WT1 and PAX-2 Podocyte Expression in Denys-Drash Syndrome and Isolated Diffuse Mesangial Sclerosis

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Denys-Drash syndrome is a rare disorder of urogenital development characterized by the association of early onset glomerulopathy caused by diffuse mesangial sclerosis, gonadal dysgenesis leading to pseudohermaphroditism in males, and a high risk of developing Wilms' tumor. The syndrome is caused by dominant negative point mutations in the *WT1* gene that encodes a tumor suppressor transcription factor normally expressed in podocytes. Mutations usually affect the zinc fingers of the *WT1* protein. The basic defect is unknown in most cases of isolated diffuse mesangial sclerosis, a disease characterized by the same glomerular changes as in Denys-Drash syndrome but possibly transmitted as an autosomal recessive trait. Here we show that the distribution of *WT1* is abnormal in most patients with Denys-Drash syndrome: *WT1* nuclear staining of podocytes is decreased or absent. This finding is consistent with the decreased DNA binding capacity of the mutated protein. One target gene of *WT1* is *PAX2*, the expression of which is down-regulated in podocytes during early stages of nephrogenesis. We demonstrate that *WT1* mislocalization is associated with abnormal podocyte expression of *PAX2* protein and RNA. We suggest that persistent expression of *PAX2* is likely to result from the loss of *WT1* dependent transcriptional repression and may participate in the pathological mechanisms leading to glomerular dysfunction. Abnormal distribution of *WT1* and *PAX2* was also observed in isolated diffuse mesangial sclerosis suggesting that a defect in *WT1* could also be operative in isolated diffuse mesangial sclerosis. Primary involvement of *PAX2* is an alternative hypothesis because persistent expression of *PAX2* in transgenic mice is associated with the occurrence of early and severe glomerulopathy. (*Am J Pathol* 1999, 154:181–192)

Denys-Drash syndrome (DDS) is a rare disorder of urogenital development characterized by the association of early onset nephrotic syndrome, male pseudohermaphroditism, and nephroblastoma (Wilms' tumor).^{1,2} Incomplete forms of the syndrome have been described consisting of glomerulopathy, a constant feature, associated with either genital abnormality or Wilms' tumor.³ In all cases, the disease progresses to end-stage renal disease usually before the age of 5 years, and the characteristic glomerular lesion is diffuse mesangial sclerosis.³ Constitutional heterozygous intragenic mutations of *WT1* have been demonstrated in nearly all patients with the complete or incomplete form of the disease.^{4–7} Isolated diffuse mesangial sclerosis (IDMS) is characterized by the same renal histological changes as in DDS but is observed in the absence of the other anomalies of the DDS triad.⁸ Patients with this disease also have early onset nephrotic syndrome progressing rapidly to end-stage renal disease with a possible autosomal recessive inheritance. The basic defect is still unknown in most cases of IDMS, but we have recently shown that *WT1* is involved in some patients with the disease.⁷

The *WT1* gene comprises 10 exons and encodes a zinc finger (ZF) protein. Exons 1 to 6 encode a proline/glutamine rich region, and exons 7 to 10 encode the four ZF of the DNA-binding domain.^{9,10} Different functional domains involved in either repression or activation of transcription,^{11,12} a region involved in homodimerisation of the protein,¹³ and an RNA binding domain¹⁴ have been characterized. Two alternative splicing regions, one corresponding to the 17 amino acids encoded by exon 5 and the other corresponding to amino acids KTS encoded by the 3' end of exon 9, lead to the synthesis of four isoforms with definite and stable proportions¹⁵ and distinct subnuclear localization revealing different functions. *WT1* plays a role in posttranscriptional processing of RNA as well as in transcription.^{14,16,17}

Formation of the metanephros, the third and permanent renal organ in mammals, starts on the 5th week of

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gestation in human. It results from interactive events between the ureteric bud, an epithelial tube off the Wolffian duct, and the metanephric blastema, a loosely organized mesenchyme located at the caudal extremity of the nephrogenic cord.^{18,19} The mesenchymal blastema induces the growth and regular dichotomous branching of the ureteric bud leading to the formation of the collecting system. In contact with the ampullar tip of ureteral bud branches, mesenchymal cells condensate and transform into epithelial cells. Condensates undergo a series of transformation and elongation, resulting in the formation of mature nephrons. Vascularization seems to be provided by a double process of angiogenesis and vasculogenesis. Successive generations of nephrons are formed from the 8th week to the 36th week of gestation. Concomitant with the nephrogenesis, there are profound and definite changes in the expression of cellular and extra-cellular matrix proteins, growth factors and their receptors, proto-oncogenes, and transcription factors, with a tight spatial and temporal regulation.

WT1 is one of the transcription factors involved in nephrogenesis.^{20–22} In the fetal kidney, it is expressed in the metanephric blastema, condensing mesenchyme, renal vesicle, and developing and mature podocytes. The other main sites are the genital ridges, gonads and mesothelium. Knock-out of the gene in the mouse resulted in the absence of both kidneys and gonads.²² The expression of WT1 in the podocyte persists into adult life. In DDS, most *WT1* mutations are *de novo* missense changes in exons 8 or 9 affecting zinc fingers 2 or 3 with a hotspot (R394W) in exon 9.^{4–7} However, rare DDS patients carry a *WT1* mutation leading to putative truncated proteins lacking part of or the entire ZF domain.^{5–7} Changes in the zinc-finger structure are thought to behave in a dominant negative fashion in patients with DDS.²³ *In vitro*, they abolish the binding of the WT1 ZF domain to its normal DNA targets.^{4,24} The gene *PAX2*, which encodes a transcription factor expressed in early kidney development, is one of the WT1 targets.^{25,26} It is down-regulated in precursor cells of the visceral glomerular epithelium, simultaneously with a marked increase in WT1 expression.²⁷ Repression of *PAX2* by WT1 has been demonstrated *in vitro* by the binding of WT1 to *PAX2* regulatory sequences and by cotransfection assays using CAT reporter constructs under the control of *PAX2* regulatory sequences showing WT1-dependent transcriptional repression.²⁷ Transactivation of the WT1 promotor by *PAX2* has also been described.²⁸ *PAX2* plays an important role in kidney development. Up-regulation of its expression in transgenic mice generates severe kidney abnormalities,²⁹ whereas *Pax2* null mutant mice lack kidneys, ureters, and genital tracts.³⁰

The aim of our study was to analyze the glomerular expression of *WT1* and *PAX2* in patients presenting with DDS. Normal fetal and postnatal kidneys, and kidneys from patients presenting various types of nephropathies were used as controls. We also analyzed the expression of these two transcription factors in patients affected with IDMS. In the present study, we show that podocyte distribution of WT1 was abnormal in the majority of patients affected with DDS, a finding consistent with the de-

creased DNA-binding capacity of the abnormal WT1 protein. Persistent expression of *PAX2* was observed in the podocyte, a finding likely to result from the loss of WT1 dependent transcriptional repression. Interestingly, the same anomalies were also found in most patients with IDMS suggesting that a defect in *WT1* could also be operative in at least some IDMS, a hypothesis recently confirmed in some patients by our molecular studies.⁷ Primary involvement of *PAX2* is an alternative hypothesis.

Patients and Methods

Patients and Kidney Tissue Specimens

Renal tissue from 10 patients with DDS and 13 patients with IDMS was obtained at biopsy performed for diagnostic purposes (15 patients) at nephrectomy because of Wilms' tumor or before renal transplantation (6 and 9 patients, respectively). Clinical and molecular data concerning these patients are presented in Table 1. Clinical data from 14 of these patients have been previously reported^{3,8} as well as results of molecular studies of DDS patients (patients (pts) 3, 4, and 5),⁴ DDS patients (pts 1, 2, and 6), and IDMS patients (pts 22, 23, and 24).⁷ Clinical and molecular findings in three patients with Wilms' tumor and abnormal genitalia but with no glomerular symptoms are also presented in Table 1.⁷ Analysis of the expression of *PAX2* and *WT1* was performed on normal renal tissue adjacent to the tumor. In this retrospective study, molecular analysis of the *WT1* gene was done only in patients observed in the most recent period.

Controls consisted of normal kidneys from four fetuses of 10 to 28 gestational weeks obtained at autopsy after spontaneous abortion or termination of pregnancy for medical reasons and from four children or adults (2 to 42 years old) (two nontransplanted kidneys and two normal renal biopsies in hematuric patients secondarily found to have lithiasis). In addition, renal biopsies or nephrectomies from 20 children 3 to 16 years old affected with various types of nephropathies including idiopathic nephrotic syndrome with minimal glomerular changes or focal and segmental sclerosis were also used as controls.

Antibodies

WT(C-19)(Santa Cruz Biotechnology, Santa Cruz, CA) is an affinity-purified rabbit polyclonal antibody raised against a peptide corresponding to amino acid residues 327–345 mapping at the carboxy terminus of the human Wilms' tumor protein. The first batch of antibody (6063) gave abnormal staining of vascular smooth muscle cell nuclei in addition to the expected normal pattern, a reaction previously observed by Charles et al³¹ with similar antibody. Faint but diffuse cytoplasmic staining of tubular cell cytoplasm was also observed. For these reasons, two other batches (D124 and H 277) were tested. Both gave the same specific labeling on normal kidneys without any staining of smooth muscle cells; tubular cell cytoplasm was strictly negative. Staining was stronger with H277.

Table 1. Mutations, Clinical Symptoms, and Family History of the 27 Patients

Patient	WT1 mutation	Exon	Protein alteration	Nephropathy		Karyo-type	External genitalia	WT year	Family history
				1st symptom years	ESRF years				
DDS									
1	1096 C-T	8	R366C	birth	0.1	46XY	F	No	No
2	1180 C-T	9	R394W	3.2	4.5	46XY	Ambiguous	No	No
3	1180 C-T	9	R394W	2.3	2.4	46XX	F	2.3	No
4	1180 C-T	9	R394W		3	46XY	Ambiguous	2.3	No
5	1180 C-T	9	R394W	0.6	1.2	46XX	F	Bil 0.9	No
6	1192 delC	9	Stop at 398	0.10	1	46XY	Ambiguous	No	No
7	nd			0.2	0.8	46XY	Ambiguous	0.7	No
8	nd			0.8	0.9	46XY	Ambiguous	0.8	No
9	No (exons 8, 9)			0.6	11.1	46XY	Ambiguous	No	No
10	nd			1	1	46XY	Ambiguous	Bil 0.11	No
IDMS									
11	nd			0.6	2.2	46XX	F	No	Yes (one sister)
12	nd			1.6	2.6	46XY	M	No	Parental consanguinity
13	nd			0.6		ND	F	No	No
14	No (exons 1 to 10)			2.3	2.3	46XX	F	No	No
15	nd			6.8	6.10	ND	M	No	No
16	nd			0.3	2	ND	M	No	Yes (one brother and one sister)
17	nd			0.9	2	ND	F	No	No
18	nd			1	2	ND	F	No	No
19	nd			2.3	5	ND	F	No	Yes (one brother)
20	nd			Birth	0.3	ND	F	No	No
21	nd			2.7	3.5	ND	M	No	No
22	1129 C-T	8	H377Y	0.6	3.10	46XX	F	No	No
23	1186 G-A	9	D396N	1.1	2.2	46XX	F	No	No
24	1147 T-C	9	F383L	0.8	3	ND	M	No	Yes (one brother)
Wilms' tumor									
25	712 G-A	4	W238X	No		46XY	Cryptorchidism	0.6	No
26	667 G-A	3	S223N	No		46XX	F	1.4	Yes (father)
27	901 T-G	7	R301X	No		46XY	Ambiguous	Bil	No

DDS, Denys-Drash syndrome; IDMS, isolated diffuse mesangial sclerosis; nd, not determined; F, female; M, male; Bil, bilateral; WT, Wilms' tumor.

Consequently, only results obtained with this last antibody are presented in this paper. The PAX2 antibodies were generated against a bacterial fusion protein containing amino acids 188–385 of the PAX2 coding region.²⁶ This region does not include the conserved paired domain. Studies in transfected cells demonstrate minimal cross-reactivity with the related proteins, PAX5 and PAX8.³² Within the PAX2 sequence used for the antigen, the mouse and human genes are 97.5% identical. These antibodies recognize PAX2 proteins from a diverse array of species, including mouse, human, zebrafish, and chick. PC10 (Pharmingen, San Diego, CA) is a purified monoclonal antibody raised against the proliferating cell nuclear antigen (PCNA). Goat anti-rabbit immunoglobulins were purchased from Biosource International (Camarillo, CA). Affinity-purified fluorescein isothiocyanate-conjugated swine anti-goat IgG(H+L) was purchased from Caltag Laboratories (Burlingame, CA). Biotinylated horse anti-mouse immunoglobulins, avidin-biotin blocking kit, VECTASTAIN Elite ABC kit, diaminobenzidine, and substrates were from Vector Laboratory (Burlingame, CA).

Methods

Both immunofluorescence and immunoperoxidase methods were used on Dubosq-Brazil or formalin-fixed, par-

affin-embedded tissues from 10 DDS patients (four biopsy and six nephrectomy specimens), 14 IDMS patients (11 biopsy and three nephrectomy specimens), three patients with Wilms' tumor, and 28 controls. In four patients (pts 6, 8, 14, and 21), a second specimen obtained later on in the course of the disease was also studied. In six patients (pts 2, 3, 5, 14, 15, and 21), immunolabeling was performed in parallel on frozen tissues.

Immunofluorescence

Indirect immunofluorescence was performed as follows: 3- μ m thick sections were mounted on 3-aminopropyl triethoxysilane pretreated slides. Deparaffinized sections were treated with microwave heating (2 $\times$ 5 minutes in 0.8 mol/L urea, pH 6.4). Ten percent normal swine serum in PBST (0.01 mol/L phosphate-buffered saline (PBS) containing 0.05% Tween-20) was used as blocking solution. WT1 and PAX2 antibodies were diluted to 1:100 and 1:500, respectively. After incubation with primary antibodies for 1 hour at room temperature, sections were rinsed three times in PBS and incubated for 30 minutes with goat anti-rabbit immunoglobulins diluted to 1:40 in PBS. After washing in PBS, they were incubated for 40 minutes with fluorescein isothiocyanate-conjugated swine anti-goat IgG(H+L) diluted to 1:40 in PBS. Then slides were washed in PBS. A mounting media containing

p-phenylene-diamine was used to delay fluorescence quenching. Labeling was examined with a Leitz Orthoplan microscope equipped for light, fluorescence, and phase contrast microscopy. Phase contrast microscopy allows the visualization of cell structure and the precise identification of cell nuclei.

Immunoperoxidase

Frozen Tissue

Renal samples were snap-frozen in liquid nitrogen using OCT compound (Miles Laboratories Inc, Naperville, IL). Four- μ m thick cryostat sections were air dried and fixed in acetone for 10 minutes. Immunoperoxidase staining was carried out using the Vectastain Elite ABC kit (Vector). After washing in fresh buffer (0.01 mol/L PBS, pH 7.4), endogenous biotin was blocked by the biotin blocking agent according to the instructions of the manufacturer. Then sections were incubated for 1 hour at room temperature in a moist chamber with the appropriate dilution of primary antibodies in PBST:WT1, 1/1000 and PAX2, 1/500. They were washed in PBS and incubated with biotinylated secondary antibody for 30 minutes. For quenching the endogenous peroxidase, sections were treated with 3% hydrogen peroxide in methanol for 5 minutes and then washed in PBS for 20 minutes. They were then incubated for 30 minutes with Vectastain Elite ABC reagent. After washing, final staining of the sections by diaminobenzidine or very intense purple, chromogenes was monitored under the microscope. Sections incubated with anti-PCNA antibodies were counterstained with hematoxylin for 5 minutes. No counterstaining was performed on other sections.

Fixed Tissue

Three- μ m thick deparaffinized sections were pretreated by microwave heating in a urea solution (2×5 minutes in 0.8 mol/L urea, pH 6.4). WT1, PAX2, and PCNA antibodies were used at a dilution of 1:1000, 1:800, and 1:1000, respectively. Staining procedures were as previously described.

Controls were performed in the same conditions, but the primary antibody was omitted. No labeling was observed in control sections.

In Situ Hybridization

The murine *Pax2* probe is a *Bam*HI-*Eco*RI fragment that corresponds to the 3' half of the *PAX2* coding sequence and does not contain the conserved paired domain. On Northern blots, this fragment recognizes two *PAX2* mRNAs of 4.2 and 4.5 kb in size but does not detect the 3.0-kb *PAX8* mRNA.²⁵ By *in situ* hybridization, the *PAX2* probe does not detect *PAX8* expression in the thyroid nor *PAX5* expression in the spleen. *PAX2* probe was subcloned into Bluescript vector (Stratagene, LaJolla, CA). The template was linearized with restriction endonuclease *Eco*RI (sense probe) or *Bam*HI (antisense probe). Sense and antisense RNA probes were transcribed from

the linearized templates with T7 (sense) and T3 (antisense) RNA polymerases (Boehringer Mannheim, Mannheim, Germany) using digoxigenin UTP (Boehringer Mannheim) according to the manufacturer's instructions. They were DNaseI-treated, purified, and stored at -80° .

Six- μ m thick deparaffinized sections were pretreated by microwave heating for 5, 1, and 5 minutes in a urea solution (0.8 mol/L urea, pH 6.4) and then washed in PBST (PBS containing 5% bovine serum albumin and 0.05% Tween 20) for 30 minutes. Prehybridization was carried out for 1 hour at 48°C in 100 μ l of hybridization solution containing 50% deionized formamide, $5\times$ SSC, $5\times$ Denhardt's solution, 250 μ g/ml of fish sperm DNA, 250 μ g/ml yeast tRNA, 4 mmol/L EDTA, and 10% dextran sulfate. Hybridization was performed at 48°C for 16 hours in 50 μ l of the hybridization buffer (50% deionized formamide, $5\times$ SSC, $5\times$ Denhardt's solution, 250 μ g/ml yeast tRNA, 4 mmol/L EDTA) containing 10 ng/ml of the denatured digoxigenin-labeled *Pax2* probe (antisense or sense). After hybridization, the slides were washed twice with $2\times$ SSC at 42°C , followed by $0.2\times$ SSC and $0.1\times$ SSC at room temperature. After blocking with 0.5% blocking reagent in the buffer (0.1 mol/L Tris-HCl, 150 mmol/L NaCl, pH 7.9) for 30 minutes at room temperature, sections were incubated overnight at 4°C with alkaline phosphatase-conjugated sheep anti-digoxigenin IgG (Boehringer Mannheim) 1:800 in the blocking solution. The color was then developed with the chromogenic agents nitro blue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mannheim), and 2 mmol/L levamisole in the dark at room temperature. After the color was fully developed, all of the sections were washed at the same time with the buffer (100 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8) to allow comparison of the hybridization signal and then was mounted in an aqueous medium.

Results

Morphological Study

Normal Human Fetal and Postnatal Kidneys

In the kidneys (10, 19, 24, and 28 gestational weeks) from the 4 fetuses, undifferentiated mesenchyme was seen in the outer cortex. Progression of nephron differentiation from condensation of mesenchymal cells around the ampullar tip of the ureteral branches (future collecting ducts) to vesicle, comma-shaped body, S-shaped body, and later stages of differentiation was observed from the outer to the deep cortex.

Denys-Drash Syndrome (10 Patients)

Diagnosis of diffuse mesangial sclerosis had been based on evaluation of renal tissue obtained by biopsy (5 pts) or nephrectomy performed because of Wilms' tumor (5 pts), in patients under 1 year of age (7 pts) or between 2 and 5 years (3 pts). Various degrees of severity of glomerular lesions were observed. In the early stages, the glomerular lesions were characterized by a fibrillar increase in mesangial matrix with no mesangial cell pro-

liferation and the capillary walls were lined by hypertrophied podocytes. In the fully developed lesion, there was a combination of thickening of the glomerular basement membranes and massive enlargement of mesangial areas leading to reduction in the patency of the capillary lumens (Figure 1a). The expanded mesangial matrix had a spongy appearance with mesangial cells embedded in a delicate, fine periodic acid-Schiff and silver positive network. In advanced stages, the contracted solidified glomeruli were still surrounded by a layer of hypertrophied and vacuolized podocytes. The deepest glomeruli were usually the least severely affected. Tubulointerstitial lesions were associated and included focal tubular dilations involving primarily proximal tubules. Dilatations were prominent in patients with fully developed or advanced glomerular lesions.

Isolated Diffuse Mesangial Sclerosis (14 Patients)

Diagnosis of diffuse mesangial sclerosis had been made on renal biopsy specimens obtained between the ages of 1 month and 5 years in 13 patients presenting with nephrotic syndrome and on nephrectomy specimen obtained at the time of transplantation in one. Glomerular and tubular lesions were similar to those described in DDS (Figure 1b).

Wilms' Tumor and Abnormal Male Genital Development (3 Patients with WT1 Mutations)

Kidney tissue excluding the Wilms' tumor was normal. No glomerular changes were detected.

WT1 and PAX2 Expression and Immunohistology

Similar results were obtained whatever the technique used (immunofluorescence or immunohistochemistry, frozen or paraffin-embedded tissues). No modification in immunolabeling was observed on renal specimens from the same patient obtained at different times in the course of the disease.

Normal Human Fetal and Postnatal Kidneys

In normal fetal kidneys (10, 19, 24 and 28 gestational weeks), WT1 was faintly expressed in nuclei of mesenchymal blastema. The nuclear expression increased in condensates, renal vesicles, and prepodocytes of comma-shaped bodies, and it persisted in podocytes of mature glomeruli. No cytoplasmic labeling was observed in these structures. Derivatives of the ureteric bud were WT1 negative. (Figure 1c). PAX2 was absent in the undifferentiated blastema. In contrast, strong nuclear expression was observed as soon as the cells condensed in contact with the ampullae. The expression persisted in the comma-shaped body and disappeared in the visceral epithelium of the future glomeruli of the S-shaped body (Figure 1d). Progressive effacement of PAX2 labeling in the prox-

imal and then the distal tubule was observed in the following stage of nephron differentiation, whereas parietal epithelial cells of the mature glomeruli remained positively labeled with PAX2 antibodies. PAX2 was expressed in the ureteral bud ramifications; the expression was maximal in the ampullar cells.

In postnatal kidneys (2 to 42 years), WT1 antibody uniformly stained the nuclei of the podocytes and the parietal epithelium lining the Bowman's capsule. The cytoplasm was consistently negative (Figure 1, e and g). No tubular structure was labeled. PAX2 was strongly expressed in the parietal epithelium of Bowman's capsules and in the collecting tubules (Figure 1, f and i) with a nuclear localization. Nuclei from distal tubules were also focally labeled. No PAX2 staining was detected in podocytes, vessels, or interstitial cells.

Denys-Drash Syndrome (10 Patients)

Marked decrease in podocyte nuclear staining with anti-WT1 antibodies was observed in eight cases with focal disappearance of the labeling in two of them (Figure 2, a and b). Compared with staining in controls, irregular podocyte staining was a striking feature with the presence within the same glomerulus of positive, negative, and faintly stained podocyte nuclei. Three patients with abnormal distribution of WT1 also showed abnormal nuclear expression of PAX2 in the podocytes (Figure 2c). In the others, no change in the expression of PAX2 was detected. In two patients, the expression of WT1 and PAX2 was normal. Normal expression of PAX2 was observed in Bowman's capsule, distal, and collecting duct cells. No neoexpression of PAX2 was detected in dilated proximal tubules (Figure 2k).

Isolated Diffuse Mesangial Sclerosis (14 Patients)

In 11 patients, abnormal distribution of the WT1 protein was observed, consisting of decrease or absence of nuclear labeling of the podocytes (Figure 2, d and e). In seven of these patients, nuclear labeling of the podocytes with anti-PAX2 antibodies was observed (Figure 2f). In two patients, the glomerular distribution of WT1 and PAX2 was normal. Findings in other renal cells were the same as in DDS patients.

Wilms' Tumor and Abnormal Male Genital Development (3 Patients with WT1 Mutations)

Focal decrease or disappearance of WT1 nuclear staining was observed in the adjacent kidney of the tumor of one patient (Figure 2, g and h). In the two other patients, the expression of WT1 and PAX2 was normal in nontumoral tissue. No glomerular symptoms were detected.

Controls (22 Patients)

Renal biopsy or nephrectomy specimens from patients presenting various types of nephropathies were studied.

No change in WT1 or PAX2 expression was observed in any of these cases.

PAX2 Expression and in Situ Hybridization

Normal Human Fetal and Postnatal Kidneys

In fetal kidney, no PAX-2 expression was seen in un-induced blastema cells. PAX-2 transcripts were detected in the condensing mesenchyme around the ampullar extremities of the developing collecting ducts and in early epithelial structures (Figure 3, a and c). Expression decreased at later developmental stages and was absent from future podocytes. Pax-2 transcripts were also seen in collecting duct cells.

In postnatal kidneys, persistent PAX-2 expression was found in the epithelial cells of Bowman's capsules and of distal and collecting tubules. No labeling was detected in podocytes, vascular, or interstitial cells.

Diffuse Mesangial Sclerosis

In two patients with positive labeling of podocyte nuclei with anti-PAX-2 antibodies, abnormal expression of PAX2 mRNA was seen in the cytoplasm of podocytes (Figure 3d). PAX-2 expression was normal in epithelial cells of Bowman's capsules, distal, and collecting tubules.

Controls

Normal expression of PAX-2 was observed in control kidneys. PAX-2 transcripts were not detected in the podocytes.

Correlations between Mutations of WT1 and Podocyte Expression of WT1 and PAX2

WT1 mutations were identified in six DDS and three IDMS patients. In five DDS and one IDMS patients, localization of WT1 was abnormal and was associated with podocyte expression of PAX2 in four patients (Table 2). In contrast, three patients with WT1 missense mutations lying in exon 9 had normal WT1 and PAX2 expression. Two of these mutations are the most frequently found in DDS and affect amino acids involved in DNA binding, arginine 394 in one patient and aspartic acid 396 in the other.

PCNA Immunolabeling

Normal Postnatal, DDS, and IDMS Kidneys

In normal mature kidneys, PCNA positive cells were not found in glomeruli, whereas very occasional tubular or interstitial cells were stained by anti-PCNA antibodies. In all patients with DDS or IDMS, marked increase in tubular (from dilated or normal tubules) and interstitial cell labeling was observed (Figure 2j). In contrast, no podocyte labeling was detected in one third of patients. In the others, PCNA-positive podocytes were focally present: they were rare and observed in 6 to 12% of glomeruli (Figure 2i) in most cases, but in two patients, one with normal, the second with abnormal PAX2 expression, they were present in 20 and 50% of glomeruli, respectively.

Discussion

Podocyte hypertrophy and vacuolization are constant features of DMS, the distinctive lesion of DDS.^{3,8} The syndrome is caused by constitutional heterozygous mutations of WT1, a tumor suppressor gene encoding a Cys2-His2 zinc-finger protein.^{9,10,34} Normally, WT1 is expressed by the podocytes from the early stages of nephrogenesis to adult life and, as expected for a transcription factor, has a nuclear localization.^{20,31,35-37} In this study, using an antibody raised against the carboxy terminus of the molecule, we documented for the first time the glomerular distribution of WT1 in 10 patients affected with DDS, six of whom having proved WT1 mutation. We compared our findings with those observed in normal fetal and postnatal kidneys and in kidneys from patients presenting various types of nephropathies.

In normal fetal kidneys, we clearly showed expression of WT1 in nuclei of uninduced blastema, confirming the initial findings of Grubb et al.³⁶ The expression eventually increased in condensing blastema, renal vesicle, comma, and S-shaped bodies and peaked in podocytes, whereas it was down-regulated in tubular structures. In normal mature kidneys, a strong, regular labeling of podocyte nuclei was observed without cytoplasmic staining.

In 8 of the 10 DDS patients studied, the distribution of WT1 protein was abnormal. Podocytes were irregularly stained with disappearance or focal decrease of the nuclear signal. These changes were not observed in the

Figure 1. **a:** Light microscopy. Periodic acid-Schiff. DDS patient: typical diffuse mesangial sclerosis showing the contracted glomerular tuft surrounded by a crown of enlarged podocytes. **b:** Light microscopy. Periodic acid-Schiff. IDMS patient: two stages of diffuse mesangial sclerosis are seen with moderate (right glomerulus) to massive increase in mesangial matrix leading to complete sclerosis of the glomerular tuft (left glomerulus). In both glomeruli, podocytes are hypertrophied and vacuolized. **c and d:** Normal fetal kidney. Immunoperoxidase with anti-WT1 (**c**) and anti-PAX2 (**d**) antibodies. WT1 is expressed in nuclei of mesenchymal blastema. The nuclear expression is more intense in condensates, renal vesicles, prepodocytes of S-shaped body, and peaked in mature podocytes. In contrast, PAX2 is absent from the uninduced blastema. Strong nuclear expression is seen in condensates and its derivatives. The expression decreased in the proximal part of the S-shaped body and is absent from the prepodocytes and podocytes. PAX2 is also expressed in the nuclei of collecting ducts and the expression is maximal in ampullae. **(e and f)** Normal kidney. 7-year-old child. Immunofluorescence. **e:** Anti-WT1 antibodies. Strong nuclear labeling of podocytes and Bowman's capsule epithelial cells. Absence of cytoplasmic staining. **f:** Anti-PAX2 antibodies. Nuclear staining of Bowman's capsule epithelial cells and of distal and collecting duct cells. Absence of podocyte staining. **g and i:** Normal mature kidney. **g:** Immunoperoxidase staining. Anti-WT1 antibodies. Strong and uniform staining of podocyte nuclei (**arrows**) and less intense staining of Bowman's capsule epithelial cells. **h:** Counterphase of **g**. The corresponding nuclei are indicated by **arrows**. **i:** Immunoperoxidase staining. Anti-PAX2 antibodies. Nuclear staining of Bowman's capsule epithelial cells and of distal tubular cells. Absence of podocyte staining. Original magnification, $\times 180$ (**a**, **e**, **g**, and **i**), $\times 160$ (**f**), $\times 80$ (**b**), $\times 40$ (**d**).

Figure 2. Immunoperoxidase staining. **a** to **c**: Consecutive sections of the same glomerulus in a DDS patient. **a**: Anti-WT1 antibodies. Faint and irregular staining of podocyte nuclei with presence in the same glomerulus of faintly positive and negative podocyte nuclei (arrows). **b**: Counterphase of **a**. **c**: Anti-PAX2 antibodies. Strong nuclear staining of podocytes (arrows). **d** and **f**: Consecutive sections of the same glomerulus in a IDMS patient. **d**: Anti-WT1 antibodies. Decrease or absence of nuclear staining of podocytes are clearly seen. **e**: Counterphase of **d**. **f**: Anti-PAX2 antibodies. Nuclei of podocytes are abnormally labeled. **g** and **h**: Normal tissue adjacent to the Wilms' tumor in a patient without glomerular symptom. **g**: Faint and irregular labeling of podocyte nuclei (arrows). **h**: Counterphase of **g**. **i** and **j**: Anti-PCNA antibodies. **i**: One PCNA positive podocyte (arrow) is present as well as one positive tubular cell. **j**: Tubular sections. Numerous PCNA-positive and mitotic cells are present. **k**: Anti-PAX2 antibodies. Outer medulla. Collecting ducts are positively labeled, whereas dilated proximal straight tubules are not labeled with anti-PAX2 antibodies (arrows). Original magnification, $\times 180$ (**a**, **c**, **d**, **f**, and **j**), $\times 120$ (**i**), $\times 40$ (**k**).

Figure 3. *In situ* hybridization with DIG-labeled PAX2 anti-sense (a, c, and d) and sense (b and e) probes. **a:** Normal fetal kidney. PAX2 mRNA is detected in condensing mesenchymal cells and their early derivatives. The expression decreases in the prepodocytes and is absent from podocytes. PAX2 mRNA is also detected in the collecting ducts and their ampullar extremities. **c:** Superficial cortex of normal fetal kidney. The reaction product has a perinuclear localization in positive epithelial cells. **d:** Diffuse mesangial sclerosis. PAX2 transcripts are observed in podocytes. **b** and **e:** No signals are detected with sense probes. Original magnification, $\times 25$ (a), $\times 80$ (c), $\times 180$ (d).

control group. Most mutations in DDS patients are missense exon 8 or 9 mutations affecting zinc fingers 2 or 3.⁴⁻⁷ We have no information on the molecular defect in four patients of our series. In the six others, a mutation

was identified. One patient has a frameshift mutation leading to a protein truncated in zinc finger 3. Five patients carry the most commonly reported DDS missense mutations resulting in the replacement of arginine 366 in

Table 2. Distribution of WT1 and PAX2 Proteins in DDS and IDMS Patients

Patients	Exons	Mutations	WT1		PAX2	
			Normal	Abnormal	Normal	Abnormal
DDS						
1	8	R356C		+	+	
2	9	R394W		+		+
3	9	R394W		+	+	
4	9	R394W		+		+
5	9	R394W	+		+	
6	9	Stop at 398		+		+
7 to 10	nd	nd	+ (1 pt)	+ (3 pts)	+ (2 pts)	
IDMS						
11 to 21	nd	nd	+ (3 pts)	+ (8 pts)	+ (4 pts)	+ (5 pts)
22	8	H377Y		+		+
23	9	D396N	+		+	
24	9	F383L	+		+	
WT						
25	3	S223N	+		+	
26	4	W238X		+	+	
27	7	R301X	+		+	

Correlations with WT1 mutations in 12 patients.

WT, patients with Wilms' tumor and abnormal genitalia but without renal symptoms; analysis was performed on nontumoral renal tissue.

PAX2 expression could not be determined in four patients for technical reasons.

zinc finger 2 (one patient) or arginine 394 in zinc finger 3 (4 patients). These highly conserved arginine residues are critical for contact with guanine in the DNA target and consequently for protein-DNA binding. The consequences of DDS mutations on the nuclear targeting and DNA binding capacity of the WT1 protein have been analyzed in cellular models. Missense mutations such as 394Arg, altering amino acids that directly interact with the DNA target^{4,24} but also mutations leading to the removal of the last zinc fingers,²⁴ abolish WT1 binding to its DNA targets. More recently, targeting signals required for nuclear localization of the WT1 protein have been identified, one within ZF 1 and another within ZF 2 and 3.³⁸ In this reported study, the WT1 product of 3T3 cells transfected with DDS WT1 mutant (CMV-PM) was unable to concentrate in the nucleus. Our immunohistochemical findings in podocytes of DDS patients are the *in vivo* morphological counterpart of *in vitro* laboratory data. However, two DDS patients (pts 4 and 10), one of them carrying the classic R394W missense mutation, had normal WT1 nuclear localization. WT1 self-associates *in vitro* and *in vivo* through the amino-terminal part of the protein and dimerisation of the mutated protein with wild-type WT1 may prevent DNA binding of the complex.^{13,16,39} The low nuclear labeling observed in most podocytes is in agreement with this dominant negative effect of the mutated protein.

One of the WT1 targets is PAX2, the expression of which is down-regulated by WT1 during normal kidney development.²⁷ In the metanephros, PAX2 RNA and protein are detected in the ureteric bud and its derivatives, and after birth, the expression persists in collecting ducts.^{26,33,40-41} PAX2 is absent from the uninduced blastema and transiently expressed in the developing nephron. It progressively disappears from the presumptive precursors of the podocytes at the S-shaped body stage and is no longer expressed in the following stages

of nephron development.^{26,27,33} In parallel with the disappearance of PAX2, podocytes stop proliferating and definitively lose the ability to divide.⁴² Using immunohistochemical and *in situ* hybridization techniques, we observed this pattern of expression in normal fetal and postnatal kidneys and in control kidneys showing various types of nephropathies. In contrast, the podocytes of three DDS patients showed PAX2 nuclear immunolabeling. These dramatic changes in PAX2 expression, which were observed in DDS patients with poor or absent WT1 nuclear staining, are likely to result from the loss of WT1 dependent transcriptional repression. No abnormal PAX2 expression was detected in patients with normal WT1 podocyte distribution. Deregulation of PAX2 expression may be implicated in the mechanisms leading from WT1 mutation to podocyte alteration and the development of DMS as PAX2 dominant gain-of-function mutation in transgenic mice leads to congenital nephrotic syndrome with severe tubular microcystic dilatations, a frequent morphological finding in DDS.^{3,29} Other target genes of WT1, such as *PDGFA*,^{43,44} *IGF2*,⁴⁵ *TGF-β1*,⁴⁶ or *nov*, encoding a putative IGFBP,⁴⁷ may also be involved in the pathological pathway leading from WT1 mutation to podocyte dysfunction and glomerular sclerosis.

Abnormal WT1 and PAX2 expression was also detected in nephrotic infants with IDMS. As glomerular lesions are identical, this clinicopathological entity may be difficult (if not impossible) to differentiate from DDS, especially in females, in the absence of other symptoms of the triad. Actually, most 46XX DDS females do not develop genital abnormalities.⁷ In addition, development of Wilms' tumor may be prevented by bilateral nephrectomy performed at the time of transplantation, which should occur early in life because of rapid progression to end-stage renal failure. However, the occurrence of IDMS in normal 46XY males, the frequent familial incidence of the

disease and the occurrence of IDMS in children born to consanguineous parents, strongly suggest that it is a separate entity with a possible autosomal recessive inheritance (Table 1).^{8,48-57} Abnormal podocyte distribution of WT1 was observed in 9 of the 14 IDMS patients studied and was associated with PAX2 nuclear expression in seven patients. This anomaly, similar to that found in DDS patients suggested that WT1 could also be implicated in the occurrence of IDMS, making IDMS a clinical variant of DDS. This hypothesis was confirmed by the finding of WT1 exon 8 or 9 mutation in three patients, one male and two females. Two of these mutations (H377Y and D396N) have been previously described in DDS patients.⁵ An alternative hypothesis is that IDMS is a multigenic disease caused by mutations of *WT1* or one of the genes involved in the regulatory pathway of *WT1*. The finding of abnormal distribution of WT1 and PAX2 proteins in one patient in whom the WT1 mutation has been excluded by sequencing of the 5' part of exon 1 and of all of the exons 2 to 10 supports this hypothesis. One candidate gene is *PAX2*, abnormally expressed in the podocyte, especially because its deregulation in transgenic mice leads to early and severe nephrotic syndrome with renal changes compatible with those observed in DMS²⁹ and because some reported familial cases of IDMS are associated with ocular defects, another target tissue of PAX2 dysfunction.^{8,48,50,52,55}

No change in the distribution of WT1 or PAX2 was detected in the nontumoral renal tissue from two patients with Wilms' tumor, genital anomalies in the male, a constitutional *WT1* missense mutation in exon 3 in the female, and stop codon in exon 7 leading to the loss of the four zinc fingers in the male. These patients had no glomerular symptoms. However, in one male patient with the same phenotype and a stop codon in exon 4, WT1 nuclear labeling of podocytes was focally absent. At this time, this 9-year-old patient has not developed glomerular symptoms.

It is noteworthy that the up-regulation of PAX2 observed in podocytes, which is known to stimulate proliferation, was associated with podocyte hypertrophy but not with patent podocyte hyperplasia. However, using PCNA as a marker of proliferation, we observed PCNA positive podocytes in two thirds of patients; they were abundant in two and rare in most cases. No glomerular expression of PCNA was found in normal controls. Interestingly, marked increase in PCNA expression was observed in tubular and interstitial cells. This finding may be correlated with the early occurrence of severe tubulointerstitial lesions with tubular dilatation in diffuse mesangial sclerosis. Recently, it has been shown that in human dysplastic kidney, PAX2 is expressed in cystic and hyperproliferative epithelia, which are thought to be malformed branches of the ureteral bud, suggesting that persistent expression of PAX2 is involved in cyst formation.³³ In diffuse mesangial sclerosis, tubular dilatations develop primarily from proximal tubules, and we did not detect any abnormal PAX2 expression in these dilated tubules, suggesting that the mechanism of tubular cell proliferation and dilatation is not PAX2 dependent in this condition. Contrarily to the findings of Winyard et al,³³ we

observed persistent expression of PAX2 in nondilated collecting tubules of patients as well as of normal mature kidneys.

In conclusion, changes in podocyte distribution of WT1 and PAX2 observed in DDS patients are consistent with the transcription role of WT1 and the WT1 down-regulation of *PAX2*. However, no correlation could be established between the presence of any particular mutation and the expression pattern of WT1 or PAX2. The same anomalies have been observed in males and females patients with IDMS suggesting that WT1 is likely to play a significant pathophysiological role not only in DDS but also in IDMS.

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