Constitutional mutations in the WT1 gene in patients with Denys–Drash syndrome

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ABSTRACT

The Denys–Drash syndrome is characterised by a typical nephropathy, genital abnormalities and also predisposes to the development of Wilms' tumor. These patients eventually go into end stage renal failure. A candidate Wilms' tumor gene, WT1, from the 11p13 chromosome region has recently been cloned. We have analysed the DNA sequence in constitutional cells from eight patients and have shown heterozygous mutations in six of them. Four of the mutations were in exon 9, all resulting in missense mutations. Three were at nucleotide position 1180 resulting in an arg>trp amino acid change. The other was at position 1186 converting an asp>asn in the predicted resultant protein. One patient had a missense mutation in exon 8, converting an arg>his. A single base pair insertion at nucleotide position 821 in exon 6 resulted in the generation of a premature stop codon in the last patient. We were unable to find a mutation in one patient despite complete sequencing of the genomic sequence of the gene. The last patient carried a constitutional deletion of the 11p13 region and no additional mutation was found. There was no obvious correlation between the type of mutation and phenotypic expression. These results further demonstrate that the WT1 gene is important in both the development of the kidney and the genito-urinary system.

INTRODUCTION

Individuals with deletions on the short arm of chromosome 11 (1) are predisposed to Wilms' tumor (WT). Detailed analysis of these deletions showed that the distal part of chromosome band 11p13 was the only consistently deleted region (2,3). These patients usually also present with the complex phenotype of aniridia, abnormal genitalia and sometimes mental retardation—the WAGR syndrome (4). Approximately 50% of WAGR patients develop WT (5) and it was assumed that genes responsible for these phenotypes were located in region 11p13. Although the region containing the aniridia gene was distinct (6), the WT and GU phenotypes were possibly caused by the same gene (7). Since the kidneys and gonads are derived from embryologically adjacent tissues it was possible that a single gene could be responsible for the abnormal development of both organs.

A candidate WT gene, from the 11p13 region, was cloned recently (8, 9) and encodes a 3.2 Kb mRNA with ten exons and was called WT1 (10). The last four exons encode, individually, for each of four zinc finger regions which are characteristic motifs for DNA binding transcription factors (8). The WT1 gene showed a very restricted pattern of tissue expression occurring predominantly in the developing gonads and kidneys (8, 11, 12). The highest level of expression was seen in mesenchymal cells making the transition to epithelial cells (12) and supports the notion that this gene is part of the regulatory pathway leading to terminal differentiation in the kidneys and gonads. Loss of function of this gene was presumed to be a critical event in deregulating this pathway (13) which was supported by observations that Wilms' tumours frequently showed loss of constitutional heterozygosity (LOH) for the 11p13 region (14, 15).

Another multiple malformation condition (16, 17), which is also associated with a high frequency of WT and genital abnormalities, is the Denys–Drash syndrome (DDS). The invariable feature of this syndrome is the complex nephropathy which results in proteinuria which is usually sufficient to cause nephrotic syndrome (18, 19). The genital abnormality most often described is male pseudohermaphroditism with children having either ambiguous external genitalia or a normal female phenotype with an XY karyotype. Other types of genital abnormalities have been described including true hermaphroditism (20) and hypoplastic (21) or streak gonads. In some cases, children have developed gonadoblastoma at a young age (21, 22), an observation also made in children with chromosome deletions known to predispose to WT (23). In one DDS patient a constitutional 11p13 deletion has been reported (24) which prompted us to look for structural rearrangements of the WT1 gene in tumours from DDS patients. Although no gene rearrangements were noted following Southern blotting (25) or gene dosage studies (24), one of the tumours showed LOH for loci on 11p13 (26). Recently, subtle abnormalities in the WT1 gene in DDS patients have been described (27) which were restricted to exons 8 and 9, coding for the second and third zinc fingers respectively. To determine whether these regions represent hot-spots for mutations resulting in DDS we performed an exon-by-exon analysis of WT1 in tumour and constitutional cells from DDS patients in our series. In this report we describe a variety of mutations in the WT1 gene which supports its role in the normal development of the uro-genital system.

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RESULTS

A total of eight DDS patients were included in our series. The clinico-pathological details of six of these patients have been described previously (19). The clinical details and blood sample from patient LVH were kindly provided by Dr. Proesmans and those from patient LB were provided by Dr. D. Walker. The clinical characteristics of the eight patients are summarised in Table 1. Apart from the nephropathy, WT developed in 6/8 patients. Seven had XY gonadal dysgenesis with an external phenotype which ranged from ambiguous genitalia to normal female.

SSCP analysis of WT1

The individual exons of WT1 from all patients were analysed using the single strand conformation polymorphism (SSCP) technique (31). Oligonucleotide primers were as designed (29) to amplify each exon and limited parts of flanking intron sequences using the polymerase chain reaction (PCR). The SSCP technique relies on the fact that DNA sequence changes are sufficient to alter the conformation of single stranded DNA which can be identified as a band shift on a polyacrylamide gel. An example of a gel showing abnormal SSCP banding patterns for exon 9 is shown in figure 1. Three patients, MA (GOS 372), HD (GOS 368) (figure 1) and LB (data not shown) showed the same abnormal banding pattern. A fourth patient, SS (GOS 378), had a different banding profile for the same exon (figure 1). The only other abnormal SSCP banding profiles were seen in exons 6, patient PM, and 8, in patient LVH.

Sequence analysis of exons showing abnormal SSCP profiles

The PCR products from those exons showing abnormal patterns were sequenced and in all cases mutations were identified. The three patients with the same abnormal SSCP banding pattern in exon 9 carried an identical mutation, a single C → T base pair change (figure 2), at position 1180 from the TAG start site (10), resulting in an amino acid change of an arginine → tryptophan residue. This C → T transition destroys a Hpa II restriction site which was then used to confirm the presence of this mutation (data not shown). Those patients with a T in position 1180 lose the restriction enzyme site and show the full length 203 bp fragment whereas those with the normal sequence produce two bands 125 and 78 bp long. The fourth patient carried a G → A transition (figure 2) at position 1186 in exon 9, resulting in an aspartic acid → asparagine change and did not affect a restriction enzyme site. Exon 8 (zinc finger 2) in patient LVH (GOS 456) showed a G → A transition (figure 3) resulting in an arginine → histidine change. The only non-zinc finger mutation identified was in exon 6 in patient PM (GOS 389). This was an insertion of a G at position 821 (figure 4) resulting in the immediate generation of a stop codon (TAA). All of these mutations were initially detected in constitutional cells and, as such were heterozygous on sequencing gels. However, in the case of patient PM, the mutation shown in Figure 4 is from the tumor and also shows two bands at each position on the sequencing ladder from the site of the insertion at position 821. The dominant mutant bands are from the tumor DNA and lighter banding is due to residual contaminating normal DNA.

Of the two remaining cases, one, which was reported previously (24), carries a constitutional deletion of the 11p12–13 region. We presume that this deletion constitutes the first mutation in this case and it is perhaps not surprising that we were unable to identify any mutation in the remaining allele in constitutional cells. The second case, DH, in addition to the nephropathy and ambiguous genitalia, had cleft palate and mental retardation. Despite complete sequencing of the gene we were unable to find a mutation although we did not sequence the non-coding promoter region.

Five of the eight patients developed WT although only three (from SS, PM and LB) were available for analysis (table 1). In all cases the same mutation, which was identified in constitutional cells, was also present in tumor cells and was also heterozygous on sequencing gels presumably due to the presence of contaminating normal cells (an example is shown from patient PM in Figure 4).

To determine whether these mutations were sporadic or inherited we analysed DNA from those parents available to us (see table 1). In no case was the mutation detected in any of the parents examined.

Comparison of phenotype and genotype

Two of the patients with the 1180 mutation in exon 9 had Wilms' tumor and genito-urinary abnormalities in addition to the nephropathy. The third patient, HD, (GOS 368) did not have WT. However, she underwent prophylactic bilateral nephrectomies at 2 years of age, once she reached end-stage renal failure (ESRF). The 4th patient (SS) with a mutation in exon 9, at position 1186, had WT and his nephropathy followed the same rapid progress to ESRF as seen in the other children with the 1180 mutation of exon 9. Patient LVH (GOS 456), with the exon 8 mutation, had a typical nephropathy, ambiguous genitalia and received a nephrectomy for WT at 9 months.

Patient PM (GOS 372) had an insertional mutation in position 826 in exon 6, generating a stop codon. He had nephropathy, bilateral WT and ambiguous genitalia. He had presented at the age of 0.5 years with a right WT for which he underwent a right nephrectomy and received vincristine. At 1.5 years he presented with a left side WT which failed to respond to cytotoxics and...
Table 1. Summary of the phenotypes and constitutional mutations in WT1 from DDS patients

<table>
<thead>
<tr>
<th>Initials</th>
<th>Patient No.*</th>
<th>GOS No.</th>
<th>Karyotype</th>
<th>Parents/</th>
<th>Wilms' tumor†</th>
<th>Affected exon</th>
<th>Mutation type</th>
</tr>
</thead>
<tbody>
<tr>
<td>MA</td>
<td>1</td>
<td>372</td>
<td>46XY</td>
<td>M</td>
<td>Yes (U)</td>
<td>9</td>
<td>1180 C-T</td>
</tr>
<tr>
<td>PD</td>
<td>11</td>
<td>400</td>
<td>46XX</td>
<td>M/F</td>
<td>Yes (B)</td>
<td>—</td>
<td>11p12—p13 deletion</td>
</tr>
<tr>
<td>DH</td>
<td>8</td>
<td>374</td>
<td>46XY</td>
<td>M</td>
<td>No</td>
<td>none detected</td>
<td>1186 G—A</td>
</tr>
<tr>
<td>SS</td>
<td>12</td>
<td>378</td>
<td>46XY</td>
<td>M/F</td>
<td>Yes (U)</td>
<td>9</td>
<td>821 Ins of G STOP</td>
</tr>
<tr>
<td>PM</td>
<td>3</td>
<td>389</td>
<td>46XY</td>
<td>M/F</td>
<td>Yes (B)</td>
<td>6</td>
<td>1180 C—T</td>
</tr>
<tr>
<td>HD</td>
<td>7</td>
<td>368</td>
<td>46XY</td>
<td>M</td>
<td>No</td>
<td>9</td>
<td>1097 G—A</td>
</tr>
<tr>
<td>LVH</td>
<td>—</td>
<td>456</td>
<td>46XY</td>
<td>M/F</td>
<td>Yes (U)</td>
<td>8</td>
<td>1180 C—T</td>
</tr>
<tr>
<td>LB</td>
<td>—</td>
<td>606</td>
<td>46XY</td>
<td>M/F</td>
<td>Yes (U)</td>
<td>9</td>
<td>1180 C—T</td>
</tr>
</tbody>
</table>

* These numbers refer to the patients as reported by Jadresic et al (19).
† M = mother, F = father—indicating in which parents it was possible to analyse the WT1 gene.
Ins Indicates insertion.

Figure 2. DNA sequencing ladders from constitutional DNA showing the two mutations identified in exon 9. In (1) a G and A residue occupy the same position on the ladder at residue 1186 in patient GOS 378. In (2) a C and T occupy the same position at residue 1180 in patient GOS 372.

radiotherapy. Moderate proteinuria, hypertension and at the time only moderately reduced renal function were detected at 2 years, shortly before being rendered anephric with a left nephrectomy for his resistant WT, at 2.1 years. The non-tumor involved areas of this kidney showed histological changes of mesangial sclerosis. However, because of the need for nephrectomy it was not possible to chart clinically the progression of his nephropathy.

Patient PP (GOS 400) with the 11p deletion, had bilateral WT, aniridia and mental retardation. Her nephropathy, which became evident by the presence of proteinuria at 2 years of age, did not progress to ESRF, until 6.5 years of age. Her sex chromosomes are XX but there is no histological information on internal genitalia. However, she has had a normal onset of menarche.

One patient, DH (GOS 374), in whom we could not find a mutation, had a typical nephropathy, preceded by proteinuria and progression to ESRF by 0.8 yrs of age. He had ambiguous genitalia but did not have WT. In addition, he had cleft palate, nystagmus and mental retardation.

DISCUSSION

We have analysed constitutional DNA from eight patients with the Denys—Drash syndrome and identified mutations in the WT1 gene in six of them. In three cases the mutation involved a single base pair change at position 1180 in exon 9 resulting in a missense mutation. A different missense mutation in exon 9 at position 1186, was also identified in another patient. The other missense mutation was in exon 8 at position 1097. All of these mutations occur within the region of the gene coding for the zinc finger motif (ZF) and are the same as those reported in a different series of patients with DDS (27). The only non-zinc finger mutation was in exon 6 at position 821 which resulted in the generation of a stop codon and has not previously been reported.

The missense mutations in exon 9 result in a change of either acidic or basic amino acid residues to neutral ones. In the case of the exon 8 mutation the charge remains basic despite the amino acid change. The WT1 gene binds to DNA, in a sequence specific
a result of heterozygous insufficiency or requires another mutation at the homologous normal locus for pathogenicity is not clear. Although we were able to analyse tumor tissue from three DDS patients, we have previously shown that the presence of infiltrating normal cells in our PCR-sequencing assays, masks the detection of any homozygous mutation (29). Thus, tumor GOS 217, which we have previously shown to have undergone loss of heterozygosity for the 11p13 region (26), was still heterozygous on the sequencing gels (Figure 4).

There is still a question about the role of WT1 in Wilms' tumorigenesis. Only 30% of tumours show allele loss for the 11p13 region (26) and only half of AGR patients carrying a chromosome deletion will actually develop the tumor (5). A small percentage of tumours show structural rearrangements of WT1 (25) and not all tumours from WAGR patients show mutations in the remaining alleles in their tumours (29). Evidence from LOH studies (26, 34) and family linkage analysis (35, 36) suggest at least two other genes involved in predisposition to WT. Not all DDS patients develop WT either. The feature common to them all is the nephropathy which may co-exist with WT or genital abnormalities, or both. XY gonadal dysgenesis is the most common genital abnormality. However, on autopsy females with 46 XX karyotypes have been shown to have abnormal development of the gonads (19). It has been suggested that the WT1 gene may be more important for the development of the gonads than the kidney (37) although the nephropathy is a characteristic feature of DDS patients. In both our study and that of others (27) the same mutation resulted in the development of WT in some patients but not in others. It may be that this gene is responsible for the abnormal development of the genito-urinary system (GU) but that another gene must cooperate with WT1 to result in tumor formation.

All previously reported cases (27) resulted in a missense mutation. By analogy with the retinoblastoma susceptibility gene (RB1) these may result in only a mild phenotype (38), whereas the generation of premature stop codons result in absence of expression of the gene (39). We have extended the observations in DDS and now report a mutation producing a premature stop codon in a patient with DDS. In this patient the natural evolution of his nephropathy could not be witnessed as he was rendered anephric very soon after the diagnosis of his nephropathy and before the onset of ESRF. It is not possible, therefore, to ascertain whether his nephropathy would have progressed differently to that classically seen in children with DDS with mutations in exons 8 or 9. He had bilateral WT, often thought to reflect a genetic susceptibility to WT. Whether this phenotype can be considered more extreme than those seen in patients with missense mutations is not clear. It was likewise not possible to correlate the development of tumours with the location or type of the mutation. In one patient, GOS 374, we were unable to demonstrate any mutation even after sequencing the whole gene with the exception of the most 5' end of the promotor region. In addition he had ambiguous genitalia and a fulminant nephropathy which culminated in ESRF by 0.8 years as well as the histological changes of diffuse mesangial sclerosis as seen in the other children with DDS. He also had a cleft palate, nystagmus and mental retardation which is not typical of DDS. This observation raises the possibility, however, of the existence of other equally important genes which, if abnormal, may have a serious influence on renal and GU development. On this point we also originally included another patient, GOS 276, in the series who was an atypical XY DDS female with ambiguous genitalia and Wilms'
tumor but no nephropathy. Using a combination of SSCP and PCR-sequencing analyses we were also unable to find a mutation in the WT1 gene in this patient. It is possible, therefore, that true DDS patients should be reclassified to include the presence of a WT1 mutation in addition to (or possibly the absence of) other typical phenotypes. Alternatively, there may be other genes which also predispose to this clinical condition.

MATERIALS AND METHODS

Tissue Samples

Tumor samples obtained from the Histopathology Department at the Great Ormond Street Hospital for Sick Children were snap frozen in liquid nitrogen soon after the time of surgical resection. All patients had features consistent with Denys–Drash syndrome. Freshly isolated lymphocytes or lymphoblastoid cell lines prepared from them were used as representitives of constitutional cells from all patients.

Genomic DNA isolation

High molecular weight DNA from tumor samples was prepared by grinding tissue to a fine powder in liquid nitrogen and resuspending in approximately 20 ml lysis buffer (50 mM NaCl, 10 mM EDTA, 150 mM Tris, 0.5% SDS). Proteinase was achieved using 50 μg/ml proteinase K for 16–24 hours at 37°C. DNA was then extracted using standard phenol/chloroform procedures (28). Peripheral blood lymphocytes or EBV transformed lymphoblastoid cell lines were resuspended in lysis buffer and the DNA extracted as described above.

PCR Amplification

The series of oligonucleotide primers which amplified the coding region of the WT1 gene, exon by exon, were as described previously (29). 5' and 3' primers for each exon were located within the adjacent intron sequence to allow amplification across splice junction sites.

PCR was carried out as described previously (29) using 40 cycles of denaturation at 96°C for 30 seconds, annealing between 57°C to 71°C for 30 seconds, followed by an extension step at 72°C for 30 seconds.

SSCP and Sequencing

Detailed methods for SSCP and direct sequencing from PCR products have been described elsewhere (29, 30). Essentially, for SSCP a normal PCR reaction was performed except that 1 /μCi (3,000 CL/mmol) of 32P-dCTP was added. DNA on streptavidin-coated magnetic 'Dynabeads' (Dynal, UK), which were then washed and eluted in water, was resuspended in 1× sequencing buffer and 15% glycerol at 94°C and incubated for 1 min. The DNA was then electrophoresed on 6% polyacrylamide, 10% glycerol, non-denaturing gels. For描述了其他方法(29, 30)。本质上，对于SSCP，正常PCR反应被进行，除了添加1 μCi（3,000 CL/mmol）的32P-dCTP。DNA在streptavidin-coated磁性‘Dynabeads’（Dynal, UK），然后被清洗并以水复溶，被悬在1×sequencing buffer和15%glycerol中，在94°C进行1 min的热变性。DNA然后电泳在6%聚丙烯酰胺，10%甘油的非变性凝胶上。