Genotype–phenotype associations in *WT1* glomerulopathy

Beata S. Lipska[1,2], Bruno Ranchin[3], Paraskevas Iatropoulos[4], Jutta Gellermann[5], Anette Melk[6], Fatih Ozaltin[7,8], Gianluca Caridi[9], Tomas Seeman[10,11], Kalman Tory[12], Augustina Jankauskiene[13], Aleksandra Zurowska[14], Maria Szczepanska[15], Anna Wasilewska[16], Jerome Harambat[17], Agnes Trautmann[2], Amira Peco-Antic[18], Halina Borzecka[19], Anna Moczulska[20], Bassam Saeed[21], Radovan Bogdanovic[22], Mukaddes Kalyoncu[23], Eva Simkova[24], Ozlem Erdogan[25], Kristina Vrljicak[26], Ana Teixeira[27], Marta Azocar[28], Franz Schaefer[2] and the PodoNet Consortium[29]

[1]Department of Biology and Genetics, Medical University of Gdansk, Gdansk, Poland; [2]Division of Pediatric Nephrology, Center for Pediatrics and Adolescent Medicine, University of Heidelberg, Heidelberg, Germany; [3]Service de Néphrologie Pédiatrique, Centre de Référence des Maladies Rénales Rares, Hôpital Femme Mère Enfant, Hospices Civils de Lyon and Université de Lyon, Bron, France; [4]IRCCS - Istituto di Ricerche Farmacologiche Mario Negri, Clinical Research Centre for Rare Diseases Aldo e Cele Daccò, Ranica, Bergamo, Italy; [5]Klinik für Pädiatrie/Nephrologie, Charité Campus Virchow-Klinikum, Berlin, Germany; [6]Pediatric Kidney, Liver and Metabolic Disease, MHH Children's Hospital, Hannover, Germany; [7]Departments of Pediatric Nephrology and Rheumatology, Hacettepe University Faculty of Medicine, Ankara, Turkey; [8]Nephrogenetics Laboratory, Hacettepe University Faculty of Medicine, Ankara, Turkey; [9]Laboratorio di Fisiopatologia dell'Uremia e UOC di Nefrologia Dialisi e Trapianto, Istituto G Gaslini, Genova, Italy; [10]1st Department of Pediatrics, University Hospital Motol, Prague, Czech Republic; [11]2nd Faculty of Medicine, Charles University, Prague, Czech Republic; [12]1st Department of Pediatrics, Semmelweis University, Budapest, Hungary; [13]Vilnius University , Vilnius, Lithuania; [14]Department Paediatrics, Nephrology and Hypertension, Medical University Gdansk, Gdansk, Poland; [15]Dialysis Division for Children, Department and Clinics of Pediatrics, Medical University of Silesia in Katowice, Zabrze, Poland; [16]Department of Pediatric Nephrology, University of Bialystok, Bialystok, Poland; [17]Service de Pédiatrie, Centre de Référence Maladies Rénales Rares du Sud Ouest, Centre Hospitalier Universitaire de Bordeaux, Bordeaux, France; [18]Division of Pediatric Nephrology, University Children's Hospital, Belgrade, Serbia; [19]Medical University Lublin, Pediatric Nephrology, Lublin, Poland; [20]Department of Pediatric Nephrology, Jagiellonian University Medical College, Krakow, Poland; [21]Department of Pediatric Nephrology, Kidney Hospital, Damascus, Syria; [22]Department of Nephrology, Institute of Mother and Child Healthcare of Serbia, Belgrade, Serbia; [23]Karadeniz Technical University, Faculty of Medicine, Pediatric Nephrology Department, Trabzon, Turkey; [24]Paediatric Department, Dubai Hospital, Dubai, UAE; [25]Department of Pediatric Nephrology, Dr. Sami Ulus Maternity and Children's Hospital, Ankara, Turkey; [26]Division of Nephrology, Department of Pediatrics, Zagreb University Hospital Centre, University of Zagreb, Zagreb, Croatia; [27]Pediatric Nephrology, University Children's Hospital, Porto, Portugal and [28]Unidad de Nefrología Infantil Hospital Luis Calvo Mackenna, Facultad de Medicina Universidad de Chile, Santiago de Chile, Chile

***WT1* mutations cause a wide spectrum of renal and extrarenal manifestations. Here we evaluated disease prevalence, phenotype spectrum, and genotype–phenotype correlations of 61 patients with *WT1*-related steroid-resistant nephrotic syndrome relative to 700 *WT1*-negative patients, all with steroid-resistant nephrotic syndrome. *WT1* patients more frequently presented with chronic kidney disease and hypertension at diagnosis and exhibited more rapid disease progression. Focal segmental glomerulosclerosis was equally prevalent in both cohorts, but diffuse mesangial sclerosis was largely specific for *WT1* disease and was present in 34% of cases. Sex reversal and/or urogenital abnormalities (52%), Wilms tumor (38%), and gonadoblastoma (5%) were almost exclusive to *WT1* disease. Missense substitutions affecting DNA-binding residues were associated with diffuse mesangial sclerosis (74%), early steroid-resistant nephrotic syndrome onset, and rapid progression to ESRD. Truncating mutations conferred the highest Wilms tumor risk (78%) but typically late-onset steroid-resistant nephrotic syndrome. Intronic (KTS) mutations were most likely to present as isolated steroid-resistant nephrotic syndrome (37%) with a median onset at an age of 4.5 years, focal segmental glomerulosclerosis on biopsy, and slow progression (median ESRD age 13.6 years). Thus, there is a wide range of expressivity, solid genotype–phenotype associations, and a high risk and significance of extrarenal complications in *WT1*-associated nephropathy. We suggest that all children with**

Correspondence: *Beata S. Lipska, Department of Biology and Genetics, Medical University of Gdańsk, Debinki Street 1, 80211, Gdansk, Poland. E-mail: b.lipska@gumed.edu.pl*

[29]See the Appendix for a list of PodoNet collaborators.

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steroid-resistant nephrotic syndrome undergo *WT1* gene screening.

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KEYWORDS: Denys–Drash syndrome; diffuse mesangial sclerosis; focal segmental glomerulosclerosis; Frasier syndrome; steroid-resistant nephrotic syndrome; *WT1*

Up to 25% of steroid-resistant nephrotic syndrome (SRNS) cases in children are caused by abnormalities in genes specifically or preferentially expressed by the podocyte.[1–4] *WT1* was the first gene shown to be mutated in SRNS.[5] Originally identified as a Wilms tumor (WT) suppressor gene, the primary physiological role of *WT1* is to control the development of the genitourinary system. In the fetal kidney, *WT1* is abundantly expressed in areas of active glomerulogenesis, supporting a major role of the gene in the development and maturation of the glomerular filtration barrier.[6] After completion of nephrogenesis, *WT1* expression is limited to podocytes.

WT1 encodes a transcription factor containing an N-terminal transactivation domain (exon 1) and four zinc-fingers at the C-terminus (exons 7–10). Germline alterations located throughout the entire coding sequence, usually truncating point mutations, predispose to WT.[7–9] A splice site at exon 9 inserts three additional amino acids between the third and the fourth zinc-finger (usually referred to as the KTS splice insert). Mutations in the KTS site result in Frasier syndrome.[10] A group of mostly missense mutations in exons 8 and 9 affect the zinc-finger domains. These variably impair the DNA-binding capacity of *WT1* and cause either Denys–Drash syndrome or Meacham syndrome, a condition associated with extrarenal congenital defects including diaphragmatic hernia.[5,7,11] Finally, large genomic rearrangements affecting chromosome 11p13 may disrupt *WT1* among several genes, resulting in WAGR syndrome (*W*ilms Tumor, *A*niridia, *G*enitourinary Abnormalities, and Mental *R*etardation).[8] *WT1*-associated disorders may include SRNS either as an initial symptom or as a later development in a patient diagnosed on the basis of extrarenal features. In addition, isolated SRNS may result from a wide range of *WT1* sequence variations, predominantly but not exclusively exonic point mutations notorious for incomplete penetrance and significantly variable expressivity.[8]

The international PodoNet registry has assembled the largest genetically screened SRNS cohort to date. In this work we used the PodoNet cohort to describe the genotypic and phenotypic spectrum of *WT1*-associated kidney disease. The 61 cases collected by the consortium represent the largest and best characterized cohort of *WT1* nephropathy analyzed to date. We provide demographic information on the relative frequency of *WT1*-associated SRNS and mutation types and perform a detailed genotype–phenotype correlation study encompassing the age and symptoms at disease onset, the long-term course of kidney function, and the incidence and spectrum of extrarenal manifestations.

RESULTS

Among the 746 consecutive SRNS cases registered in the PodoNet cohort who underwent *WT1* gene screening, 46 patients (6%) from 23 clinical centers in 12 European and Middle East countries were found to be positive for a *WT1* mutation. These included 2 out of 25 (8%) SRNS families with autosomal dominant inheritance reported to the Registry. In addition, information on 15 patients, including 2 additional families, diagnosed with *WT1*-associated SRNS before the formation of the PodoNet consortium was included in the analysis, yielding a cohort of 61 patients (Figure 1). *WT1*-positive patients did not cluster to any specific region, country, or era. Results of 17 (28%) patients have been previously published;[3,12–22] however, for the purposes of this study the most recent follow-up data were provided.

WT1 mutation screening

All patients in the PodoNet cohort underwent mutational screening of exons 8 and 9 and their intronic junctions. Seven patients with clinical features highly suggestive of *WT1*-associated disease (concomitant WT or ovarian dysgerminoma) and no mutation in the *hot spot* region underwent extended screening of the entire coding sequence of *WT1*, yielding a causative point mutation in four cases. The remaining three patients were tested by array comparative genomic hybridization for large genomic deletions at chromosome 11p13. No deletion at the *WT1 locus* and/or its adjacent chromosomal region was detected.

A total of 30 different *WT1* mutations were detected (Figure 2), including 5 recurrent mutations: c.1288C>T, c.1372C>T, c.1384C>T, c.1432+4C>T, and c.1432+5 G>A (previously referred to as R362X, R390X, R394W, IVS9+4C>T, and IVS9+5G>A, respectively). Five mutations are novel: c.1017C>A, c.1063A>T, c.1192T>C, c.1249+3A>G, and c.1337C>T (detailed evaluation of their pathogenic effect is in Supplementary Material S2 online).

A total of 55 (90%) patients carried mutations in the *hot spot* region. Mutations were categorized as truncating mutations (5 mutations in 9 subjects), missense mutations affecting amino acids in DNA-binding positions (14 mutations in 24 subjects), other missense mutations ($n=7$), intronic KTS mutations (3 mutations in 19 subjects), and other intronic mutations (1 mutation in 2 family members). The most common mutation, found in nine nonrelated cases, was c.1384C>T, leading to the p.Arg462Trp substitution (previously referred to as R394W).

Initial manifestation and clinical course of disease

The clinical characteristics of the SRNS patients with and without *WT1* mutations were compared (Table 1). Patients with *WT1*-associated disease were usually less symptomatic at the time of diagnosis with less marked edema and hypoalbuminemia, but more commonly presented with impaired glomerular filtration rate as compared with *WT1*-negative cases. The initial diagnosis was isolated SRNS in 56% of cases

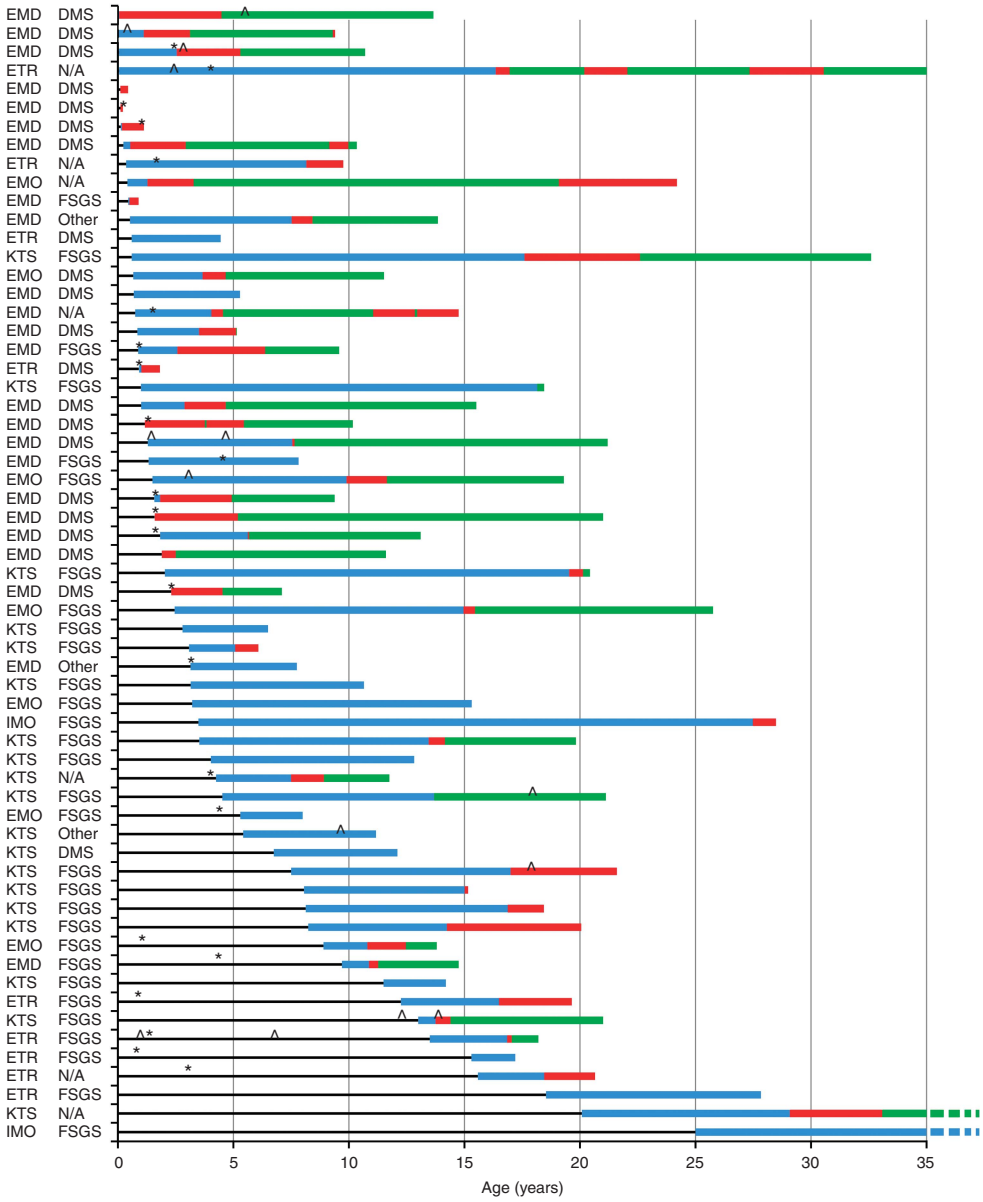

Figure 1 | Synopsis of clinical disease course in 61 steroid-resistant nephrotic syndrome (SRNS) patients with *Wilms tumor 1* (*WT1*) mutations. The first column indicates the mutation type: EMD, exonic mutation affecting the DNA-binding site; EMO, other exonic missense mutation; ETR, exonic truncating mutation; IMO, other intronic mutation; KTS, intronic mutation affecting the KTS splice site. The second column describes the type of histopathological lesion: DMS, diffuse mesangial sclerosis; FSGS, focal segmental glomerulosclerosis; other, other histopathological lesion; N/A, data not available. The black line denotes the time before first clinical disease manifestation, the blue bars denote the period with proteinuria/chronic kidney disease (CKD), the red bars show dialysis periods, and the green bars show transplantation periods. The symbols denote the time of Wilms tumor (WT) diagnosis (*) and gonadectomy (^).

($n = 34$). In retrospect, however, only 28% ($n = 17$) of the patients could be considered as having isolated SRNS when accounting for urogenital anomalies, sex reversal, and late-onset WTs detected subsequent to *WT1* mutation. All patients with isolated SRNS were genotypic and phenotypic females, whereas the syndromic cases encompassed both male and female phenotypic patients. Patients with *WT1*-associated SRNS attained end-stage renal disease (ESRD) at, on average, 3 years younger age than patients with other genetic causes of SRNS ($n = 88$; 66 *NPHS2*, 13 *NPHS1*, 5 *PTPRO*, 3 *LAMB1*, and 1 *MYO1E*; $P = 0.001$) and at almost 10 years younger age than patients without detectable genetic cause ($P < 0.0001$; Figure 3).

Grouping of mutations according to location revealed notable phenotypic differences. Patients with exonic mutations were significantly younger at diagnosis, presented with more severe proteinuria, edema, and hypertension, and progressed more rapidly to ESRD (Table 2). The average time interval from diagnosis to end-stage kidney disease was 2.7 versus 9.2 years ($P < 0.001$). Further subgrouping

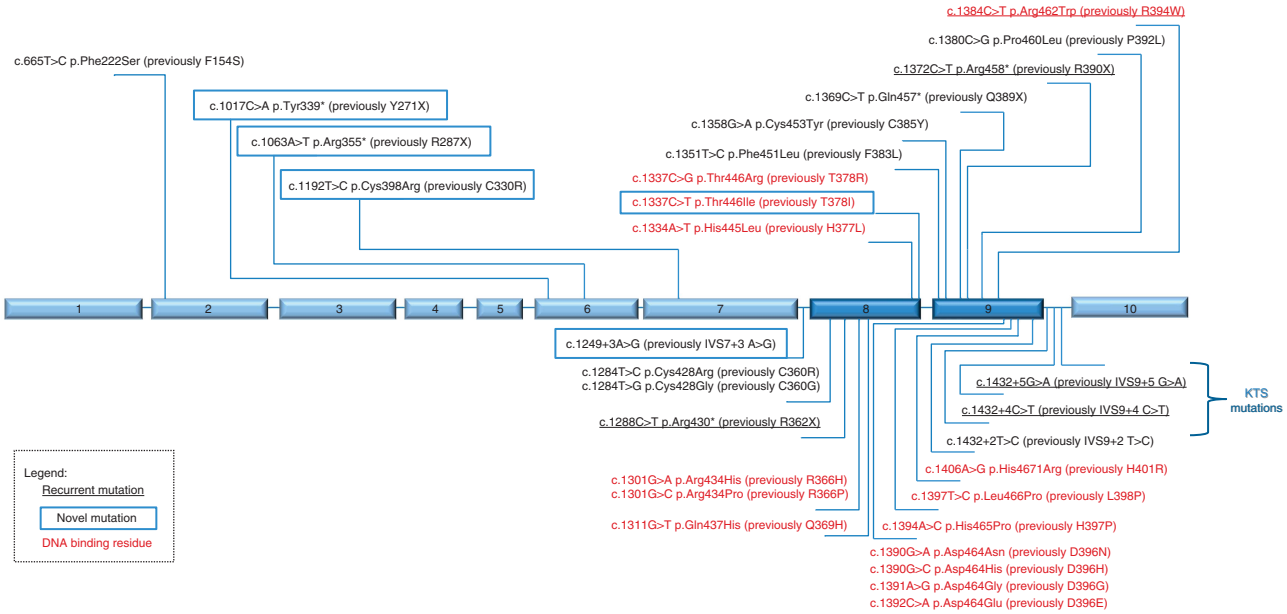

Figure 2 | Localization of the *Wilms tumor 1* (*WT1*) mutations detected in the analyzed cohort of steroid-resistant nephrotic syndrome (SRNS) patients. As the reference sequence, the NCBI 517aa isoform D (NM_024426.4, GRCh37 assembly) of *WT1* corresponding to the ENSEMBL transcript ENST00000332351 (ENSG00000184937) is used. In addition, the traditional nomenclature referring to the 449aa isoform is given for ease of comparison with previous publications on the subject matter. The underlined text indicates recurrent mutations detected in at least three unrelated patients from different ethnic groups; the tagged (blue frame) portion indicates novel mutations described for the first time in this study; and red text indicates missense mutations affecting nucleotides coding for residues important for interaction with target DNA: 434–449 (previously 366-381) in exon 8 and 461–469 (previously 393–401) in exon 9 (Swiss-Prot: P19544).

by mutation type showed distinct differences between patients with missense mutations affecting DNA-binding amino acid residues and those with truncating mutations (Table 3 and Figures 1 and 4). Mutations in DNA-binding positions were associated with the earliest disease onset and the most rapid progression to ESRD ($P<0.001$). Among the 9 patients with truncating mutations, 4 developed SRNS before 2 years and 5 beyond 10 years of age. Patients with other missense mutations showed an intermediate disease course.

Among 9 children with late-onset SRNS (age >10 years), 5 had truncating *WT1* mutations; 4 of these were long-term WT survivors. The other four late-onset patients had intronic mutations (thereof 3 KTS); these patients manifested with isolated proteinuria without extrarenal symptoms. Of the 7 peripubescent children with exonic mutations, 6 had undergone unilateral ($n=5$) or subtotal bilateral nephrectomy ($n=1$) for WT 5.3–13.6 years before the onset of SRNS.

The 9 patients with the p.Arg462Trp mutation showed SRNS onset between 3 weeks and 3.1 years of age (median 9.5 months). Of the 9 subjects, 5 progressed to ESRD before their fourth birthday, 1 at age 7, and 3 still had preserved kidney function at age 5–8 years.

Histopathology findings

Similar to the non-*WT1*-associated SRNS, the most common histopathological finding in *WT1* disease was focal segmental glomerulosclerosis (FSGS; Table 1). Diffuse mesangial sclerosis (DMS), the second most common histology, was highly predictive of *WT1* mutations: *WT1*-positive patients accounted for 60% of all DMS cases reported in the PodoNet Registry (odds ratio = 21; 95% confidence interval: 9.7–50; $P<0.0001$). Among the *WT1* patients, DMS was six times more likely to be present in children diagnosed before the age of 2 years than in older children (60% vs. 10%) and associated almost exclusively with exonic missense mutations (Tables 2 and 3). Conversely, FSGS was observed mainly in patients with first disease manifestation beyond 2 years of age (74% vs. 23%) and was present in almost 90% of patients with intronic mutations. Among the six patients with a p.Arg462Trp mutation who underwent kidney biopsy, DMS, FSGS, and membranoproliferative glomerulonephritis were reported in two cases each.

Malignancies

Cancer was more common in patients with exonic mutations (73% vs. 19%), the highest rate of neoplasia being observed among patients with truncating mutations (78%).

Wilms tumors. WTs developed in 23 patients (Tables 2 and 3). Of them, 22 were carriers of exonic *WT1* mutations who were cumulatively followed-up for 199 years (that is, one case per 9 years at risk). In contrast, only a single WT occurred in a patient with a KTS mutation (incidence 1 per 343 years at risk). The median age at WT diagnosis was 1.6 (range 0.1–4.5) years. In six patients, SRNS preceded WT by a median of 1.7 years (range 2 weeks–3.9 years); 8 children first presented with SRNS at the time of WT diagnosis, and in 9 patients SRNS developed after a median of 8 years (range 4

Table 1 | Clinical characteristics at the time of diagnosis and prospective kidney survival rates of 61 children with SRNS related to *WT1* mutations versus 700 patients screened negative in *WT1*

	WT1 SRNS	Non-*WT1* SRNS
N	61	700
Age at diagnosis (years)	2.0 (0.7–6.8)***	4.4 (1.9–10.1)
Asymptomatic, incidental diagnosis	27.9%**	12.6%
Edema (none/mild/moderate/severe)	46/19/26/9%	14/8/31/22%
Proteinuria (small/gross)	19/81%	16/84%
Hematuria	27.3%	37.5%
Hypertension	39.0%***	15.5%
eGFR (ml/min per 1.73 m^2)	74 (60–106)**	103 (71–144)
Serum albumin (g/l)	27 (19–35)*	21(16–28)
Wilms tumor	37.7%***	0.3%
Gonadoblastoma	4.9%**	0.1%
Sex reversal	14.7%***	0.1%
Genital abnormalities in subjects with concordant gender	31.1%***	0.1%
Urinary tract abnormalities	11.4%***	1.3%
Histopathology subtype		
FSGS	47.5%	43.7%
DMS	34.4%***	1.6%
MesGN	3.3%	10.0%
MCN	1.7%	15.3%
Other/no data	13.1%	21.8%
Kidney survival from diagnosis		
2-Year survival rate	70.1 ± 8.2%***	92.0 ± 1.1%
5-Year survival rate	55.2 ± 7.4%***	72.1 ± 7.9%
10-Year survival rate	25.0 ± 3.5%***	51.5 ± 3.4%

Abbreviations: DMS, diffuse mesangial sclerosis; eGFR, estimated glomerular filtration rate; FSGS, focal segmental glomerulosclerosis; MCN, minimal change nephropathy; MesGN, mesangioproliferative glomerulonephritis; SRNS, steroid-resistant nephrotic syndrome; *WT1*, *Wilms tumor 1*.
Data are given as median (interquartile range) or %. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

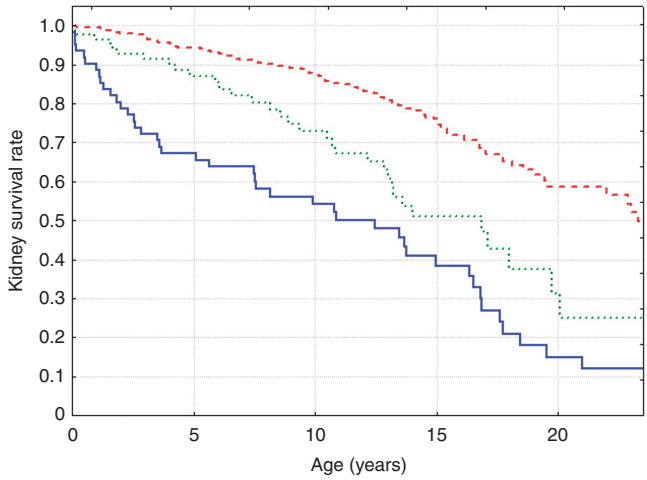

Figure 3 | Actuarial kidney survival analysis of 61 patients with steroid-resistant nephrotic syndrome (SRNS) due to *Wilms tumor 1* (*WT1*) mutations (solid blue line) compared with 88 patients with other monogenic forms of SRNS (dotted green line; $P<0.001$) and 597 mutation-negative patients (dashed red line; $P<0.0001$). Patients with prophylactic bilateral nephrectomy were censored at the time of surgery.

months–13.6 years) after diagnosis and treatment of a WT. Bilateral tumors were more common in patients with truncating mutations (odds ratio = 18.4; $P=0.01$).

Gonadoblastoma. Gonadoblastoma, including one bilateral case, occurred in three of nine 46,XY individuals with sex reversal within a cumulative observation time of 91 years.

Two patients developed Epstein–Barr virus–associated post-transplant lymphoproliferative disease (B-cell lymphoma) during adolescence. Both patients had previously undergone cyclophosphamide treatment for SRNS, were receiving ciclosporin A and mycophenolate mofetil when post-transplant lymphoproliferative disease developed, and had donor-positive/recipient-negative high-risk Epstein–Barr virus status at the time of transplantation.

Sex reversal, urogenital abnormalities, and reproductive function

Male-to-female sex reversal was detected in nine subjects (1/3 of 46,XY individuals), all of whom were already diagnosed with a *WT1* mutation. In two phenotypic girls with SRNS, primary amenorrhea had prompted *WT1* screening. Sex reversal occurred exclusively in patients with KTS mutations and DNA-binding site mutations (Table 3).

All 46,XY individuals, except one, presenting with a male phenotype had various abnormalities of the external genitalia, the most common being cryptorchidism and hypospadias, present in 83% and 67%, respectively (Table 2). Furthermore, 3 of the 34 female patients with 46,XX presented with genital abnormalities (Table 2).

The median age at menarche in the 46,XX female patients was 13 (range 10–15) years. All adolescent 46,XY individuals with male-to-female sex reversal achieved menstruation on hormone replacement therapy. To date, two adult female patients have mothered children.

Among four boys with the same p.Arg462Trp mutation, genital abnormalities varied and included penoscrotal hypoplasia, hypospadias, and cryptorchidism; one girl with the mutation presented with uterus bicornus.

Urinary tract abnormalities were noted in seven patients (11%, Tables 2 and 3). Of them, three were female, three (phenotypic and karyotypic) were male, and one was a karyotypic male with sex reversal. Five of the patients had combined urinary tract and genital anomalies.

Prophylactic nephrectomy and/or gonadectomy

A total of 27 patients (44%), including 4 with intronic mutations, underwent bilateral nephrectomy. Half of them, all with exonic mutations, underwent the surgery before their fifth birthday. Nephrectomy was performed electively before transplantation ($n=18$), because of bilateral WT ($n=5$) or because of suspicious sonographic findings ($n=4$).

In 14 (52%) 46,XY individuals, both with exonic (9/18) and intronic (5/9) mutations, preemptive bilateral gonadectomy before 20 years of age was undertaken. On histopathological evaluation, mixed gonadal dysgenesis was the most

Table 2 | Comparison of patients with exonic versus intronic mutations in *WT1*

	Exonic mutations	Intronic mutations
N	40	21
Age at SRNS onset (years)	1.1 (0.4–2.8)***	4.5 (3.2–8.2)
Asymptomatic, incidental diagnosis	40.0%**	85.7%
Edema (none/mild/moderate/severe)	35/18/32/15%**	65/20/15/0%
Proteinuria (small/gross)	15/85%	25/75 %
Hematuria	31.3%	23.8%
Hypertension	50%**	19%
Chronic renal failure at diagnosis	23%**	0%
Histopathology subtype		
FSGS	13***	17
DMS	20***	1
Other	2	1
Failed/no data	5	2
Cancer[a]	24/33[a] (73%)***	4/21 (19%)
Wilms tumor[a]	22 (17 Uni-/5 bilateral)***	1 (Unilateral)
Gonadoblastoma	1	2
Other	1 (Lymphoma)	1 (Lymphoma)
Sex reversal	3/40 (8%)* (3/18 46,XY*)	6/21 (29%) (6/9 46,XY)
Genital abnormalities in phenotypic males		
Cryptorchidism	13/15 (93%)	2/3 (67%)
Hypospadia	9/15 (60%)	3/3 (100%)
Bifidus/hypoplastic scrotum	6/15 (40%)	2/3 (67%)
Rudimentary vagina and/or uterus	5/15 (33%)	0/3 (0%)
Genital abnormalities in phenotypic females	1, Uterus bicornus	1, Polypose uterus; 1, hypertrophic ovary
Urinary tract abnormalities	1, Duplex kidney; 1, horseshoe kidney; 2, VUR > II grade	1, Horseshoe kidney; 1, kidney malrotation; 1, PUJ stenosis
Kidney survival from diagnosis		
2 Years	57.1 ± 8.3 %***	90.5 ± 6.4%
5 Years	35.8 ± 5.7 %***	85.3 ± 8.9%
10 Years	18.2 ± 2.7 %***	37.0 ± 11.0%

Abbreviations: DMS, diffuse mesangial sclerosis; FSGS, focal segmental glomerulosclerosis; PUJ, pelviureteric junction; SRNS, steroid-resistant nephrotic syndrome; VUR, vesicoureteral reflux; *WT1*, *Wilms tumor 1*.
*$P<0.05$, **$P<0.01$, ***$P<0.001$. Data are given as median (interquartile range) or %.
[a]Seven cases with prophylactic bilateral nephrectomy performed before age 5 years excluded from the analysis.

Table 3 | Genotype–phenotype association for *WT1* mutation subtypes

	Exonic			Intronic	
Type of mutation	Truncating mutations	DNA-binding site missense mutations	Other missense mutations	KTS (intron 9) mutations	Other intronic mutations
N	9	24	7	19	2
Age at SRNS onset	12.3 (0.6–15.3)	0.9 (0.2–1.6)	2.4 (0.7–5.3)	4.5 (3.1–8.1)	14.2 (3.5–25)
Age at 50% kidney survival[a]	16.5	2.5	10.8	13.6	NA
Renal histopathology[b]					
DMS	2/6 (33%)	17/23 (74%)	4/6 (67%)	1/17 (6%)	0/2
FSGS	4/6 (67%)	4/23 (17%)	2/6 (33%)	15/17 (88%)	2/2
Isolated SRNS	1/9 (11%)	5/24 (21%)	2/7 (29%)	7/19 (37%)	2/2
Sex reversal[c]	0/5 (0 %)	3/11 (27%)	0/2 (0 %)	6/9 (67%)	0/0
Genital abnormalities in subjects with concordant gender	6/9 (67%)	9/21 (43%)	2/7 (29%)	4/13 (31%)	0/2
Wilms tumor	7/9 (78%)	13/24 (54%)	2/7 (29%)	1/19 (5%)	0/2
Thereof bilateral	4/9 (44%)	1/24 (4%)	—	—	—
Other neoplasia	0/9 (0%)	2/24 (8%)	0/7 (0%)	3/19 (16%)	0/2
Urinary tract malformation	2/9 (22%)	1/24 (4%)	1/7 (14%)	3/19 (16%)	0/2

Abbreviations: DMS, diffuse mesangial sclerosis; FSGS, focal segmental glomerulosclerosis; NA, not available; SRNS, steroid-resistant nephrotic syndrome; *WT1*, *Wilms tumor 1*.
Data are given as median (interquartile range) or %.
[a]Kaplan–Meier median estimate.
[b]Only successful biopsies considered.
[c]Only patients with 46,XY karyotype considered.

common finding; however, in three patients asymptomatic gonadoblastoma was detected and in three patients no gonads were found.

DISCUSSION

In this large unselected cohort, we found *WT1* mutations in 6% of sporadic SRNS patients, in keeping with the prevalence

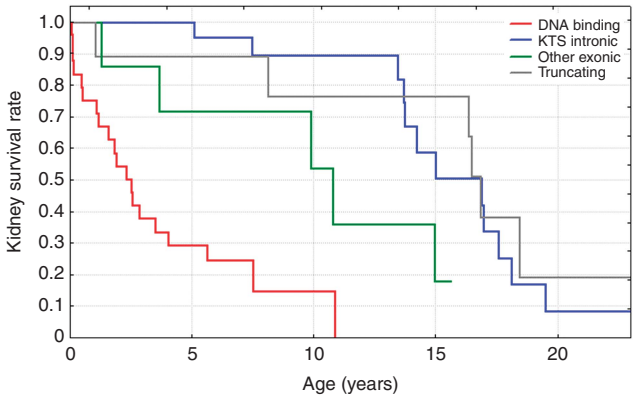

Figure 4 | Actuarial kidney survival analysis by type of *Wilms tumor 1* (*WT1*) mutation. Two patients with non-KTS intronic mutations were not considered. $P < 0.001$.

figures reported in previous smaller studies ranging from 2.7 to 7%.[4,11,23,24] *WT1*-positive cases were equally common in all pediatric age groups, reconfirming comparable figures reported separately for congenital/infantile and adolescent SRNS.[2,3]

Compared with non-*WT1*-associated SRNS, patients with *WT1* nephropathy were less often overtly nephrotic but more often presented with chronic kidney disease at the time of diagnosis. *WT1* disease tended to progress more rapidly than SRNS from other causes, with ESRD occurring at an almost 9 years younger age on average. FSGS was the most frequent histopathological finding in *WT1* nephropathy and was equally common in non-*WT1*-related SRNS, whereas the diagnosis of DMS was largely specific for *WT1*-associated disease.

Furthermore, *WT1* mutations were associated with a wide spectrum and expressivity of extrarenal phenotypes concerning urogenital development and the development of tumors. These were largely specific for *WT1*-associated disease, although a few cases of SRNS with WT or gonadoblastoma were noted in patients in whom no abnormalities in *WT1* could be detected.

Both renal and extrarenal phenotypes were clearly associated with the type and location of the causative mutation. The two largest subgroups, encompassing 70% of the cohort, comprised cases with exonic *mutations affecting nucleotides coding for DNA-binding residues* and *KTS mutations* in intron 9, that is, the abnormalities classically associated with Denys–Drash and Frasier syndrome, respectively.

The former group of mutations comprised amino acids 434, 437, 445, 446, 462, and 464–467, positions of established relevance for DNA and RNA binding[25,26] (Swiss-Prot: P19544). Patients with mutations in these positions uniformly showed early-onset renal disease and rapid progression to renal failure. DMS was found in the majority of patients, although one out of six subjects presented with FSGS. Although 54% of patients developed WT, the male-to-female sex reversal acclaimed to be typical of Denys–Drash syndrome was observed in 3 of 11 karyotypic male patients only.

Subjects with *KTS mutations* were characterized by a slightly later disease onset, almost invariably FSGS on biopsy, and a relatively slow progression pattern typically leading to ESRD in adolescence. Two-thirds of the karyotypic male patients showed the sex reversal typical of Frasier syndrome.

The phenotype of other exonic mutations was determined by the type of lesion: most subjects with *truncating mutations* developed WT in early life, whereas SRNS occurred later, typically in the second decade after nephrectomy. Only two patients with truncating mutations developed early-onset SRNS with DMS on biopsy. It is tempting to speculate that persistent glomerular hyperfiltration secondary to surgically reduced renal mass may have contributed to the late development of proteinuria, FSGS, and progressive chronic kidney disease in these patients with mutations with an otherwise low intrinsic capacity to cause podocyte damage. Missense mutations affecting amino acids *in non-DNA-binding* positions were characterized by an intermediate renal phenotype manifesting within the first 5 years of life, usually presenting with DMS on biopsy, and progressing to ESRD at $\sim$10 years of age on average. The more severe renal phenotype associated with missense as compared with truncating mutations may be explained by the production of a dominant-negative mutant *WT1* protein interfering with the action of wild-type protein, whereas truncating mutations exert a dosage effect only.

In contrast to the consistent associations of mutation type and clinical disease manifestation, considerable variability was found regarding the *histomorphological appearance*. The fraction of children displaying DMS or FSGS varied only gradually between truncating, DNA-binding site, and other exonic missense mutations. Remarkable histopathological heterogeneity was noted even among carriers of the same genetic abnormality (p.Arg462Trp), in which DMS, FSGS, and even membranoproliferative glomerulonephritis were observed with equal frequency despite uniformly early disease onset and mostly rapid progression to renal failure.

Major phenotypic variability was also observed with respect to the extrarenal manifestations of *WT1*-associated disease. As expected, male-to-female *sex reversal* was exclusively associated with KTS and DNA-binding site mutations, although $<50\%$ of karyotypic male patients with one of these mutations presented this feature characteristic of Frasier and Denys–Drash syndrome. Furthermore, a wide variety of genital and urinary tract abnormalities were noted at similar frequency in patients with any type of mutation, ranging from hypospadias and unilateral cryptorchidism to global penoscrotal hypoplasia. Subtle genital anomalies were even observed in a few karyotypic female patients. Taken together, genital malformations were detected in 50% of all patients. Our findings lend support to the concept that the interference of *WT1* variants with genital development represents a continuum ranging from normal sexual differentiation to complete sex reversal.[27] In this context, the impact of KTS and DNA-binding site mutations is most disruptive but by no means exclusive.

Wilms tumors

WTs, which occurred in 38% of the patients, are believed to develop as a consequence of somatic 'second hits' to the *WT1* gene.[7] Consistent with this two-hit model and previous observations, patients with truncating *WT1* mutations showed a very high risk for WT, in particular bilateral disease, and developed tumors earlier (at median age 19 months) than reported for *WT1* mutation–negative WT patients (median 36 months).[8,9] The tumor risk progressively decreased in subjects with mutations altering DNA binding and in those with other exonic mutations, and was as low as 1 in 19 in subjects with intronic (KTS) mutations. The one patient with a KTS mutation diagnosed with a WT adds to a single further case reported in the literature.[28] The very low tumor risk associated with KTS mutations is considered because of the fact that the two WT1 protein isoforms translated in patients with the KTS insert have equal antioncogenic properties.[29]

Germ cell tumors

Germ cell tumors occur much less frequently than WTs in patients with *WT1* mutations. As these tumors are primarily related to gonadal dysgenesis rather than to defective postnatal WT1 expression, they are more likely to develop in patients with sex reversal that occurs more commonly with intronic mutations. Indeed, we observed three cases of gonadoblastoma, all among the nine patients with sex reversal. Two cases were associated with KTS mutations and one with a missense mutation affecting DNA binding. Hence, our findings suggest that gonadoblastoma may be equally likely in subjects with sex reversal as part of the Denys–Drash and Frasier syndrome phenotypes, at variance with previous studies associating gonadoblastoma risk mainly with KTS mutations.[7,12] With an observed incidence of one gonadal tumor in 30 years at risk, surgical removal of the dysgenetic gonads in patients with sex reversal is certainly a recommendable, although not an urgent, necessity.

The variable expressivity and wide range of renal and extrarenal disease phenotypes in *WT1*-associated SRNS have several important clinical implications. Approximately 28% of all patients with a *WT1* mutation displayed isolated sporadic SRNS without any associated comorbidities, and in another 28% the extrarenal features were so subtle that they were detected only by additional examinations performed in the light of the genetic diagnosis. *WT1* nephropathy may become manifest at any time in childhood, depending on the type of mutation. Although severe nephrotic syndrome may be present at the time of diagnosis, particularly in children with disease onset in early infancy, clinically asymptomatic proteinuria with mildly impaired glomerular filtration rate and hypertension is the more common initial presentation, occurring more frequently than in non-*WT1*-related SRNS. Although we found *WT1* to be causative of only 6% of all SRNS cases, the high prevalence of truly or apparently isolated SRNS emphasizes the need to consider *WT1* disease in all children and adolescents with proteinuria. Our findings provide a rationale for routine screening of *WT1*, in addition to *NPHS2*, in children with isolated, sporadic SRNS. A notable subgroup are adolescent girls with SRNS and primary amenorrhea, who should promptly undergo *WT1* screening and karyotyping.

It should be noted that the 6% prevalence of *WT1* mutations found in our SRNS cohort may be an underestimation related to the genetic screening paradigm applied. Only the *hot spot* region was sequenced in all patients, and only subjects presenting with signs and symptoms suspicious of *WT1* disease with negative hot spot screening underwent sequencing of the entire *WT1* gene. This extended screening turned out positive in four of seven cases. Hence, it is possible that an unknown number of subjects with isolated SRNS due to *WT1* mutations may have been missed. Efforts to sequence all *WT1* exons in the entire PodoNet cohort as part of a targeted next-generation sequencing panel covering all known SRNS genes are currently underway.

In conclusion, *WT1* mutations constitute a rare but important genetic cause of SRNS, with a wide spectrum of renal and extrarenal phenotypes. Establishing the genetic diagnosis appears particularly important for *WT1* disease in view of its unique associated features and complications, including the genotype-specific risk of altered urogenital differentiation and WT and germ cell tumor development.

MATERIALS AND METHODS

Study population

Among 1374 SRNS patients consecutively enrolled in the PodoNet Registry (see Supplementary Material S1 online for further information), 746 underwent genetic evaluation of exons 8 and 9 of the *WT1* gene. In addition, 15 previously diagnosed cases of *WT1*-associated SRNS were included in the study. Seventeen cases evaluated in this study had been included in previous publications.[3,12–22]

Statistics

Statistical analyses were performed using Statistica 9.1 (StatSoft, Tulsa, OK) and SAS 9.2 (Cary, NC). The frequencies were compared using χ^2 tests with adequate corrections and the Fisher exact probability test with Freeman–Halton extension for 2×3 and 2×4 tables when applicable. The effect on long-term kidney survival rates was analyzed using the Kaplan–Meier survival probability estimates and log-rank tests. Three cases who underwent prophylactic bilateral nephrectomy before reaching ESRD were censored at the time of nephrectomy.

Mutational screening

DNA was extracted from peripheral blood following the standard phenol–chloroform protocol or using commercially available kits (Qiagen, Hilden, Germany). Exons 8 and 9 of *WT1* with their adjacent intronic junctions, and in selected cases also the remaining exons, were analyzed by direct sequencing using the ABI3130 Genetic Analyser (Applied Biosystems, Foster City, CA). The NCBI 517aa isoform D of *WT1* (NM_024426.4), corresponding to the ENSEMBL transcript ENST00000332351 (ENSG00000184937), was used as the reference sequence. In addition, the traditional nomenclature referring to the 449aa isoform is given for ease of comparison with previous publications.

Array comparative genomic hybridization

Array comparative genomic hybridization analysis of the selected patients was performed using the 3×720K Whole-Genome Tiling Array (NimbleGen, Roche, Basel, Switzerland). Arrays were scanned with a MS200 Microarray Scanner and analyzed using NimbleScan, SignalMap, and Deva v1.2.1 software (NimbleGen, Roche). All identified genomic imbalances were verified in the database of genomic variants (DGV; http://projects.tcag.ca/variation; last accessed July 2013).

In silico analyses of the effect on protein structure and function

Selected bioinformatics tools were used to assess the effect of sequence variants on the structure and function of the protein. The three-dimensional model of WT1 zinc domains (PDB: 2PRT) was analyzed using Rasmol software (www.rasmol.org) to verify the effect of amino acid substitutions on the protein structure. In order to identify conservative amino acid residues, multiple-alignment analysis of the orthologs from different species was performed using the ClustalW algorithm (www.clustal.org). The consequence on the splicing process was evaluated *in silico* using Human Splicing Finder[30] for evaluation of exon/intron boundaries and ESEFinder[31] for detection of putative exonic splicing enhancers/silencers.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Supplementary 1. Description of PodoNet Consortium and Registry.
Supplementary 2. Pathogenicity assessment of the identified novel *WT1* sequence variants.
Table S1. Summary of bioinformatic analyses of the detected novel sequence variants in the *WT1* gene.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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APPENDIX
PODONET COLLABORATORS

Chile: Marta Azocar, Santiago; Lily Quiroz, Santiago; Colombia: Lina Maria Serna Higuita, Medellín; Czech Republic: Jiří Dušek, Prague; France: Bruno Ranchin, Lyon; Michel Fischbach, Strasbourg; Georgia: Tinatin Davitaia, Tbilisi; Germany: Jutta Gellermann, Berlin; Jun Oh, Hamburg; Anette Melk, Hannover; Franz Schaefer, Heidelberg; Marianne Wigger, Rostock; Greece: Nikoleta Printza, Thessaloniki; Hungary: Peter Sallay, Budapest; Iran: Alaleh Gheissari, Isfahan; Italy: Marina Noris, Bergamo; Andrea Pasini, Bologna; Gian Marco Ghiggeri, Genova; Gianluigi Ardissino, Milano; Elisa Benetti, Padova; Francesco Emma, Rome; Lebanon: Bilal Aoun, Beirut; Pauline Abou-Jaoudé, Byblos; Lithuania: Augustina Jankauskiene, Vilnius; Poland: Anna Wasilewska, Bialystok; Ewa Gacka, Chorzow; Aleksandra Zurowska, Gdansk; Dorota Drozdz, Krakow; Marcin Tkaczyk, Lodz; Halina Borzecka, Lublin; Magdalena Silska, Poznan; Tomasz Jarmolinski, Szczecin; Agnieszka Firszt-Adamczyk, Torun; Joanna Ksiazek, Warsaw; Elzbieta Kuzma-Mroczkowska, Warsaw; Anna Medynska, Wroclaw; Maria Szczepanska, Zabrze; Portugal: Alberto Caldas Afonso, Porto; Helena Jardim, Porto; Serbia: Amira Peco-Antic Belgrade; Radovan Bogdanovic, Belgrade; Sweden: Rafael T Krmar, Stockholm; Switzerland: Giacomo D Simonetti, Bern; Syria: Bassam Saeed, Damascus; Turkey: Ali Anarat, Adana; Ayse Balat, Gaziantep; Z Esra Baskin, Ankara; Nilgun Cakar, Ankara; Ozlem Erdogan, Ankara; Birsin Özcakar, Ankara; Fatih Ozaltin, Ankara; Onur Sakallioglu, Ankara; Oguz Soylemezoglu, Ankara; Sema Akman, Antalya; Faysal Gok, Gulhane; Salim Caliskan, Istanbul; Cengiz Candan, Istanbul; Sevinc Emre, Istanbul; Sevgi Mir, Izmir; Ipek Akil, Manisa; Pelin Ertan, Manisa; Ozan Özkaya, Samsun; Mukaddes Kalyoncu, Trabzon; United Arab Emirates: Eva Simkova, Dubai; Entesar Alhammadi, Sharjah; Ukraine: Roman Sobko, Lviv.