A Novel WT1 Gene Mutation in a Three-Generation Family with Progressive Isolated Focal Segmental Glomerulosclerosis

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Background and objectives: Wilms tumor-suppressor gene-1 (WT1) plays a key role in kidney development and function. WT1 mutations usually occur in exons 8 and 9 and are associated with Denys-Drash, or in intron 9 and are associated with Frasier syndrome. However, overlapping clinical and molecular features have been reported. Few familial cases have been described, with intrafamilial variability. Sporadic cases of WT1 mutations in isolated diffuse mesangial sclerosis or focal segmental glomerulosclerosis have also been reported.

Design, setting, participants, & measurements: Molecular analysis of WT1 exons 8 and 9 was carried out in five members on three generations of a family with late-onset isolated proteinuria. The effect of the detected amino acid substitution on WT1 protein’s structure was studied by bioinformatics tools.

Results: Three family members reached end-stage renal disease in full adulthood. None had genital abnormalities or Wilms tumor. Histologic analysis in two subjects revealed focal segmental glomerulosclerosis. The novel sequence variant c.1208G>A in WT1 exon 9 was identified in all of the affected members of the family.

Conclusions: The lack of Wilms tumor or other related phenotypes suggests the expansion of WT1 gene analysis in patients with focal segmental glomerulosclerosis, regardless of age or presence of typical Denys-Drash or Frasier syndrome clinical features. Structural analysis of the mutated protein revealed that the mutation hampers zinc finger-DNA interactions, impairing target gene transcription. This finding opens up new issues about WT1 function in the maintenance of the complex gene network that regulates normal podocyte function.


Wilms tumor-suppressor gene-1 (WT1) encodes a transcription factor that plays a crucial role in kidney and genital tract development. In the developing kidney, WT1 is predominantly expressed in maturing podocytes, but its expression persists after birth in glomerular visceral epithelial cells, suggesting a role for WT1 in the function of the differentiated podocyte (1).

WT1 gene maps on chromosome 11p13, is composed of 10 exons, and encodes a 449-amino acid zinc finger protein. Each zinc finger (Zf) consists of cysteine and histidine residues linked to a zinc atom. A basic amino acid, often an arginine, is located at the top of the finger. Alternative splicing occurs at exon 5 (±17 amino acids) and exon 9 (+3 amino acids; KTS, i.e., Lys-Thr-Ser). The correct ratio of the resulting four isoforms is required for normal gene function during both nephrogenesis and adult life. Depending on splice isoform and the cellular context, WT1 may indeed act as a transcriptional factor, transcriptional cofactor, or posttranscriptional regulator (2).

Constitutional missense and splice-site mutations of WT1 gene are the cause of Denys-Drash syndrome (DDS) and Frasier syndrome (FS).

DDS (MIM 194080) is characterized by diffuse mesangial sclerosis (DMS) and renal failure with early onset, XY pseudohermaphroditism, and a high risk of developing Wilms tumor (3). DDS is caused by heterozygous missense mutations in the Zf-encoding exons of the WT1 gene. These mutations seem to act in a dominant-negative manner, hampering WT1 activity in cells (4).

FS (MIM 136680) is characterized by focal segmental glomerulosclerosis (FSGS), XY pseudohermaphroditism, and gonadoblastoma. Donor splice site mutations in WT1 intron 9 have been described as the molecular defect of FS. These mutations
result in a deficiency of the usually more abundant KTS-positive isoforms and a reversal of the normal KTS positive-to-negative ratio (5).

Nevertheless, increasing evidence seems to suggest that DDS and FS may represent two facets of the same disease, with overlapping clinical and molecular features (6–10). In the literature, sporadic cases of WT1 mutations in isolated DMS or FSGS have also been reported (6,11).

We report a novel sequence variant of WT1 gene, identified in five members on three generations of an Italian family with isolated non-nephrotic proteinuria. The reported clinical and molecular picture raises the hypothesis that WT1 is associated with a wider spectrum of phenotypes, and WT1 gene may play a more complex role in podocyte function than previously reported.

**Materials and Methods**

**Patients**

Five members of three generations of an Italian family were ascertained. The proband was a 16-year-old boy who underwent clinical assessment and renal biopsy for persistent, isolated non-nephrotic proteinuria, occasionally discovered at the age of 15 years. The other four investigated family members had non-nephrotic proteinuria, with progression to chronic kidney disease in three. None had genital abnormalities or Wilms tumor. All participants provided informed consent to molecular analysis. The study was also approved by our Institutional Review Board.

**Molecular Analysis of WT1 Gene**

Blood samples from the proband and his relatives were collected. Genomic DNA from fresh whole blood was extracted, and PCR amplification and direct sequencing reaction of coding exons 8 and 9 of the WT1 gene and their intron-exon junctions was carried out. Sequencing data were analyzed using the Sequencher software v.4.9 (Genecodes Corp., Ann Arbor, MI).

**Structural Analysis for R403K Mutation**

The crystal structure of WT1 was downloaded from the Protein Data Bank with code 2PRT and was visualized with PyMol.

**Results**

**Clinical Data**

The proband is an Italian 16-year-old boy (III.2 in Figure 1) who was referred to our unit for persistent non-nephrotic proteinuria. His personal and past medical histories were negative: he was born at term after an uncomplicated pregnancy to unrelated parents and had always been healthy. At the age of 15 years, the boy was discovered with isolated proteinuria (75 mg/dl) during regular annual physical examination. Further standard urinalysis confirmed a proteinuria of approximately 50 mg/dl. He was thus referred to our unit for a full nephrologic evaluation. On admission, physical examination was completely normal: weight and stature were at the 50th and 90th percentiles for age, respectively, BP was in the normal range for sex and age (126/69 mmHg), the cardiothoracic and abdominal examination was normal, and there were no abnormalities of genital apparatus. Blood laboratory investigations showed normal hemoglobin (14.9 g/dl), blood urea nitrogen of 45.6 mg/dl, and serum creatinine level of 1.1 mg/dl (clearance according to Schwartz formula 85 ml/min per 1.73 m²). Serum electrolytes were within the normal range, serum albumin was normal (44 g/L), and there were no abnormalities in cholesterol and triglyceride levels (184 mg/dl and 94 mg/dl, respectively). Immunoglobulins and the complement components were normal, and autoantibodies (anti-neutrophil cytoplasmic antibody, antinuclear antibody, anti-dsDNA antibody, anti-myeloperoxidase antibody) were negative. Diuresis was 1800 ml/24 h, with urinary-specific gravity of 1017, urinary pH of 7, and proteinuria of 1.46 g/24 h (corresponding to 33 mg/kg per day). Proteinuria was present and approximately the same in both orthostatic and clinostatic urinary collection, excluding orthostatic proteinuria. Renal ultrasound showed normal-sized kidneys (11.3 cm left and 10.4 cm right), with normal corticomedullary differentiation and no anomalies of the urinary tract.

A renal biopsy was performed (Figure 2). On light microscopy, 30% of sampled glomeruli showed adhesion of glomerular tuft to Bowman’s capsule and 10% presented sclerotic lesions, whereas tubuli and interstitium were normal. Immunofluorescence stain testing for IgG, IgA, C3, C4, C1q, fibrinogen, and HBsAg was negative. Electron microscopy showed extensive foot process effacement and mesangial matrix expansion in the involved glomeruli, consistent with the diagnosis of FSGS. Considering that the markers of autoimmunity were

![Figure 1. Pedigree of the family.](image1)

![Figure 2. Light microscopy of the proband, showing focal segmental glomerulosclerosis (periodic acid-Schiff; magnification, ×40).](image2)
negative, that renal biopsy showed an FSGS with negative immunostaining, and the boy’s family history was positive for a still undefined progressive renal disease in several members (see below), we accounted this pattern as more compatible with a genetic form of proteinuria than with an immune-mediated one. Therefore, we found no indications for immunosuppressive therapy in this patient, and angiotensin-converting enzyme inhibitor therapy (Ramipril, 5 mg/d) was undertaken to reduce proteinuria and preserve renal function. At last follow-up, conducted at the age of 17 years, proteinuria was approximately 1 g/24 h, and renal function was still preserved.

The boy’s family history was indeed very considerable because his father (II.2), born in 1961, was diagnosed with proteinuria, hypertension, and chronic renal failure at the age of 43 years. Renal ultrasound showed small hyperechoic kidneys, with loss of corticomedullary differentiation compatible with chronic kidney disease stage, but no other peculiar anomalies. Renal biopsy was not performed. After angiotensin-converting enzyme inhibitor therapy, he reached ESRD and underwent hemodialysis at the age of 46 years.

The proband’s aunt (II.1), born in 1963, developed hypertension at the age of 40 years. Laboratory investigations showed proteinuria and chronic renal failure, but renal biopsy was not performed. By age 44 years, ESRD was reached and hemodialysis was undertaken.

The proband’s grandfather (I.1), born in 1934, developed proteinuria when he was 59 years old. At the age of 64 years, he underwent a renal biopsy, which showed focal glomerular sclerosis, obliteration of capillary lumina with hyalinosis, increased matrix, and areas of tubular atrophy. By age 69 years, he developed ESRD, and he started peritoneal dialysis and received a renal transplant 1 year after this.

Considering the complex family history, we suggested the uninvestigated family members undergo laboratory tests and ultrasound examination, which revealed isolated non-nephrotic proteinuria in the 18-year-old cousin (III.1) of the index patient.

**Molecular Analysis of WT1 Gene**

We carried out WT1 gene exon 8 and 9 analysis by direct sequencing of blood DNA of the proband. WT1 sequencing revealed nucleotide substitution in position c.1208G>A in exon 9 (GenBank no. M74917), resulting in the substitution of a highly conserved arginine residue with a lysine in the 403 position (p.R403K) of the third Zf domain of the protein. This sequence variant was not observed in 336 control chromosomes. Molecular analysis was then extended to the other family members, and c.1208G>A variant was detected in the father, aunt, grandfather, and cousin (Figure 1).

**Structural Analysis for R403K Mutation**

From a structural point of view, replacing Arg with Lys has two effects: the position of the charged residue is shifted, and the charge is somewhat more concentrated (it is spread over three nitrogen atoms in Arg and concentrated at the “tip” on Lys) (Figure 3).

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**Discussion**

We describe an Italian family with isolated FSGS associated with a novel sequence variant in WT1 gene exon 9. In our study, we tested the detected sequence variant in 336 chromosomes and did not detect it (0 of 168 subjects). According to the literature, a sequence variant is regarded as a polymorphism if minor allele frequency is >1% in normal population. It is universally accepted that a control population of at least 100 subjects is enough to define whether a variant is or is not a polymorphism. In addition, the detected variant has never been observed in the cases of FSGS/DMS or in the somatic mutations associated with Wilms tumor reported in the literature. However, segregation in all the affected members of the family and its absence in a control population suggest that it may be a disease-causing mutation.

We applied bioinformatics tools to predict the effect of the amino acid substitution on WT1 protein structure. c.1208G>A substitution changes an arginine residue located in a strategic position of Zf3 to lysine (p.R403W). Although conservative, this kind of amino acid substitution in critical residues is hypothesized to be of functional significance (11,12). Zf4 is important for binding, and the presence/absence of the “KTS” insertion seems to switch between DNA binding (−KTS) and RNA binding (+KTS) (13,14). The residue Arg403 is located in the alphahelix of Zf3. It points toward the DNA but is not in direct contact with it (Figure 2). Analysis of the residue’s surroundings reveals that it is sterically largely unhindered but forms a salt bridge interaction with Glu408, which is in the linker region between Zf3 and Zf4. Arg403 appears to be anchoring the linker between Zf3 and Zf4 to position Zf4, close to the DNA molecule. Small movements in this conformation would probably make Zf4 swivel out of position. Interestingly, the position of the KTS insertion is exactly between Gly407 and Glu408. Because this insertion is known to affect the position of Zf4 and significantly modify the interactions of WT-1 (DNA versus RNA preference), it can be speculated that even small variations of the salt bridge geometry may affect the DNA-binding affinity. Therefore, the sequence alteration would be expected to hamper Zf-DNA interactions, resulting in target gene transcription impairment. Further studies will be requested to confirm the functional effect of the detected variant.
In the literature, WT1 alterations associated with nephropathy are generally of two types: mutations occurring in exon 8 or 9 (often missense), with patients showing DDS, and mutations at the intron 9 splice donor site, associated with FSGS in the context of FS phenotype. However, intron 9 splice donor site mutations have also been reported in patients with DDS, pseudohermaphroditism, or gonadal dysgenesis, with or without gonadoblastoma, and exon 8 or 9 mutations have been described in association with FSGS and gonadal dysgenesis (6,8–10). Our patients carried an exon 9 variant and presented FSGS, but they lacked Wilms tumor or genitourinary anomalies. This phenotype is very unusual, especially in male patients who usually show genital abnormalities, but it does actually agree with previously reported cases of isolated FSGS or DDS associated with WT1 intron 9 mutations, as well as isolated DDS or FSGS associated with exon 8 or 9 mutations (6,7,14–19). Although few, taken together these cases highlight that phenotypic variability in WT1 alterations is probably higher than previously described, suggesting the need for reconsidering and expanding genotype-phenotype correlation in WT1 alterations.

Another peculiar aspect is that the sequence variant was transmitted among three generations of a family in which all members had proteinuria (with eventual progression to chronic kidney disease) and no associated genital abnormalities or tumor. In the literature, four cases of familial transmission of WT1 mutation are reported. Denamur et al. (7) described a splice site mutation in WT1 exon 9 in a 9-year-old girl (karyotype 46, XY) with nephrotic syndrome and DDS, and in her mother, who had proteinuria since the age of 6 years and FSGS. A novel familial read-through mutation in WT1 exon 10 was detected by Zirn et al. (20) in a 22-year-old woman with Wilms tumor and ureter duplex in infancy, as well as slow progressive nephropathy; in her younger brother, who had hypertension but normal renal function; and in their mother, with late-onset nephropathy and ESRD. Regev et al. (21) recently reported the transmission of a mutation in exon 1 from a mother with Wilms tumor in infancy to her son with genitourinary anomalies and gonadal dysgenesis with gonadoblastoma foci. Transmission of a substitution in exon 9 from a mother with ESRD to her two daughters (one with nephrotic syndrome and the other healthy) was also reported by Mucha et al. (19). These observations suggested that WT1 alterations may be associated not only with interindividual but also with intrafamilial variability. Differently from these reports, all members of our family displayed the same phenotype of isolated proteinuria due to FSGS. Furthermore, in our patients the onset of proteinuria was not in early life, and ESRD developed in full adulthood, differently from most cases of the literature, in which clinical manifestations commonly occur in infancy. These peculiarities suggest that WT1 gene analysis is to be taken into consideration in the assessment of patients with FSGS-associated proteinuria, regardless of age or presence of typical DDS or FS clinical features.

Several studies have shown that the target genes potentially regulated by WT1 include genes that code for transcription factors (such as PAX2), growth factors or their receptors (EGFR, IGFR1R, TGF-β1, IGF2, IGFR, PDGF-A, VEGF), as well as podocyte proteins, such as nephrin and podocalyxin (2). Because the filtration barrier’s function requires the integration of multiple signaling pathways between endothelial, mesangial, and podocyte cells, correct WT1 interaction with target genes seems to be crucial to the maintenance of such a complex and dynamic structure. Furthermore, a proteomic study of DDS podocytes showed that they misexpress proteins associated with cytoskeletal architecture (including coflin, calponin, elﬁn, hsp27, and vinculin), and total levels of ﬁlamentous actin were also reduced (22). WT1 has also been demonstrated to regulate the intermediate ﬁlament protein nestin, whose reduced expression was associated with podocyte dysfunction (23,24). These ﬁndings suggested that in addition to its traditional role in regulation of proliferation, WT1 can also inﬂuence cytoskeletal architecture, accounting for the development of proteinuria and the lack of genitourinary abnormalities or Wilms tumor in some patients. The maintenance of regularly spaced and interdigitated podocyte foot processes with their associated slit diaphragms is indeed essential to filtration barrier integrity, and the loss of podocyte cytoskeletal architecture and slit diaphragms results in its dysfunction. In summary, normal podocyte function is maintained by a complex and dynamic gene network in which WT1 seems to exert a crucial role, so that its mutations may result in a broad range of phenotypic alterations. Furthermore, our ﬁnding of a novel WT1 mutation in a family with isolated proteinuria suggests extending WT1 gene mutational screening to patients with FSGS, which will contribute to a better understanding of WT1 functions in podocytes.

Disclosures
None.

References


