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Two distinct *WT1* mutations identified in patients and relatives with isolated nephrotic proteinuria

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ABSTRACT

Wilms' tumor type 1 gene (*WT1*) encodes a zinc-finger transcription factor that plays a key role during genitourinary development and in adult kidney. Mutations in exons 8 and 9 are associated with Denys-Drash Syndrome, whereas those occurring in the intron 9 donor splice site are associated with Frasier Syndrome. Familial cases of *WT1* mutations are rare with only few cases described in the literature, whereas cases of *WT1* mutations associated with isolated nephrotic proteinuria with or without focal segmental glomerular sclerosis (FSGS) are even rarer. Exons 8 and 9 of *WT1* gene were analyzed in two non-related female patients and their parents. Patient 1, who presented with isolated nephrotic proteinuria and histologic pattern of FSGS, is heterozygous for the mutation c.1227 + 4C > T. This mutation was inherited from her mother, who had undergone kidney transplant due to FSGS. Patient 2 is heterozygous for the novel c.1178C > T transition inherited from her father. The putative effect of this nucleotide substitution on WT1 protein is p.Ser393Phe mutation located within the third zinc-finger domain. The patient and her father presented, respectively, isolated nephrotic proteinuria and chronic renal failure. These data highlight the importance of the inclusion of *WT1* gene mutational analysis in patients with isolated nephrotic proteinuria, especially when similar conditions are referred to the family.

1. Introduction

Wilms' tumor type 1 gene (*WT1*, OMIM *607102) encodes a zinc-finger transcription factor that plays a key role during kidney and genital development. *WT1* cellular expression is tissue-specific and also depends on the growth stage of the organism. During mammalian embryogenesis, *WT1* is predominantly expressed in kidneys, gonads, spleen and mesothelium [1]. After birth, its expression persists in the glomerular visceral epithelial cells, where WT1 protein probably contributes to functional maintenance of differentiated podocytes [2]. *WT1* gene maps on 11p13 and contains ten exons. Exons 1–6 encode the N-terminal part of the protein, which contains a proline/glutamine-rich region involved in transcriptional repression or activation [3] and a domain involved in protein homodimerization [4]; exons 7–10 encode four C_2-H_2 zinc fingers that form a DNA-binding domain at the protein C-terminal part [5].

Several WT1 isoforms result from different combinations of alternative splicings, RNA editing and alternative start sites. There are two alternative splicings of particular interest that produce four isoforms: one includes or excludes 17 amino acids encoded by exon 5; the other involves the use of two different donor splice sites in intron 9 leading to the presence or absence of three amino acids between zinc fingers 3 and 4: lysine, threonine and serine (KTS), designated as (+) KTS an (−) KTS isoforms, respectively [6]. The correct ratio of the resulting four isoforms is believed to be stable temporally and spatially and is required for normal gene function during both nephrogenesis and adult life [7].

Constitutional heterozygous mutations in *WT1* gene have been described in both Denys–Drash Syndrome (DDS) and Frasier Syndrome (FS). DDS (OMIM #194080) is caused by heterozygous missense mutations in the hot-spot located in exons 8 and 9 that code for zinc fingers 2 and 3, respectively [8]. These mutations seem to act in a dominant-negative manner, interfering with WT1 activity in cells [9]. DDS is characterized by the triad of

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ambiguous genitalia, progressive glomerulopathy with typical histologic renal finding of diffuse mesangial sclerosis (DMS) and high risk of developing Wilms' tumor (WT) [10,11]. However, incomplete forms of the syndrome have been reported, consisting of early glomerulopathy associated with either urogenital abnormalities or WT [12].

Heterozygous mutations in *WT1* intron 9 donor splice site have been described as the molecular cause of FS (OMIM #136680) [13]. They result in the decrease of the usually more abundant (+) KTS isoform and, consequently, in a reversal of the normal 2(+) KTS: 1(−) KTS ratio [14]. 46,XY patients with FS present the association of complete XY gonadal dysgenesis, nephrotic syndrome with focal segmental glomerular sclerosis (FSGS) and the development of gonadoblastoma, while female patients with a 46,XX karyotype have normal genital development and normal puberty, usually presenting isolated nephropathy [15]. According to the literature, increasing evidence indicates that DDS and FS represent two sides of the same disease, since patients sharing clinical and genetic characteristics of both disorders have been described [16].

We report here two non-related patients with similarities in their clinical history but with distinct *WT1* mutations.

2. Material and methods

2.1. Subjects

Two patients and their parents were included in the study. Patient 1, a 46,XX female, has a personal history of diabetes mellitus type 1 since she was two years old, same age when she was first diagnosed with asymptomatic nephrotic proteinuria, without edema or hypoalbuminemia. She underwent renal biopsy at 3 years of age and the histologic outcome was mesangial proliferative glomerulonephritis (MPGN). Neither steroids nor cyclosporine could be administered due to her diabetes and also because her mother had history of kidney transplant and development of dysgerminoma after cyclosporine administration. As her renal function deteriorated significantly, a second biopsy was performed when she was 8 years old showing histology of FSGS. Upon treatment with angiotensin-converting enzyme inhibitor (12.5 mg, 3 times a day) her proteinuria improved. However, this treatment had been suspended because her renal function failed and continued to fail even after withdrawal of enzyme inhibitors. Currently she is 10 years old and reached stage 5 of Chronic Kidney Disease (CKD) therefore an arteriovenous fistula for future dialysis has been already performed. Patient 2, also a 46,XX female, was diagnosed as having non-nephrotic proteinuria when she was 7 years old. Renal biopsy did not reveal FSGS, whereas this condition had been referred to one of her paternal uncles. In addition, her father was referred to have chronic kidney disease. Treatment with angiotensin-converting enzyme inhibitor (25 mg, 3 times a day) was introduced and currently, with 22 years of age, her renal function is normal, maintaining the same proteinuria dosage she presented in the first evaluation (2.5 g/day – Table 1). Neither patient 1 nor 2 had genital abnormalities or Wilms' tumor. Table 1 shows clinical data for both patients. Informed consents for the study were obtained from patients and their parents. This study had been approved by the local Ethics' Committee.

2.2. Molecular analysis of WT1 gene

Genomic DNA was isolated from blood samples using standard methods [17]. The *WT1* 8–9 hotspot fragment was amplified by PCR using specific primers and PCR conditions described elsewhere [18]. Before sequencing, PCR products were purified using the Wizard® SV Gel and PCR clean-up system (Promega, Madison, WI, USA). Further direct sequencing using ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing Kit (ABI PRISM/PE Biosystems, Foster City, CA, USA) was carried out in four reactions, using sense and antisense primers. Sequences were obtained in an automatic sequencer ABI PRISM 3130 DNA Analyzer (ABI PRISM/PE Biosystems). Free softwares such as Chromas Pro v.1.5 and CLC Sequence Viewer v.6.6.2 were used to analyze and compare sequences with the reference *WT1* sequence at Ensembl database (ENSG00000184937, http://www.ensembl.org).

2.3. Bioinformatic analysis

ClustalW2 multiple alignment was applied to evaluate WT1 conservation among species. The structure of WT1 zinc finger domain in complex with DNA obtained by NMR spectroscopy was downloaded from the Protein Data Bank (PDB: 2PJA) [19] and used as template for modeling by SWISS MODEL web-served program (http://www.swissmodel.expasy.org).

We used PyMOL® and STING Millennium Blue Star Sting (http://www.cnptia.embrapa.br/), softwares available for free download and access, to perform structural analysis of modeled images.

3. Results

Direct sequencing of *WT1* gene 8–9 hot spot fragment for patients and their parents revealed two different mutations. Patient 1 was heterozygous for the mutation c.1227 + 4C > T. This cytosine to thymine transition is located at position + 4 in intron 9 and impairs splicing at the second donor splice site. As a consequence, the WT1 (+) KTS isoform is not produced and an imbalance of the normally 2(+) KTS:1(−) KTS ratio occurs. *WT1* analysis of her parents

Table 1
Clinical features and evaluation of the patients.

Patients	Initial evaluation		Karyotype	Serum complement C3/C4	Renal biopsy	Therapy	Final evaluation	
	Clinical features	Renal function					Proteinuria	Renal function
1	Nephrotic proteinuria Onset: 3 years old SI[a]: 0.22 Proteinuria: 1.8 g/day	Normal (121 ml/min/ 1.73m^2)	46,XX	Normal	2006: MPGN[b] 2010: FSGS[c]	6 months: mycophenolate sodium Calcium channel blocker	2.3 g/day	(10 ml/min/ 1.73m^2)
2	Non nephrotic proteinuria Onset: 7 years old SI[a]: 0.24 Proteinuria: 2.54 g/day	Normal (100 ml/min/ 1.73m^2)	46,XX	Normal	MPGN[b]	Captopryl	2.5 g/day	Normal (108 ml/min/ 1.73m^2)

[a] SI: Selectivity Index (Selective $\leqslant 0.1$; non-selective $\geqslant 0.2$).
[b] MPGN: Membranoproliferative Glomerulonephritis.
[c] FSGS: Focal Segmental Glomerulus Sclerosis.

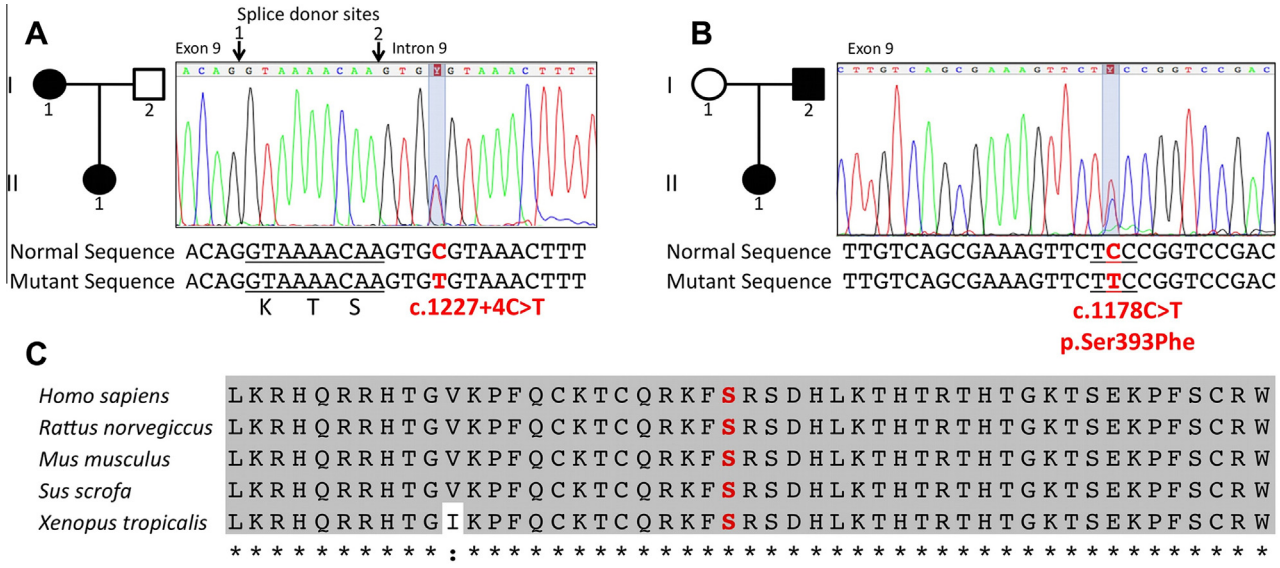

Fig. 1. Patients' pedigrees, mutation electropherograms and protein sequence alignment. (A) Patient 1 pedigree; and, partial electropherogram showing the heterozygosis for c.1227 + 4C > T transition in intron 9; codons for KTS (lysine, threonine and serine) are underlined. (B) Patient 2 pedigree; and, partial electropherogram showing the heterozygosis for c.1178C > T transition in exon 9; codon 393 is underlined in both normal and mutant sequence; c.1178C > T nucleotide change results in p.Ser393Phe mutation. (C) ClustalW2 multiple species alignment for a WT1 region surrounding the serine 393 residue denoted in red. Asterisks (*) indicate positions which have a single, fully conserved residue; Colon (:) indicates conservation between groups with strongly similar properties.

revealed the mother as being heterozygous for the same mutation whereas no *WT1* mutation was found in the father (Fig. 1A). The heterozygous c.1178C > T nucleotide transition within exon 9 was found in patient 2 (Fig. 1B). This nucleotide change results in the replacement of the serine on residue 393 by a phenylalanine (p.Ser393Phe) within the third zinc-finger domain of the protein. We also analyzed her parents and the p.Ser393Phe variant was identified in her father as well (Fig. 1B). This sequence variation has not been annotated in the *WT1* reference sequence (ENSG00000184937). In order to evaluate if the amino acid substitution is deleterious, three predictive methods were compared. PolyPhen (Polymorphism Phenotyping) that gives scores ranging from 0 (neutral) to a positive (damaging) value; SIFT (Sorting Intolerant From Tolerant) whose scores range from 0 (damaging) to 1 (neutral); and Aling GV-GD that classifies the amino acid change into classes ranging from C0 to C65, where C0 is considered tolerant and C65 deleterious [20,21]. The protein bearing the p.Ser393Phe mutation resulted in scores 1.0 and 0 for PolyPhen and SIFT, respectively, and Aling GV-GD classified it into class C65, therefore all three algorithms indicated an impairment in the WT1 function.

To verify if c.1178C > T could be a variant commonly found in Brazilian population, 200 alleles from healthy controls were analyzed and the T variant was not observed (data not shown).

3.1. Structural analysis for p.Ser393Phe mutation

ClustalW2 multiple species alignment of WT1 sequences surrounding serine 393 indicated that the affected residue is conserved among all vertebrates compared (Fig. 1C). In the human protein, Ser393 is located within a loop just before the alpha-helix in zinc-finger 3. Structural analysis showed that Ser393 normally establishes hydrophobic interactions with Phe383 (Fig. 2A and B). When Phe replaces Ser in residue 393 it establishes additional contacts with Lys381 and His397 (Fig. 2A and B). Fig. 2C shows Ser393 positioned in such a way that it suggests a direct interaction with DNA when WT1 binds to its target sequence.

4. Discussion

We report two non-related patients with *WT1* heterozygous mutations: c.1227 + 4C > T that had been described before by Barbaux et al. [13]; and, p.Ser393Phe described here for the first time. Each mutation was inherited from relatives who also carried a renal condition. Patient 1, a female child, presented isolated nephrotic proteinuria and hystologic finding of FSGS, and her mother had already undergone kidney transplant. Patient 2, a young adult female, presented isolated nephrotic proteinuria without FSGS, and her father presented with renal failure.

Missense mutations within exons 8 and 9 are usually described in patients with DDS presenting diffuse mesangial sclerosis (DMS), whereas intron 9 donor splice site mutations are commonly found in FS patients with FSGS hystologic pattern. However, overlapping clinical and molecular characteristics in each syndrome have been reported. The classical definition of FS includes patients with 46,XY karyotype and normal female external genitalia, FSGS and gonadoblastoma [22]. Nevertheless, 46,XX patients with isolated glomerulopathy may present splice site or missense mutations in *WT1* gene as well. Examples of such conditions are female patients with *WT1* mutations presenting membrano-proliferative glomerulonephritis [14], minimal-change nephrotic syndrome and Wilms' tumor [23], and isolated nephropathy [24]. Furthermore, Demmer et al. [15] described the c.1227 + 4C > T mutation in a 46,XX patient with normal female development and FSGS. Studying large cohorts, Ruf et al. [25] and Mucha et al. [26] described either intron 9 splice site or exon 8–9 missense mutations as causing SRNS in pediatric patients. Their results demonstrated that the majority of *WT1* mutations have been identified in genetically female patients. Our results are in agreement with those described above since we identified different *WT1* gene mutations in two non-related 46,XX patients, both with isolated nephrotic proteinuria and absence of genital abnormalities.

Because the great majority of mutations described so far in *WT1* gene are *de novo* mutations, an interesting feature of our findings is that both index cases inherited *WT1* mutations from one of their parents. There are only few reports on familial *WT1* mutations. Although they present a dominant inheritance, there are reports

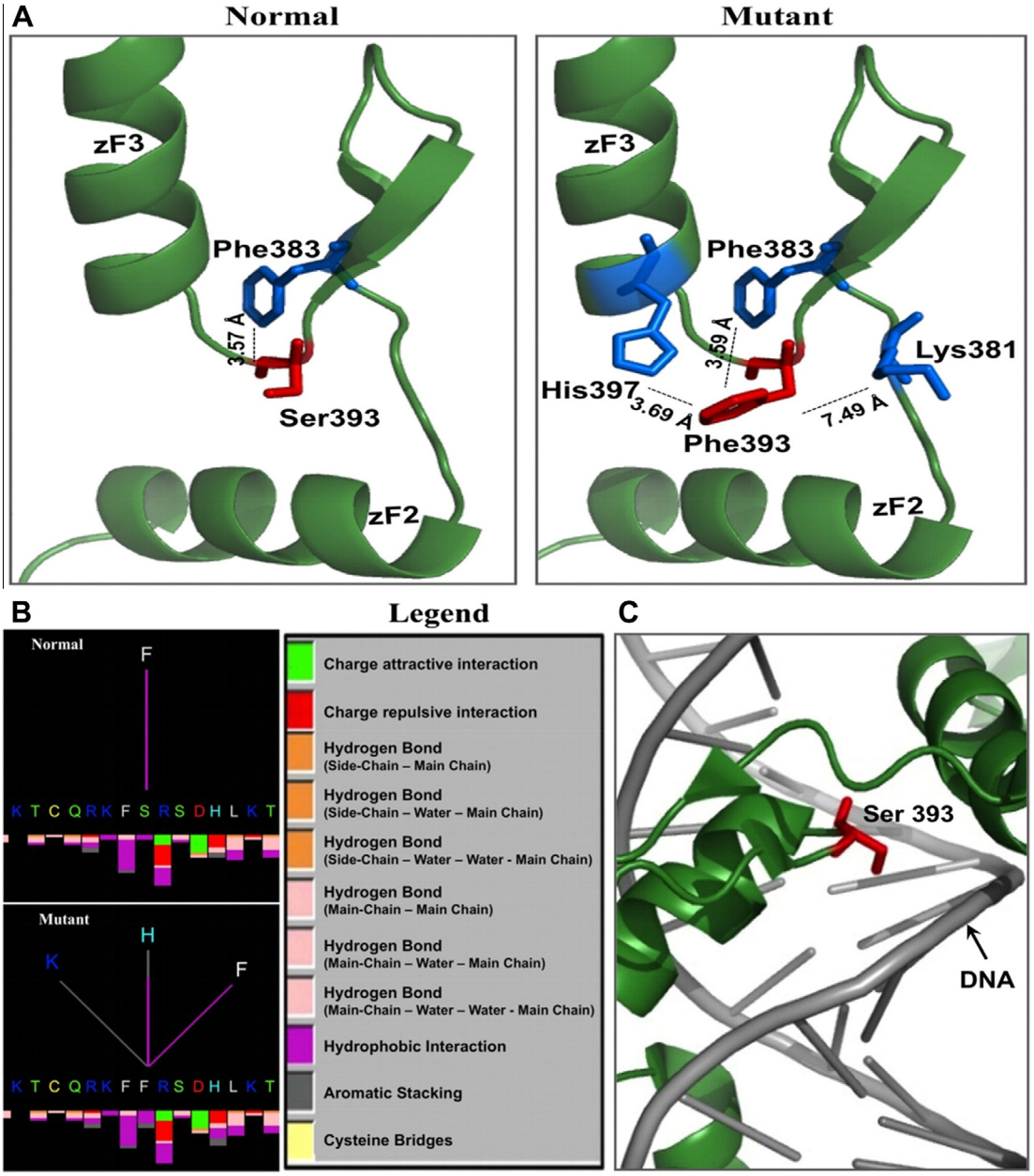

Fig. 2. *In silico* analysis wild type and mutant WT1. (A) Modeled structures of normal and mutant WT1 zinc finger 2 and 3 region generated by the PyMOL® program. Dashed lines and distances in Å are shown for internal contacts in normal Ser393 and mutant Phe393 residues. (B) Analysis of internal contacts generated by STING Millennium Graphical Contacts program (http://www.cnptia.embrapa.br/): Normal Ser393 residue is in contact with Phe383 to which it maintains hydrophobic interactions; mutant Phe 393 is in contact with Lys381 and His397, besides hydrophobic interactions with Phe383; a color legend for different kind of internal contacts is provided. (C) The structure of WT1 complex with DNA obtained by RMN spectroscopy (PDB:2PJA) was downloaded from the Protein Data Bank; after edition with PyMOL® program, Ser393 is shown in contact with DNA molecule.

on siblings with WT who inherited heterozygous truncating *WT1* mutations from phenotypically unaffected parents [27,28]. Variable expressivities of the same mutation are equally odd but had been described fifteen years ago by Denamur et al. [29] in a family where the mother, who had typical FSGS with normal internal and external genitalia, transmitted a *WT1* splice site mutation to her daughter, who had nephrotic syndrome with early-onset diffuse mesangial sclerosis and a XY gonadal disorder of sex development. Six years later, Zirn et al. [30] presented a *WT1* point mutation in the stop codon located in exon 10 in three members of one family: the index case was a female who had WT and ureter duplex diagnosed at the age of 9 and subsequently developed slow progressive nephropathy, her mother had late-onset nephropathy that led to end-stage renal failure, and her brother had normal renal function. Regev et al. [31] reported the vertical transmission of a nonsense mutation in exon 1, from a mother with WT in infancy to her son with genitourinary anomalies, gonadal dysgenesis and gonadoblastoma foci. Fencl et al. [32] described a family in which the normal father transmitted p.Arg343Pro in exon 9 to his monozygotic twins who died because of complications of congenital nephrotic syndrome. Finally, there is a report on a mother with end-stage of renal disease who transmitted an amino acid substitution in exon 9 to her two daughters, one had nephrotic syndrome and the other was healthy [26]. Differently, Benetti et al. [33] described a missense mutation in exon 9 in three generation of a family in which all affected members presented isolated

proteinuria due to FSGS. Phenotype variability due to *WT1* mutations was not observed among the affected members within each family we describe.

The novel p.Ser393Phe mutation due to c.1178C > T variation is located in the third zinc finger of the WT1 protein. The T variant was not detected in alleles of 100 healthy controls (n = 200 alleles) discarding the possibility of being a frequent polymorphism in the Brazilian population. Mutant WT1 functional aspects have been analyzed *in silico*. WT1 protein belongs to C_2H_2 zinc finger family where two cysteines and two histidines coordinate a zinc ion. The following arrangement describes this kind of zinc finger: #-X-C-X[1–5]-C-X3-#-X5-#-X2-H-X[3–6]-(H/C), where X can be any amino acid, and numbers in brackets indicate the number of residues. The positions marked as # are those that are important for the stable fold of the zinc finger. The final position can be either His or Cys. The C_2H_2 zinc finger is composed of two short beta strands followed by an alpha helix, and binds to the major groove of the DNA double helix (accession number PF00096). The sequence of the third zinc-finger in WT1 is as follows: FQ**C**KT**C**QRK**F**_**S**_RSDHLKT**H**TRT**H**. Ser393 (in bold) is next to Phe392 (underlined), an important residue for the zinc finger stable folding, and interacts with Phe383 (first residue on zinc-finger sequence). The aromatic side chain in phenylalanine is generally involved in stacking interactions with other aromatic side chains [34]. The Ser to Phe substitution in residue 393 creates novel interactions with Lys381 and His397 residues indicating a structural change in zinc-finger 3 domain.

As a transcriptional factor, WT1 undergoes post-transcriptional modifications, such as phosphorylation, by which its activity is regulated. Several potential phosphorylation sites for PKA and PKC were identified in the human WT1 C-terminal domain [35]. Similarly to the crystal structure of the Early Growth Response 1 (EGR1) protein reported by Pavletich and Pabo [36], Ser365 and Ser393 residues in WT1 protein located, respectively, within zinc finger 2 and 3, are adjacent to arginine residues involved in contacts with the guanine in DNA-binding sites. Additionally, Sakamoto et al. [37] demonstrated that WT1 DNA-binding activity is modulated *in vivo* by PKA phosphorylation of Ser-365 and Ser-393, which inhibits DNA binding and, consequently, decreases WT1 transcriptional repression activity. They also showed that phosphorylation at both sites was required for complete neutralization of repression activity.

A possible molecular explanation by which phosphorylation of Ser393 inhibits DNA binding comes from the structure of WT1 zinc fingers bound to DNA. It shows that a steric clash and electrostatic repulsions may be created between the phosphorylated serine and the DNA backbone and thereby impair binding [19]. As Phe has substituted Ser in residue 393, phosphorylation in residue 393 will not occur on the WT1 isoform produced. The result might be an increased binding of WT1 to its podocyte gene targets. In addition, the p.Ser393Phe substitution creates novel intra-molecular interactions as demonstrated in Fig. 2, leading to a different regional conformation. Those data indicate possible mechanisms by which this mutation could cause a functional effect upon WT1 leading to the phenotype of patient 2 and her father. However, further functional assays will be necessary to confirm the real effect of p.Ser393Phe variant.

As previously discussed by Benetti et al. [33], in addition to its traditional role in regulation of cell 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. Normal podocyte function is maintained by a complex and dynamic gene network in which *WT1* seems to play a crucial role, so that mutations of its sequence may result in a broad range of phenotypic alterations, not only in the context of FS and DDS. These observations confirm the importance of molecular analysis of *WT1* gene in patients with isolated nephrotic proteinuria associated or not with FSGS, especially those with familial history regardless the age or the presence of typical DDS or FS clinical features. Therefore, data discussed in the present paper strongly support the need of *WT1* mutational analysis in young girls with glomerulopathy in order to diagnose properly the condition and to avoid potentially treatment with harmful drugs.

Disclosure

No potential conflict of interest relevant to this article was reported.

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