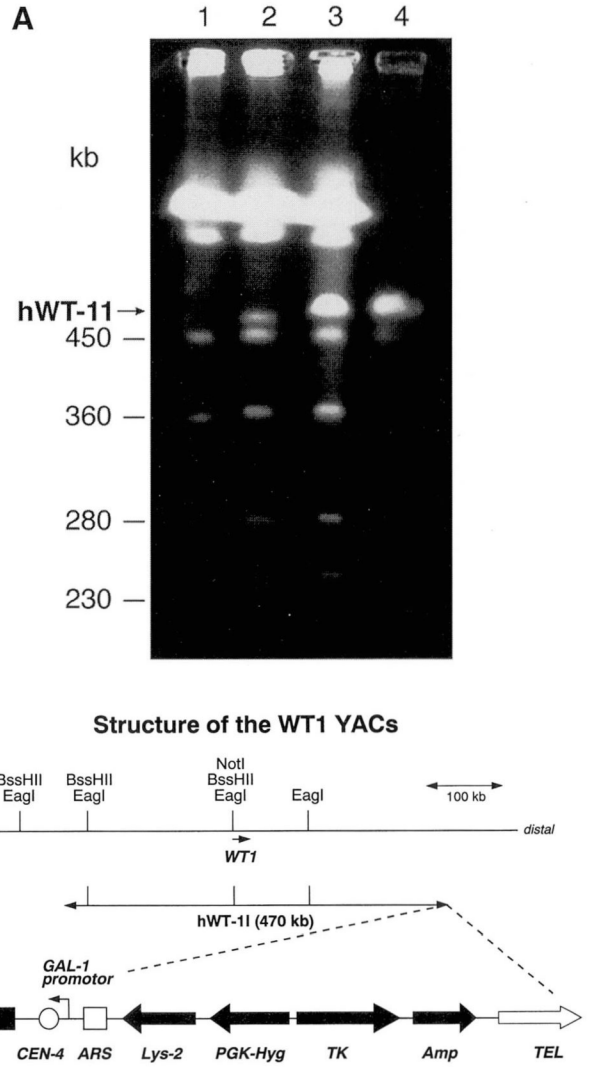

Fig. 2. Amplification and isolation of a large YAC containing the human WT1 gene. (A) Pulsed field gel electrophoresis of the YAC before and after purification; lane 1, chromosomes from *S. cerevisiae*; lane 2, chromosome preparation from *S. cerevisiae* containing the human WT1 YAC (hWT-1I) - see extra band at 470 kb; lane 3, human WT1 YAC after amplification stage (see Schedl et al., 1993); lane 4, the hWT-1I YAC cut out of the gel (as in lane 3) and rerun. (B) Map showing the position of the WT1 gene (70 kb) within the human insert together with features of the vector (see Schedl et al., 1993).

tissue. So far no effect of ectopic WT1 expression on the differentiation of these cells has been obtained; on the one hand there is no inhibition of differentiation into muscle, on the other hand we find no evidence of activation of epithelial markers that would have shown a direct effect of WT1 on mesenchyme to epithelial transition.

The only problems with these experiments were: (i) that we may not have been expressing sufficiently high levels of WT1 protein in these cells; and (ii) that the WT1 gene normally expresses four different forms of mRNA through alternative splicing. The ratio of these different forms is invariant in different mammals and in different tissues so that all the forms

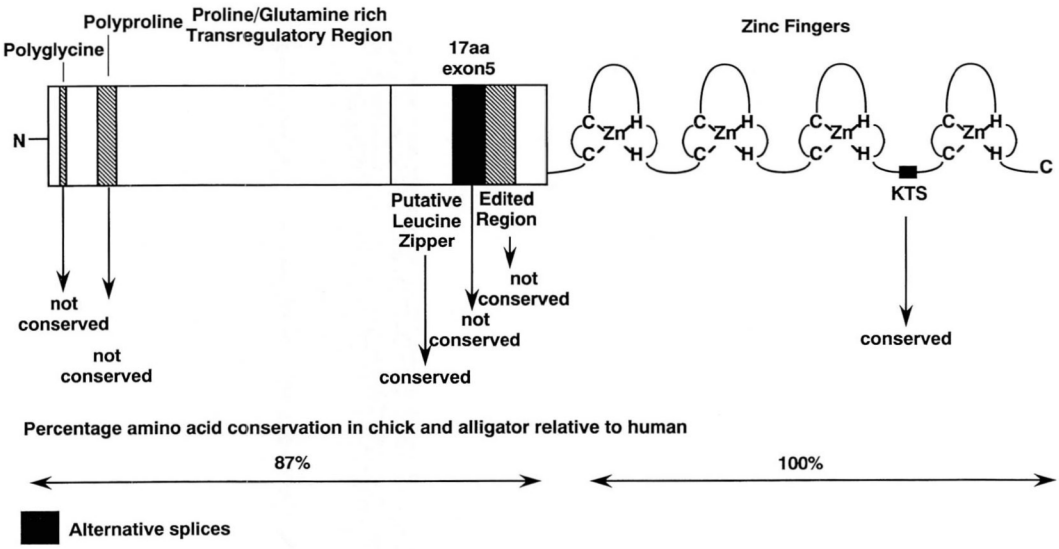

Fig. 3. Evolutionary comparison of the chick/alligator and human WT1 proteins.

may be necessary for WT1 to function, perhaps as a complex. If so, expression vectors encoding individual spliced forms may not be able to provide WT1 function. To circumvent this problem we are introducing the whole WT1 gene, which should express all the spliced forms on a YAC vector, both into cells in culture and into mice.

THE USE OF WT1 GENE-CONTAINING YACS FOR FUNCTIONAL STUDIES

As discussed above WT1 encodes four different splice forms of mRNA. All of these may be essential for function. Hence it is desirable to use the whole gene encoding all the different forms of the protein for expression studies. The problem here is that the complete gene is at least 70 kb in size and therefore too large to be included in conventional vectors. Hence we are using YAC vectors, which contain the human WT1 gene and up to 200 kb of flanking sequences upstream and downstream of the gene. If such YACs are introduced into mice or cells they should contain all the regulatory regions intact and should be able to carry out regulated splicing correctly (Schedl et al., 1993). Hence we have introduced one such YAC with a 500 kb insert into mice by direct pronuclear injection (Fig. 2). We have obtained a number of transgenic animals and are now waiting to see if these will transmit the YAC. If so the YAC will be transferred by conventional breeding to WT1 null mice to see if it can rescue the complete development of the mice. We will then create a whole range of modified WT1 YACs using the power of homologous recombination in yeast to generate mutations. Hence we are replacing the WT1 promoter with a constitutive promoter so that we can test whether ectopic expression of WT1 can both suppress muscle formation and direct the mesenchyme to epithelial transition in mice. Also we are making mutations that will disrupt individual splice forms to test the role of these forms during development. Finally, we are intending to create WT1 forms with missense mutations that are similar to those found in patients with DDS. Using these we would like to see whether we can create a mouse model for this condition.

THE EVOLUTION OF THE WT1 GENE AMONGST VERTEBRATES

We have been able to isolate full-length WT1 cDNAs from chicken and alligators and partial cDNAs including the zinc finger region from fish and amphibians (*Xenopus*). Sequencing of these cDNAs has shown that WT1 is highly conserved throughout its length but particularly in the zinc finger region, which maintains 99% identity throughout all the vertebrates (Fig. 3). This is particularly striking as the WT1 zinc fingers share 50-80% identity with the fingers of other known transcription factors. The zinc fingers of WT1 seem to be interchangeable with the related fingers of these other transcription factors. If this is the case why have the zinc fingers been constrained to diverge so much less during evolution? It suggests that there are additional functions for the zinc finger region, other than DNA binding, which we have not yet unravelled.

The +KTS/−KTS differential splice is conserved throughout all vertebrates proving that this must be functionally very important. However, the second alternative splice (±17 amino acids) could only be detected in mammals and not in birds or reptiles (Fig. 3) suggesting that this is a mammal-specific increase in complexity. Hence two WT1 isoforms can be encoded by chickens and alligators compared with four in all the mammals studied. Could the additional forms be involved in directing the production of mammal-specific structures in the genitourinary system? To test this we hope to introduce the WT1 gene from different vertebrates into WT1 null mice to see if they can rescue all the structures.

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