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Homozygous Inactivation of *WT1* in a Wilms' Tumor Associated With the WAGR Syndrome

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Wilms' tumor is a childhood nephroblastoma that is postulated to arise through the inactivation of a tumor suppressor gene by a two-hit mechanism. A candidate 11p13 Wilms' tumor gene, *WT1*, has been cloned and shown to encode a zinc finger protein. Patients with the WAGR syndrome (Wilms' tumor, aniridia, genitourinary abnormalities, and mental retardation) have a high risk of developing Wilms' tumor and they carry constitutional deletions of one chromosome 11 allele encompassing the *WT1* gene. Analysis of the remaining *WT1* allele in a Wilms' tumor from a WAGR patient revealed the deletion of a single nucleotide in exon 7. This mutation likely played a key role in tumor formation, as it prevents translation of the DNA-binding zinc finger domain that is essential for the function of the *WT1* polypeptide as a transcriptional regulator. *Genes Chrom Cancer 7:131–136 (1993).* © 1993 Wiley-Liss, Inc.

INTRODUCTION

Individuals with the WAGR syndrome (Wilms' tumor, aniridia, genitourinary abnormalities, and mental retardation) frequently carry cytogenetically visible, constitutional deletions involving chromosome band 11p13 (Francke et al., 1979). The molecular dissection of this genomic region ultimately led to the isolation of the *WT1* Wilms' tumor gene (Call et al., 1990; Gessler et al., 1990). The gene is transcribed into a 3 kb mRNA that can be differentially spliced, giving rise to four different protein isoforms (Haber et al., 1991; Gessler et al., 1992). The translation product is a zinc finger protein that binds to DNA in a sequence specific manner, and it is involved in transcriptional regulation (Rauscher et al., 1990; Madden et al., 1991).

A small fraction of sporadic Wilms' tumors exhibit homozygous deletions of this gene, some of them intragenic, supporting the hypothesis that *WT1* can function as a tumor suppressor gene (Call et al., 1990; Gessler et al., 1990; Cowell et al., 1991; Huff et al., 1991; Royer-Pokora et al., 1991; Ton et al., 1991). In most of the tumor samples tested, however, no deletions were found. RNA transcripts of this gene could still be detected in many cases at levels comparable to those in developing kidney. This apparent discrepancy may in part be explained by the presence of two other loci (*WT2* and *WT3*) postulated to play a role in an unknown fraction of Wilms' tumors (Grundy et al., 1988; Huff et al., 1988; Koufos et al., 1989; Reeve et al., 1989). Alternatively, more subtle mutations of *WT1* (Pelletier et al., 1991a) may be present in some cases.

In this report we describe the cytogenetic and molecular characterization of the *WT1* locus in normal tissue and Wilms' tumor cells from a patient with WAGR syndrome. The extent of the WAGR deletion has been determined as well as the nature of the second genetic event leading to a homozygous inactivation of *WT1* function in tumor cells.

MATERIALS AND METHODS

Patient

The patient, a boy, was born April 19, 1979, to healthy and normal parents. He had bilateral aniridia, hypospadias, and cryptorchidism, suggesting the presence of the WAGR syndrome. He was therefore kept under observation for the development of Wilms' tumor. At the age of almost 7 yrs he presented with gross hematuria, low back pain, and a palpable mass in the right upper abdomen. The tumor was removed by right radical nephrectomy and diagnosed as a Wilms' tumor. Follow-up at age 12 showed no evidence of recurrent tumor in the patient.

Cytogenetic Analysis

Lymphocytes from a peripheral blood sample were stimulated with phytohemagglutinin and arrested in early cell division with ethidium bromide followed by Colcemid treatment (Ikeuchi, 1984).

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Chromosomes were G-banded using trypsin treatment followed by Giemsa staining (GTG-banding).

Isolation of Genomic DNA and Gene Dosage Analysis

DNA from white blood cells and pulverized frozen tumor tissue was isolated according to standard procedures (Sambrook et al., 1989). Southern blot filters with exactly 4 μg of *Eco*RI-digested DNA per lane were used for hybridization in the gene dosage studies. Hybridization conditions and evaluation of gene dosage results have been described previously (Gessler et al., 1989). All probes used in this study have been characterized by Gessler et al. (1989, 1990). For studies on loss of heterozygosity at the *HRAS1* and *TH* loci, primers and amplification conditions were as described (Tanci et al., 1992; Polymeropoulos et al., 1991).

Preparation of RNA and RT-PCR Analysis

RNA was isolated from frozen tumor tissue by the guanidinium-isothiocyanate/cesium chloride method (MacDonald et al., 1987). Single-stranded cDNA was prepared using the first strand kit (Stratagene, Heidelberg), followed by PCR amplification of the *WT1* fragment (nucleotides 779–1605) as described by Gessler et al. (1992). Subfragments suitable for sequencing were cloned into pBluescript and sequenced using vector or internal cDNA primers. Sequence comparison was done using the HUSAR/UWGCG sequence analysis package (DKFZ, Heidelberg).

SSCP Analysis and Direct Sequencing of PCR Products

Exon 7 DNA was amplified using primers corresponding to nucleotides 156–175 and 389–408 of the genomic sequence at an annealing temperature of 60°C (X61636, Gessler et al., 1992). For SSCP analysis the products were labelled by ^{32}P-dCTP incorporation and separated on non-denaturing polyacrylamide gels at 4°C and room temperature (Orita et al., 1989).

For direct sequencing, amplification products were chloroform-extracted and purified by glass powder adsorption (Gene-clean, Bio101). DNA from one-tenth of a standard amplification reaction was used for sequence determination using the fmol kit (Promega, Heidelberg) and one of the end-labelled PCR primers.

RESULTS

Clinical Findings

Based on histology and electron microscopy, the Wilms' tumor (S86-1334) was classified as a blastemal tumor with focal tubular differentiation. No nephroblastomatosis was identified. Extensive changes were seen in the renal parenchyma, with interstitial fibrosis and chronic inflammation. Segmental to global thickening of the mesangium and capillary loops was seen in a fairly diffuse fashion, together with deposits of IgM via immunofluorescence studies in the mesangium.

There appears to be no impairment of intellectual functioning of the patient, as is the case for a fraction of WAGR patients. Although no formal IQ testing was done, the patient was rated as having average or above average intelligence by neurologic examiners. He does quite well at school and at 13 yr of age is in the seventh grade, an age-appropriate level.

Cytogenetic Findings

The cytogenetic analysis from white blood cells revealed a male karyotype with a deletion on the short arm of one chromosome 11. The deletion was originally identified as del (11)(p13p14.2). Reexamination using high resolution banding (Fig. 1) suggested a slightly smaller deletion, interpreted as del (11)(p13p14.1). This is consistent with the presence of the WAGR syndrome in this patient.

Molecular Characterization of the WAGR Deletion

The chromosome 11 deletion was detected at the molecular level during the screening of Wilms' tumor DNAs for submicroscopic deletions of the *WT1* locus without prior knowledge of the patient's phenotype. Using DNA probes covering most of chromosome region 11p13-p14, a hemizygous deletion was identified in tumor DNA with the *FSHB* gene, the *AN2* locus, and the *WT1* Wilms' tumor gene showing reduced gene dosage (Fig. 2). The flanking markers M16 and catalase (*CAT*) on the centromeric side and 74 on the telomeric side are present in two copies per cell. By comparison with the well established physical map of this region, a deletion size of approximately four million basepairs could be deduced.

Examination of constitutional DNA from white blood cells of the patient showed the same hemizygous deletion. In DNA from the patient's father, however, no reduction of gene dosage for any of the markers tested could be found. There was no DNA available from the mother. Given the ab-

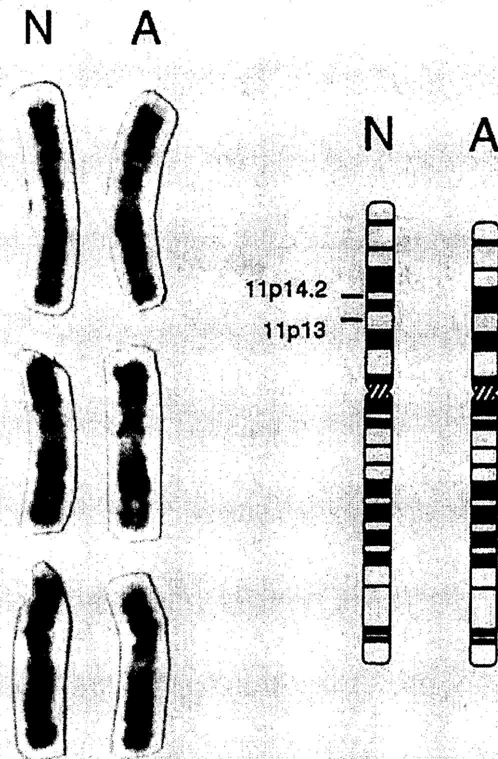

Figure 1. Partial karyotypes and idiogram of chromosome 11, illustrating the normal (N) and the deletion of material from bands 11p13 and 11p14.1 (A).

sence of clinical signs like aniridia in both parents, it is likely that the deletion detected in the patient represents a new mutation.

Analysis of the Remaining *WT1* Allele in Tumor DNA

The presence of a constitutional deletion of one *WT1* allele suggested that inactivation of the second *WT1* allele may have caused tumor formation in this case. By Southern blot analysis, no evidence for alteration in any of the genomic DNA fragments of the remaining *WT1* allele could be found. Northern blot analysis with RNA from tumor tissue revealed the presence of a normal size transcript for *WT1* (data not shown), leaving the possibility that a more subtle mutation might interfere with the expression of a functional protein product.

We therefore examined the coding region of the *WT1* mRNA by sequence analysis. A fragment corresponding to nucleotides 779–1605 of the *WT1* sequence was amplified from reverse transcribed tumor RNA and subcloned. Sequence analysis of the entire fragment revealed the presence of a single mutation in exon 7 of the *WT1* gene (Fig. 3). The deletion of nucleotide 1284 leads to a translational frameshift resulting in a premature termination of the reading frame. In this case, the protein product would likely be nonfunctional as it is devoid of the essential zinc finger domain which mediates DNA binding. The mutation has been confirmed on several independent cDNA clones as well as by direct sequencing of exon 7 amplified from tumor DNA (not shown).

The mutant genomic exon, amplified from tumor DNA, produces a variant banding pattern upon SSCP analysis. In constitutional DNA from this patient, however, only the normal DNA fragments are seen. Thus, the deletion of one nucleotide from the remaining allele of the *WT1* gene is a tumor-specific event.

To assess a possible loss of heterozygosity at chromosome band 11p15 (i.e., the *WT2* locus), we analyzed polymorphic repeat sequences at the *HRAS1* and *TH* loci (Tanci et al., 1992; Polymeropoulos et al., 1991). For both loci, tumor DNA was heterozygous, thus ruling out loss of a larger genomic segment during tumor formation.

DISCUSSION

The patient described in this study developed Wilms' tumor rather late, at the age of 83 months. The median age at presentation for the subgroup of cases that are associated with hypospadia and cryptorchidism is 21.5 months (Breslow et al., 1988). These genitourinary malformations are likely caused by the absence of one *WT1* allele in constitutional DNA of our patient, since mutations at the *WT1* gene can exert a dominant effect on the development of the urogenital system (Pelletier et al., 1991a).

Similarly, bilateral aniridia is presumably due to the reduction in gene dosage for the entire AN2 locus as tested by probe 495. Interestingly, the patient shows apparently normal mental development. Approximately two-thirds of WAGR patients with cytogenetic deletions are mentally retarded (Russell and Weisskopf, 1986), but a putative locus for this component of the WAGR syndrome has not yet been mapped. The large size of the deletion in our patient implies that such a gene may be located at a significant distance from the other two WAGR genes, *WT1* and *AN2*, which are found within less than one million basepairs (Compton et al., 1988; Gessler and Bruns, 1989).

The WAGR-associated deletion spans a considerable genomic distance and must include a large number of other genes. This would likely prevent inactivation of the second *WT1* copy by chromo-

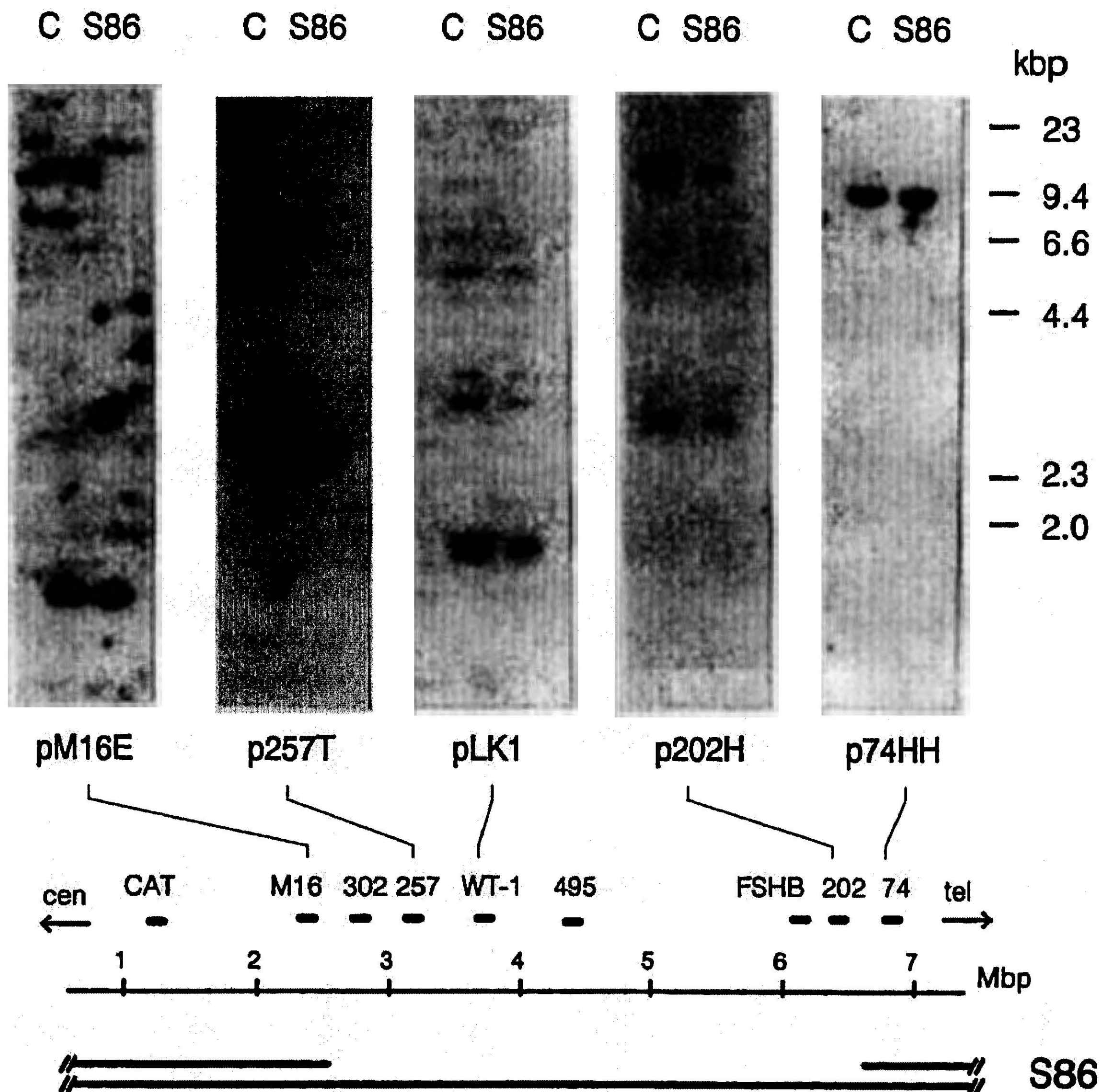

Figure 2. Gene dosage analysis of DNA from Wilms' tumor S86-1334. Southern blot filters prepared with exactly 4 μg of EcoRI digested normal human control DNA (lanes C, 6697 cells, Gessler et al., 1989) and S86-1334 tumor DNA (S86) were hybridized sequentially with the probes listed. For probes p257T, pLK1 (*WT1*), and p202, an approximately 50% reduction of the hybridization signal compared to control DNA was seen. Probe 495 marks the *AN2* aniridia locus, which is included in the deletion. The schematic diagram depicts the location of all probes used within the consensus physical map that has been established by pulsed field gel analysis (Compton et al., 1988; Gessler and Bruns, 1989).

some loss or mitotic recombination which is frequently detected as loss of heterozygosity (LOH) in other tumors. There are several reported cases of Wilms' tumors with two independent, large deletions on both chromosome 11 alleles (Call et al., 1990; Gessler et al., 1990; Royer-Pokora et al., 1991; Ton et al., 1991). The resulting region of homozygous deletion, however, was always limited to a few hundred kilobase pairs around the *WT1* locus.

The second event in our case was the deletion in the *WT1* coding region of a single nucleotide, detected in DNA and RNA from the patient's tumor. The mutation changes the translational reading frame. The altered transcript encodes a polypep-

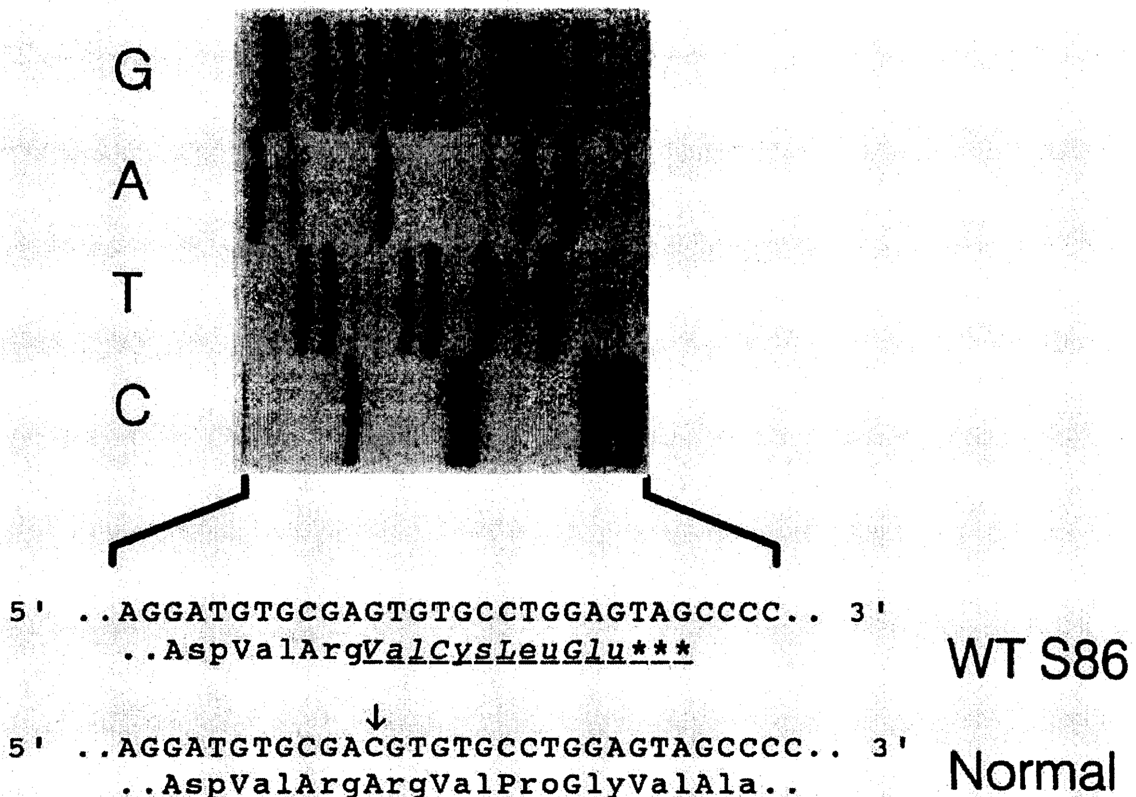

Figure 3. Sequence analysis of the *WT1* transcript from Wilms' tumor S86-1334. Deletion of a single nucleotide (C) is seen. The shift in reading frame leads to four altered amino acid residues followed by a premature stop codon. The truncated protein is missing the zinc finger domain encoded in exons 7 to 10.

tide lacking the essential DNA-binding domain of four consecutive zinc fingers. This would likely prevent its normal functioning and lead to a complete loss of *WT1* activity, consistent with the two hit model for Wilms' tumor development (Knudson and Strong, 1973). Similar small deletions or point mutations of *WT1* in Wilms' tumors have been described recently in non-WAGR patients (Pelletier et al., 1991a; Little et al., 1992). In all cases, the region encoding the zinc fingers is altered or preceded by stop codons.

Henry et al. (1989) could demonstrate loss of heterozygosity limited to the 11p15 region—the location of the postulated *WT2* gene—in Wilms' tumor DNA from a WAGR deletion patient, suggesting that both loci, *WT1* and *WT2*, may be affected in the same tumor. It is interesting to note that, in our case loci at chromosome band 11p15 remained heterozygous in tumor DNA. This does not rule out, however, that other alterations at 11p15 have occurred during tumor formation.

Microscopical examination of the kidney adjacent to the tumor showed features that are compatible with a broader definition of Denys-Drash syndrome (Eddy and Mauer, 1985). Recently, Pelletier et al. (1991b) have reported a striking correlation between this syndrome and highly specific point mutations altering a single amino acid of the *WT1* zinc finger domain, which exert a strong dominant effect on kidney development. These data suggest that the kidney changes seen in our case may be based on mechanisms different from those acting in Denys-Drash syndrome.

The homozygous inactivation of the *WT1* gene in this tumor further supports the postulated role of this gene as a tumor suppressor gene and provides a direct explanation for the high frequency of Wilms' tumor formation in individuals with WAGR-associated deletions.

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