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Genetics

Role for the Wilms tumor gene in genital development?

(kidney/gonads/cancer/pulsed-field gel electrophoresis/deletion on human chromosome 11)

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ABSTRACT **Detailed molecular definition of the *WAGR* region at chromosome 11p13 has been achieved by chromosome breakpoint analysis and long-range restriction mapping. Here we describe the molecular detection of a cytogenetically invisible 1-megabase deletion in an individual with aniridia, cryptorchidism, and hypospadias but no Wilms tumor (WT). The region of overlap between this deletion and one associated with WT and similar genital anomalies but no aniridia covers a region of 350–400 kilobases, which is coincident with the extent of homozygous deletion detected in tumor tissue from a sporadic WT. A candidate *WT* gene located within this region has recently been isolated, suggesting nonpenetrance for tumor expression in the first individual. The inclusion within the overlap region of a gene for WT predisposition and a gene for the best-documented WT-associated genitourinary malformations leads us to suggest that both of these anomalies result from a loss-of-function mutation at the same locus. This in turn implies that the *WT* gene exerts pleiotropic effect on both kidney and genitourinary development, a possibility supported by the observed expression pattern of the *WT* candidate gene in developing kidney and gonads.**

Wilms tumor (WT) is a kidney malignancy—the most frequently seen solid tumor of childhood. Most cases are sporadic and unilateral, with no associated anomalies. Bilateral and familial tumors, comprising approximately 7% and 1% of the incidence, respectively (1), have an earlier age of onset than unilateral cases. On the basis of such statistics, the two-hit hypothesis formulated originally for retinoblastoma was extended to WT (2). This model suggests that two mutational steps are required for the development of these childhood tumors. In the hereditary (bilateral and familial) cases, the first predisposing mutation is postulated to be present already in the germ-line and therefore present constitutively in all cells of the target tissue—hence, the earlier age of onset. In sporadic cases, two mutations have to accumulate postzygotically within the same target cell. Ninety-five percent of all WT cases have presented by the age of 10 years (1), suggesting that the target-cell population disappears as development to mature form is completed.

Apart from rare familial incidence, the most clear-cut evidence for the presence of a predisposing DNA-level change has come from the unexpectedly frequent association (1 in 100) of WT with aniridia (absence of iris) (3). After this observation, the diagnosis of sporadic aniridia in newborn infants, especially when accompanied by other anomalies, became a prognostic indicator for the possible development of WT. In many such cases, additional genitourinary abnormalities and mental retardation are also present—hence, the acronym WAGR for this syndrome. Karyotypic analysis of WAGR cases revealed the frequent presence of a deletion on one chromosome 11 homolog, always including part of band 11p13 (4). Only about 60% of the cases with a visible deletion develop WT, demonstrating that there is incomplete penetrance—presumably because no second mutation has occurred, although it should be borne in mind that deletions that do not include the *WT* locus may exist. However, genitourinary abnormalities, particularly in affected males, are seen at increased frequency, even in cases of sporadic WT, where no evidence of deletion can be found (M. M. Weil and D. A. Compton, personal communication; D. E. Housman, T. M. Glaser, and J. Pelletier, personal communication). There is also strong statistical evidence that genital- and kidney/urinary-system abnormalities are associated with early-onset (more frequently bilateral) WTs and with intralobar nephroblastomatosis (1, 5). These associated anomalies are all hallmarks of the "hereditary" tumor arising when there is a preexisting germ-line mutation. Therefore, their existence may result from the dominant effects of constitutional mutation—the first "hit" at the *WT* locus.

In the course of localizing accurately and eventually cloning the genes of the *WAGR* region, using a number of deletion and translocation breakpoints, we have carried out molecular analysis of the 11p13 region in a child with incomplete WAGR syndrome. His abnormalities comprise aniridia, genitourinary abnormalities, and mental retardation but no WT. Hybrid cell analysis and pulsed-field gel electrophoresis (PFGE) with previously mapped markers for the region revealed the loss of around 1 megabase (Mb) from one chromosome 11 homolog in this case, although no cytogenetic abnormality was visible.

Detailed molecular analysis also has been carried out on a second patient with a visible deletion at chromosome 11p13, who had WT, genitourinary abnormalities, but no aniridia (6–8). The smallest region of overlap between these two deletions coincides with the extent of a rare WT-associated homozygous deletion region (9, 10) and includes the putative *WT* locus, for which a candidate gene was recently isolated (11). From the clinical observations in these two constitutional deletion patients, the overlap region must also contain the gene(s) whose deletion leads to the associated genitourinary abnormalities.

On the basis of these results, we suggest that the *WT* gene may have pleiotropic effects on both kidney and genital

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Abbreviations: WT, Wilms tumor; WAGR, WT with aniridia, genitourinary anomalies, and mental retardation triad; *WT*, gene for WT; *AN2*, chromosome 11p13-associated aniridia 2 gene (without WT, genitourinary anomalies, and mental retardation); PFGE, pulsed-field gel electrophoresis; *FSHB*, gene for follicle-stimulating hormone β; *CALC*, gene for calcitonin; *CAT*, gene for catalase.

development, at least in males. This idea is supported by the elevated frequency of genitourinary anomalies accompanying WT and by the common embryological origins of the genital system and the metanephric kidney. The argument in favor of this dual role for the *WT* gene in the development of both the kidney and genital system is further strengthened by the pattern of expression of the recently isolated candidate gene for the *WT* locus.

MATERIALS AND METHODS

Clinical Summary and Cytogenetic Analysis. The patient, PAZO, presented initially with sporadic aniridia. Subsequently cryptorchidism (undescended testes) and hypospadias (misplaced penile external urinary orifice) were noted. Mild mental retardation and developmental delay were observed. No WT has been found in this boy, now age 12. Karyotype analysis of chromosome preparations from peripheral blood and later from the Epstein–Barr virus-transformed lymphoblastoid cell line (12) revealed no abnormalities. In particular no deletion was detected on the chromosome 11 short arm.

The second patient, DAR, has been described in detail (13). He presented with WT at age 21 months. There was no sign of aniridia or cataract. He required surgery to correct abnormal external genitalia: chordee (abnormally curved penis) and bilateral cryptorchidism. Karyotype analysis of chromosomes from peripheral blood cultures revealed deletion of most of band 11p13. Catalase activity was reduced, suggesting deletion of the locus *CAT* encoding this enzyme.

Somatic Cell Hybrids. Fusions were carried out between Epstein–Barr virus-transformed lymphoblastoid cells from the patients and mouse myeloma cell lines as described (6, 12). Stable cell lines were selected by fluorescence-activated cell sorting and subcloning of live cells with high-level expression of appropriate human chromosome 11-encoded cell surface markers (6, 14). The newly produced hybrids from the PAZO cell line were designated PAX and were analyzed for heterozygous marker alleles to ensure the presence of each chromosome 11 homolog in independent hybrids. DAR 15.4 has been described (6).

DNA Markers. The derivation and characterization of the DNA markers used in these analyses have been described in detail as follows: probes specific for follicle-stimulating hormone β gene (*FSHB*), for the calcitonin gene (*CALC*), for *CAT*, and for monoclonal antibody markers for *MIC11* and *MIC4* (14); *D11S16* (15); L65-6/74 (*D11S112*), C65-6/6 (*D11S102*), L65-6/22B (*D11S107*), and P9RH (16); 582 (*D11S317*), 495 (*D11S310*), M20 (*D11S377*) (7); p5 (*D11S323*) (17); S1 (*D11S87*) (9); NBC12 (*D11S294*) (8); LP2G4 (*D11S48*), LP4F11 (*D11S82*), LP11F9 (*D11S49*) (18); and *D11S9* (19).

Repeat-free probes used in this study have been described (8). Additionally, JM44 is a 4.4-kilobase (kb) *Bam*HI fragment from L65-6/74 DNA marker subcloned into pBluescribe.

PFGE. High molecular weight DNA was prepared, and PFGE was performed as described (8). Size markers were oligomers of phage λ DNA and chromosomes of *Saccharomyces cerevisiae*, strain YPH 148.

RESULTS

A series of PAX hybrids were produced from the PAZO lymphoblastoid cell line known to be heterozygous for the polymorphic marker *CALC*. Analysis of these hybrids revealed that one allele was present in PAX 1 and the alternate allele in PAX 17, thus demonstrating the separation of the two chromosome 11 homologs (Fig. 1*a*).

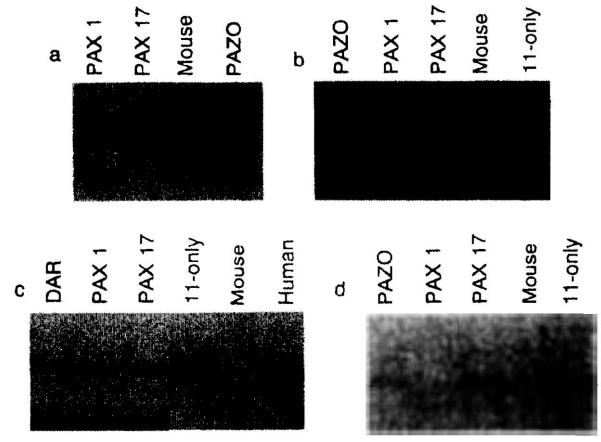

FIG. 1. Deletion mapping by DNA blot analysis in somatic cell hybrids. Digested DNA was loaded for hybrids (10 μg) and species controls (5 μg). "11-only" denotes a control somatic cell hybrid with chromosome 11 as its sole human component. *Thermus aquaticus Taq* I digests were probed for *CALC* polymorphism (*a*), and *Bam*HI digests were probed for DNA markers NBC12 (*b*), L65-6/74 (*c*), and S1 (*d*).

Initial hybridization with available markers for the 11p13 region showed no evidence of deletion, even with DNA markers such as NBC12 and L65-6/74, which were located close to the *WT* and *AN2* loci (8) (Fig. 1 *b* and *c*). As further markers became available the hybrids were examined for their presence. In PAX 1, but not PAX 17, the markers S1 (Fig. 1*d*) and 495 were deleted as well as the intervening marker p5 (data not shown). Results are summarized in Fig. 2, which illustrates the extent of deletions in the two patients described here.

The physical map of the *WAGR* region, summarized in Fig. 3, suggested that the DNA markers NBC12 and L65-6/74 should closely flank the deletion in PAZO DNA (8). Characterization of PAZO DNA by PFGE demonstrated that the proximal deletion breakpoint disrupts the 500-kb *Not* I fragment hybridizing to NBC12 (Fig. 4*B*), although the proximal 90-kb region of this fragment was unaltered (data not shown). In a similar manner, the distal deletion breakpoint interrupts the 1.4-megabase (Mb) *Bss*HII fragment [these CpG island sites are coincident with *Not* I sites (8, 20)] hybridizing to L65-6/74 (Fig. 4*A*). The presence of an unaltered *Sac* II fragment restricts the position of this breakpoint to the centromeric 400 kb of the *Bss*HII (*Not* I) fragment (20). This fragment directly abuts the AN2 translocation, which has been studied in detail (21).

The distal deletion breakpoint in the DAR parent lymphoblastoid line cannot be localized precisely. No altered *Not* I fragment could be detected with probe p5, which therefore places the deletion breakpoint proximal to the 325-kb fragment on which p5 resides (17).

DISCUSSION

Molecular analysis of 11p13 has allowed several groups to delineate a consensus map of the DNA in the *WAGR* region (7, 8, 17, 20). In this study we have demonstrated the presence of a 1-Mb deletion in a patient with aniridia, hypospadias, cryptorchidism, and mental retardation but no WT. It is worth noting that a karyotypically undetectable deletion can involve loss of such a large amount of DNA.

The smallest region that must contain the *WT* locus has been refined by the deletions in DAR and in WiT13 DNAs. DAR is an individual with WT, cryptorchidism, and mild hypospadias but without aniridia—the case that clearly demonstrated the separate identities of the *WT* and *AN2* genes,

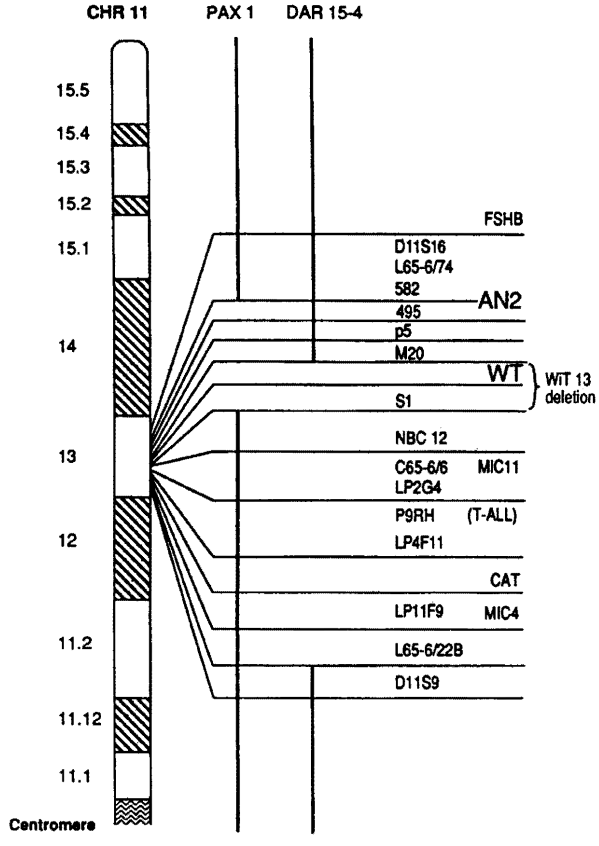

FIG. 2. Map defining the smallest region of overlap between the DNA deletions in PAZO and DAR cells and the extent of the DNA deletion in WiT13 tumor cells. The markers are described in the text. Horizontal divisions delineate the separate compartments, which have been defined by independent breakpoints. T-ALL, T-cell acute lymphocytic leukemia.

since the latter is considered to be a fully penetrant dominant mutation (7, 8, 22). The position of the *AN2* gene has also been defined at the molecular level in aniridia-associated translocations (21, 22). WiT13 is a tumor cell line from a

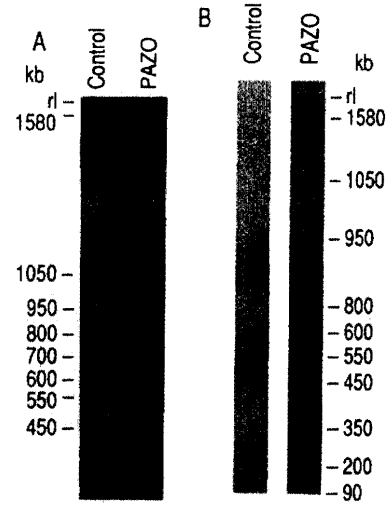

FIG. 4. PFGE analysis of the PAZO DNA breakpoints. Position of the yeast size markers is indicated. (*A*) *Bss*HII digest was probed with the single-copy subclone of L65-6/74: homozygosity for the normal 1.4-Mb fragment is seen in control DNA, while PAZO DNA is heterozygous for the normal fragment on one homolog and a new smaller fragment on the deletion chromosome. (*B*) *Not* I digest was probed with the single-copy subclone of NBC12: homozygosity for the normal 500-kb fragment is seen in control DNA, while PAZO DNA is heterozygous for the normal fragment and an ≈1.5-kb altered fragment.

sporadic WT in a patient with no constitutional chromosome 11 deletion. However, the tumor itself was shown to be homozygously deleted for the 11p13 marker S1 (9). Subsequent analysis of the two chromosome 11 homologs revealed independent overlapping deletions (10). The region of homozygous deletion is presumed to include the *WT* locus, and its existence permitted the isolation of a strong candidate for the *WT* gene (11). Recently, homozygous deletions within the same small region were described in two further sporadic tumors and a second cloning of the same candidate *WT* gene was reported (23).

It is only in the light of the location of these homozygous tumor-associated deletions that we could conclude that the

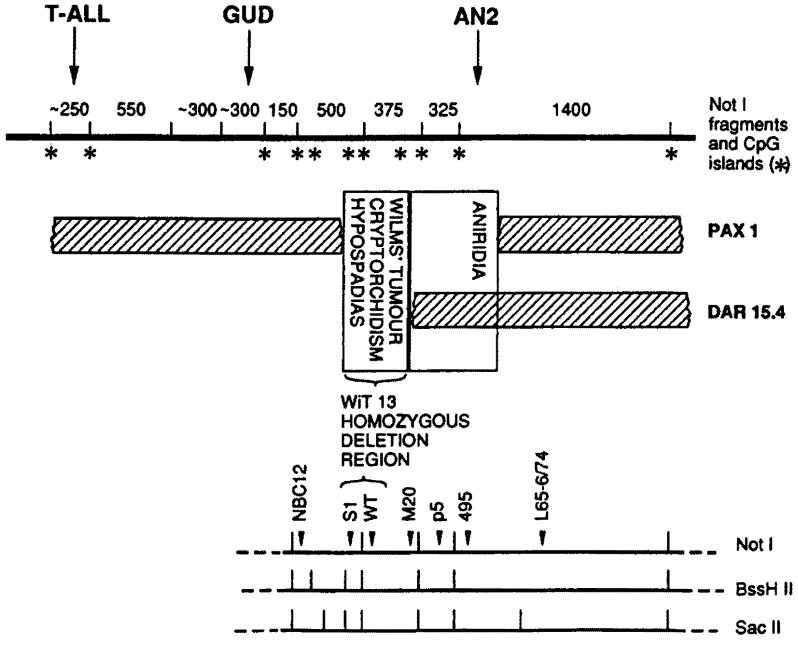

FIG. 3. The consensus long-range map of the chromosome 11p13 region. The size of the normal *Not* I fragments in kb is shown, with the positions of well-characterized translocation breakpoints marked by arrows. Asterisks denote the site of CpG islands associated with the ends of genes. The regions that must contain genes responsible for the observed phenotypes in the two deletions are shown as boxes. The coincidence between the homozygous WiT13 tumor DNA deletion and the region of deletion overlap between PAZO and DAR DNAs is marked. The expected sizes of the altered PFGE fragments in PAZO DNA can be deduced from the position of the restriction sites shown. T-ALL, T-cell acute lymphocytic leukemia; GUD, genitourinary dysplasia.

patient PAZO, although nonpenetrant for tumor expression, must lack the *WT* locus. Therefore, the smallest region of overlap between the proximal breakpoint in PAZO DNA and the distal one in DAR DNA includes the *WT* gene and covers almost exactly the same 350-kb region as the homozygous deletion in WiT13 DNA (10). It is interesting to note that patients PAZO and DAR also share the hypospadias–cryptorchidism phenotype. This implies that a gene involved in aspects of male genital development also lies within the region of deletion overlap. Could it be that the gene involved in predisposition to WT also plays a role in genital development?

The candidate *WT* gene of Call *et al.* (11) was generously made available to us. We have been studying the expression pattern of this gene in normal development of early embryos. At the level of RNA blot analysis, expression is clearly seen in fetal testis and ovary in addition to developing kidney (11, 29). mRNA *in situ* hybridization in early embryos confirms the tissue distribution, showing strong expression in both metanephric and mesonephric kidney and in the early gonad (29). These results are consistent with the hypothesis that the *WT* gene may have pleiotropic effects on both kidney and genital development. Since correct early gonadal function influences subsequent genital development, mutation in this gene could account for the high frequency of cryptorchidism and hypospadias in male WT patients with or without aniridia (1, 5).

In the case of aniridia-associated genitourinary abnormalities (four of five males among our patients with 11p13 deletion-associated aniridia, with or without WT, have cryptorchidism and/or hypospadias), the presence of a deletion (and therefore by definition a null allele) is implicated. It seems reasonable to suggest that similar genitourinary abnormalities seen in association with apparently "sporadic" WT are the result of germ-line or early zygotic mutation within the 11p13 region and perhaps within the single *WT* candidate locus. Since the candidate *WT* gene covers a genomic distance of only ≈50 kb, there is room for other genes with influence on genital development in the 350- to 400-kb overlap region between the deletions of the PAZO and DAR DNAs. However, the pleiotropy hypothesis can now be tested by looking for germ-line mutations at the *WT* locus in appropriate patients.

The possible involvement of the WT candidate gene in gonadal development should be borne in mind when considering the documented lack of affected offspring among 179 children of 99 surviving unilateral WT patients (24). Such a large number of cases should include at least some with germ-line mutations (2). Absence of WT transmission could be explained by suggesting that such a constitutional change might render the carrier infertile or the carrier gametes nonviable. Consistent with this idea, where linkage studies of familial WT have been done, no 11p13 association has been seen (25, 26). Counter to this suggestion, however, there is evidence of at least partial fertility from observations of the Dickie Smalleye (Sey^D) mouse, which carries a *WAGR*-like chromosome 2 deletion encompassing loci that include the *WT* candidate and *Sey*, which may be the mouse aniridia gene homolog (27). However, the validity of the mouse model for human disease may be questioned because the Sey^D mouse also shows no predisposition to kidney tumors (27). In addition, genetic control of fertility may differ in mouse and man: for example, X0 mice are usually fertile, while human X0 individuals are virtually always sterile (28).

To confirm involvement of the *WT* candidate gene in tumorigenesis and in genitourinary development, mutation analysis is required (*i*) at the mRNA or DNA level in tumor tissue (11) and (*ii*) at the germ-line level in candidate patients with WT accompanied by genitourinary anomalies. Only detailed functional analysis of this candidate gene—including the search for upstream control elements and downstream targets—will allow us to make the final conclusions about its biological role in both kidney and genital development. Meanwhile, the search continues for other genes of interest in this region of chromosome 11p13.

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