Am. J. Hum. Genet. 62:824–833, 1998

Identification of Constitutional WT1 Mutations, in Patients with Isolated Diffuse Mesangial Sclerosis, and Analysis of Genotype/Phenotype Correlations by Use of a Computerized Mutation Database

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Summary

Constitutional mutations of the WT1 gene, encoding a zinc-finger transcription factor involved in renal and gonadal development, are found in most patients with Denys-Drash syndrome (DDS), or diffuse mesangial sclerosis (DMS) associated with pseudohermaphroditism and/or Wilms tumor (WT). Most mutations in DDS patients lie in exon 8 or exon 9, encoding zinc finger 2 or zinc finger 3, respectively, with a hot spot (R394W) in exon 9. We analyzed a series of 24 patients, 10 with isolated DMS (IDMS), 10 with DDS, and 4 with urogenital abnormalities and/or WT. We report WT1 heterozygous mutations in 16 patients, 4 of whom presented with IDMS. One male and two female IDMS patients with WT1 mutations underwent normal puberty. Two mutations associated with IDMS are different from those described in DDS patients. No WT1 mutations were detected in the six other IDMS patients, suggesting genetic heterogeneity of this disease. We analyzed genotype/phenotype correlations, on the basis of the constitution of a WT1 mutation database of 84 germline mutations, to compare the distribution and type of mutations, according to the different symptoms. This demonstrated (1) the association between mutations in exons 8 and 9 and DMS; (2) among patients with DMS, a higher frequency of exon 8 mutations among 46,XY patients with female phenotype than among 46,XY patients with sexual ambiguity or male phenotype; and (3) statistically significant evidence that mutations in exons 8 and 9 preferentially affect amino acids with different functions.

Received December 9, 1997; accepted for publication January 30, 1998; electronically published March 20, 1998.

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0002-9297/98/6204-0014$02.00

Introduction

The WT1 gene encodes a zinc-finger transcription factor involved in kidney and gonadal development (Pritchard-Jones et al. 1990). In fetal kidney, it is expressed in the condensing mesenchyme, the renal vesicle, and developing podocytes. The other main sites are the genital ridge, fetal gonads, and the mesothelium. Knockout of this gene in mouse resulted in the absence of renal and gonadal development (Kreidberg et al. 1993). The gene has 10 exons, of which exons 1–6 encode a proline/glutamine-rich transcriptional-regulation region and exons 7–10 encode the four zinc fingers of the DNA-binding domain. Different functional domains involved either in repression or in activation of transcription (Wang et al. 1993, 1995) and a region involved in homodimerization of the protein (Moffett et al. 1995) 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, allow synthesis of four isoforms with conserved relative amounts (Haber et al. 1991), different binding specificities (Bickmore et al. 1992; Drummond et al. 1994), and different subnuclear localizations (Englert et al. 1995; Larsson et al. 1995). The exon 5–containing isoforms contain an additional dominant repressor domain (Wang et al. 1995), and their relative proportions may vary during renal development (Renshaw et al. 1997). The −KTS isoforms bind to a consensus sequence identical to that for EGR1 and present in numerous genes, including IGF2 (Drummond et al. 1992), PDGFA (Wang et al. 1992), and WT1 (Rupprecht et al. 1994), in agreement with a role in the regulation of transcription. Transient transfection assays have confirmed that WT1 may activate or suppress these genes, but the physiological and functional significance of these effects is still not known. The +KTS proteins associate with splicing factors and may have a role in RNA splicing (Larsson et al. 1995).

Constitutional heterozygous intragenic mutations of

WT1 have been described in most patients with Denys-Drash syndrome (DDS) (MIM 194070 and 194080) (for a review, see Little and Wells 1997). This syndrome includes a specific nephropathy characterized by diffuse mesangial sclerosis (DMS), male pseudohermaphroditism, and/or Wilms tumor (WT) (Denys et al. 1967; Drash et al. 1970). DDS patients with a 46,XY karyotype present with ambiguous or female external genitalia and dysgenic gonads. In contrast, 46,XX children have a normal female phenotype. Among the five 46,XX patients whose gonads were tested, four had apparently normal ovaries, and only one had dysgenic gonads (Jeanpierre et al. 1998). No data concerning puberty in the 46,XX DDS patients are available. Most of the mutations in the DDS patients are missense changes in either exon 8, coding for zinc finger 2, or exon 9, coding for zinc finger 3, with a hot spot at position 1180 in the codon for arginine 394, which is predicted to be involved in the interaction with the consensus DNA sequence.

The reasons for heterogeneity of the clinical features of patients with WT1 mutations are still poorly understood. One way to elucidate the role of constitutional mutations, in the expression of the different clinical features, is to analyze WT1 in patients presenting with different combinations of these symptoms. Mutations have been described (1) in male patients with cryptorchidism and/or hypospadias and WT (Pelletier at al. 1991; Huff et al. 1995; Nordensköld et al. 1995*b*) and (2) in patients presenting with WT only, either unilaterally or bilaterally, including one familial case (Little et al. 1992; Akasaka et al. 1993; Coppes et al. 1993; Kaplinsky et al. 1996; Schumacher et al. 1997). However, no mutations have been identified in patients with isolated XY gonadal dysgenesis (Nordenskjöld et al. 1995*a*). No data concerning patients presenting with isolated DMS (IDMS) are available. This early-onset nephropathy, which most often progresses to end-stage renal failure (ESRF) at ~3 years of age, can occur in the absence of other abnormalities, and there is no histological difference between this form and that in DDS patients (Habib et al. 1993).

Analysis of WT1 in patients with IDMS would be helpful not only in deciphering the role of WT1 in renal and genital development but also in providing a molecular definition of IDMS related to DDS. We therefore sequenced the WT1 gene from each of 10 patients with IDMS. We identified mutations in four cases, suggesting that IDMS could be a clinical form of DDS. However, no mutation was detected in the six other patients, suggesting genetic heterogeneity of IDMS. We also characterized WT1 mutations in 10 new patients with DDS and in 4 patients with urogenital abnormalities and/or WT and no nephropathy.

We recently developed software and a database for the analysis of WT1 mutations (Jeanpierre et al. 1998). The mutations we describe in this article were added to this database. Using this tool, we performed computerized analyses of the distribution and type of mutations in the gene, according to clinical features. These analyses suggest that exon 8 and exon 9 mutations may not be equivalent for genital development impairment associated with DMS, in DDS patients. With the accumulation of data, genotype/phenotype-correlation analyses based on utilization of this database and the associated software will help in the understanding of the roles of different isoforms and regions of the WT1 protein, in development and cancer.

Patients and Methods

Patients

Ten patients with IDMS were selected on the basis of the presence of DMS in the absence of urogenital abnormality and WT (table 1). Patients P2, P8, and P10 were male children; patients P1, P3–P7, and P9 were 46,XX female children. One male and three female patients underwent normal pubertal development, whereas the other patients are still too young. WT was not found during follow-up exams performed within 2 mo–12.5 years. There were no other congenital abnormalities and no family history of developmental abnormalities or renal problems, for any of these patients. The parents of patient P6 were consanguineous.

Ten patients presenting with DMS in a context of DDS also were studied (table 1). Patients P11–P13 and P15–P20 had the 46,XY karyotype and genital abnormalities ranging from testicular ectopia to female phenotype. Patient P14 had the 46,XX karyotype and streak ovaries. Unilateral WT was diagnosed in two patients, and gonadoblastoma was diagnosed in two other patients.

For these 20 patients with DMS, the age at which the first symptoms of nephrotic syndrome were observed varied, from birth to 4.3 years. Age at ESRF was 18 d–4.5 years, except for patient P19, who developed ESRF at age 11 years 6 mo. Patients P11 and P13 died of ESRF during the 1st mo of life. Patients P17 and P18, who were 6 years old and 2.9 years old, respectively, had not yet developed ESRF. DMS was ascertained histologically in all these patients.

Two 46,XY patients (P22 and P23) with urogenital abnormalities and WT but no DMS, a 46,XY patient (P24) with ambiguous genitalia, WT, and mild proteinuria, and a 46,XX patient (P21) with WT only also were studied (table 1). The parents of patients P1–P4, P13, P14, P17, and P21 were available for study. The appropriate informed consents were obtained.

Table 1

Summary of Phenotypes and Constitutional WT1 Mutations in Patients

Group and Patient[a]	WT1 Mutation[b]	Exon or Intron	Protein Alteration	Nephropathy			Karyotype[c]	External Genitalia	Puberty (age [years/mo])/ Gonads	WT (Age at Onset [years/mo])[d]	Age at Nephrectomy[e] (years/mo)	Age at Follow-up without WT[f] (years/mo)	Parent(s) Studied[g]
				Histology	Age at First Symptoms (years/mo/d)	Age at ESRF (years/mo/d)							
A:													
P1	1129C→T	Exon 8	H377Y	DMS	0/6/…	3/10/…	46,XX	Female	Normal puberty	No	4/2 (L), 4/7 (R)		F/M
P2	1147T→C	Exon 9	F383L	DMS	0/8/…	3/…/…	ND	Male	Normal puberty	No	5/… (L), 6/… (R)		F/M
P3	1186G→A	Exon 9	D396N	DMS	1/…/…	2/3/…	46,XX	Female	? (10/7)	No	2/… (L), 3/… (R)		M
P4	1228+4C→T	Intron 9	Splicing (?)	DMS	0/4/…	1/2/…	46,XX	Female	Normal puberty	No	4/… (R)	12/5 (L)[e]	F/M
P5	None (exons 1–10)	…	…	DMS	2/…/…	2/5/…	46,XX	Female	Normal puberty	No	4/… (L), 6/… (R)		
P6	None (exons 1–10)	…	…	DMS	2/4/…	2/5/…	46,XX	Female	? (9/…)	No	3/… (L/R)		
P7	None (exons 1–10)	…	…	DMS	4/3/…	4/5/…	46,XX	Female	Normal puberty	No	5/… (L/R)		
P8	None (exons 8 and 9)	…	…	DMS	0/8/…	0/10/…	46,XY	Male	? (2/…)	No	1/2 (L/R)		
P9	None (exons 8 and 9)	…	…	DMS	1/3/…	3/2/…	46,XX	Female	? (8/…)	No	3/8 (L), 4/1 (R)		
P10	None (exons 8 and 9)	…	…	DMS	0/2/…	No ESRF (death)	ND	Male	? (No necropsy)	No	No nephrectomy	0/2 (death)	
B:													
P11	1096C→T	Exon 8	R366C	DMS	Birth	0/1/…	46,XY	Female	? (No necropsy)	No	No nephrectomy	0/1 (death)	
P12	1180C→T	Exon 9	R394W	DMS	3/2/…	4/5/…	46,XY	Ambiguous	Gonadoblastoma[h]	No	5/… (L), 6/… (R)		
P13	1180C→T	Exon 9	R394W	DMS	0/0/3	0/0/18	46,XY	Ambiguous	? (No necropsy)	No	No nephrectomy	18 d (death)	F/M
P14	1180C→T	Exon 9	R394W	DMS	0/3/…	2/1/…	46,XX	Female	Streak dysgenic ovaries	U (2/…)	2/1 (L/R)		F/M
P15	1181G→A	Exon 9	R394Q	DMS	0/5/…	0/7/…	46,XY	Testicular ectopia	? (No necrospy)	U (0/7)	No nephrectomy		
P16	1192delC	Exon 9	Stop at 398	DMS	0/10/…	1/1/…	46,XY	Ambiguous	Sertoli cells testis (R), streak testis (L)[e]	No	1/1 (L), 1/5 (R)		
P17	1193T→C	Exon 9	L398P	DMS	0/8/…	No ESRF at 6/…/…	46,XY	Ambiguous	Dysgenic testes	No	No nephrectomy	6/…	F/M
P18	1228+5G→A	Intron 9	Splicing	DMS	Birth	No ESRF at 2/9/…	46,XY	Female	Bilateral gonadoblastoma	No	No nephrectomy	2/9	
P19	None (exons 8 and 9)	…	…	DMS	0/5/…	11/6/…	46,XY	Ambiguous	Dysgenic testes	No	11/10 (L/R)		
P20	None (exons 8 and 9)	…	…	DMS	0/0/6	0/10/…	46,XY	Cryptorchidism	Atrophic testes	No	2/6 (L), 4/7 (R)		
C:													
P21	667G→A	Exon 3	S223N	…	No symptoms	…	46,XX	Female	? (2/…)	U/L (1/4)[e]	…		Fmut/M
P22	712G→A	Exon 4	W238X	…	No symptoms	…	46,XY	Testicular ectopia hypospadias	Dysgenic testes	U/L (0/6)[e]	…		
P23	901C→T	Exon 7	R301X	…	No symptoms	…	46,XY	Ambiguous	Dysgenic testes	Bi	…		
P24	1019T→G	Exon 7	L340X	…	? (Mild proteinury)	…	46,XY	Ambiguous	Dysgenic testes	U (4/…)	…		

[a] A = patients with IDMS; B = patients with DDS; and C = patients with urogenital abnormalities and/or WT and no DMS.
[b] As indicated in the study by Beaudet and Tsui (1993).
[c] ND = not determined.
[d] U = unilateral; Bi = bilateral.
[e] L = left; R = right.
[f] When kidney in position.
[g] F = father; M = mother; and Fmut = mutation present in the father's DNA.
[h] Bilateral castration at 4 years 9 mo.

DNA Sequencing

For exon 1, we amplified a 277-bp fragment, using primers WT256 (5′-AGC CAG AGC AGC AGG GAG TC-3′) and WT532R (5′-AAC GAC CCG TAA GCC GAA GC-3′), which correspond to positions −125 bp and 152 bp, respectively, from the site of translation initiation (Huff et al. 1995). Exons 2–10 were amplified by use of primers corresponding to the preceding and following intronic sequences. The primers and product lengths were as follows: for exon 2, C147 (5′-AAG CTT GCG AGA GCA CCG CTG-3′) and C148 (5′-TAA TTT GCT GTG GGT TAG GAA TTC-3′), 259 bp; for exon 3, C149 (5′-GGC TCA GGA TCT CGT GTC TC-3′) and C150 (5′-CCA AGT CCG CCG GCT CAT G-3′), 271 bp; for exon 4, C486 (5′-AAA CAG TTG TGT ATT ATT TTG TGG-3′) and C152 (5′-ACT TTC TTC ATA AGT TCT AAG CAC-3′), 224 bp; for exon 5, C153 (5′-CAG ATC CAT GCA TGC TCC ATT C-3′) and C154 (5′-CTC TTG CAT TGC CCC AGG TG-3′), 176 bp; for exon 6, C178 (5′-AAG CTT CAC TGA CCC TTT TTC CCT TC-3′) and C177 (5′-GAA TTC CAA AGA GTC CAT CAG TAA GG-3′), 221 bp; for exon 7, WT1-7S (5′-AAG ACC TAC GTG AAT GTT CAC A-3′) and WT1-7AS (5′-TAC AAC ACC TGG ATC AAG ACC T-3′), 351 bp; for exon 8, C323 (5′-CCT TTA ATG AGA TCC CCT TTT CC-3′) and 796 (5′-GGG GAA ATG TGG GGT GTT TCC-3′), 391 bp, or WT89S (5′-TCC AGC GAA GTG CCT TAG GC-3′) and WT11 (5′-CCT AGC CCA AGG GAA CAC AG-3′), 257 bp; for exon 9, 798 (5′-TGC AGA CAT TGC AGG CAT GGC AGG-3′) and 801 (5′-GCA CTA TTC CTT CTC TCA ACT GAG-3′), 349 bp, or WT12 (5′-CCT CAC TGT GCC CAC ATT GT-3′) and WT89R (5′-TCC CTC TCA TCA CAA TTT CAT TC-3′), 221 bp; and, for exon 10, C911 (5′-ACT TCA CTC GGG CCT TGA TAG-3′) and C912 (5′-GTG GAG AGT CAG ACT TGA AAG-3′), 234 bp.

PCR fragments were purified on microspin S400 columns (Pharmacia) or on Wizard columns (Promega Biotec). Sequence reactions were performed by use of either the same, but fluorescently labeled, primers and the Thermo-Sequenase fluorescent labeled–primer cycle sequencing kit (Amersham) for resolution on an ALF sequencer (Pharmacia) or the ABI Prism Dye terminator sequencing kit for resolution on an ABI sequencer (Perkin Elmer).

Restriction Analysis

For analysis of the exon 1 polymorphism, 5 μl of the PCR products (primers WT256 and WT532R) were digested with 2 U of *Bbv*I (Biolabs). The fragments were resolved on 10% acrylamide gels, transferred onto a Biodyne membrane (Pall), and probed, for hybridization, with one of the radiolabeled primers.

Database

To investigate genotype/phenotype correlations, we recently developed software and a computerized WT1 mutation database containing 70 germ-line mutations from the literature (Universal Mutation Database [http://www.umd.necker.fr]; Jeanpierre et al. 1998). In brief, for each mutation, information was provided at the gene level (exon and codon number, wild-type codon, and mutant codon), at the protein level (wild-type and mutant amino acid), and at the clinical level, for the clinical features that developed in patients (nephropathy, karyotype/external genitalia/internal reproductive organs, and presence of unilateral or bilateral WT). Routines have been developed that allow analysis of the type and distribution of mutations, according to the clinical features.

Among the 70 registered mutations, 50 were described in patients with nephropathy, and 17 were described in patients without nephropathy (4 patients with genital abnormalities and WT and 13 patients with WT only, 1 of whom is a familial case). Three patients—two with WT and nephrotic syndromes not associated with DMS (Bardeesy et al. 1994; Schmitt et al. 1995) and one with WT, maldescended testis, and proteinuria (Schumacher et al. 1997)—were difficult to classify. These patients were not included in the genotype/phenotype-correlation analyses. Major rearrangements and mutations in introns were omitted, since they cannot be accommodated in the present version of the software.

Results

Characterization of WT1 Mutations

Patients with IDMS.—Of the 10 patients with IDMS, 4 were shown to carry a mutation in one WT1 allele. These mutations involved exon 8 (patient P1), exon 9 (patients P2 and P3), or intron 9 (patient P4) (table 1). The missense mutation F383L in exon 9 (patient P2) has not been described previously. The H377Y (patient P1) and the D396N (patient P3) changes have been described in several DDS patients. The C→T transition at position +4 of the splice-donor site within intron 9 (patient P4) has been reported in two patients with Frasier syndrome (focal and segmental glomerulosclerosis, pseudohermaphroditism, and gonadoblastoma) (Barbaux et al. 1997). None of these mutations were identified in DNA from the parents. In patients P5–P7, sequencing of the *5* part of exon 1 and of the entirety of exons 2–10 did not identify any mutation. We ascertained the presence of two copies of the WT1 gene in patient P5, who was heterozygous for a T→C polymorphism in intron 9, at position 156 of sequence M80232 (Tadokoro et al.

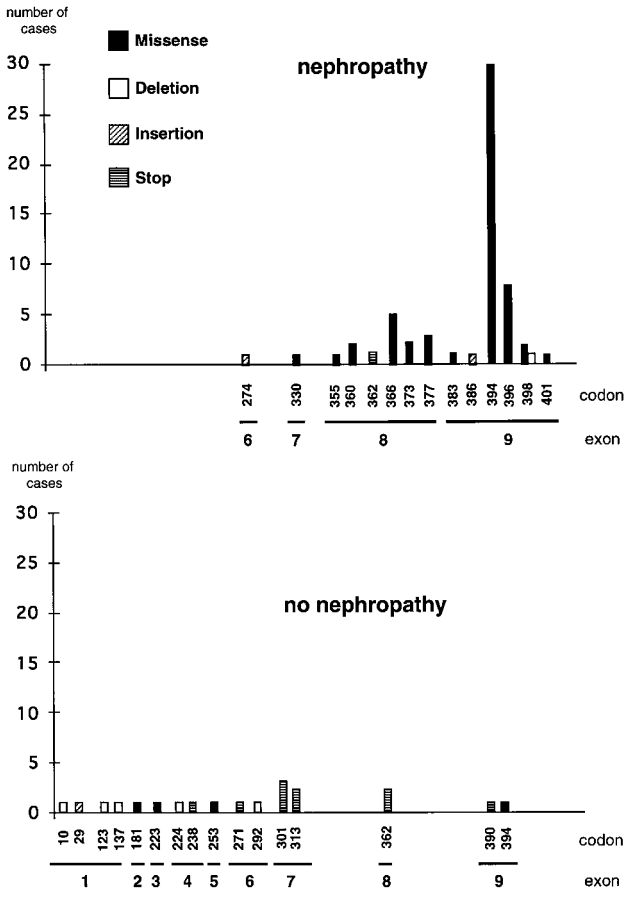

Figure 1 Analysis of distribution of WT1 mutations, according to the presence or absence of nephropathy, by use of the WT1 mutation database and associated software. Records were selected by use of the following categories: (1) DMS or nephropathy (60 cases) or (2) no nephropathy (20 cases). The data provided by the routine "binary comparison" were exported to the program Excel, to construct the diagrams. The amino acids and exons affected by the mutations are indicated below each diagram.

1993). In patients P8–P10, only exons 8 and 9 were investigated, and no mutation was identified.

Patients with DDS.—Of the 10 patients with DDS, 8 carried WT1 mutations (table 1). The mutations involved exon 8 (patient P11), exon 9 (patients P12–P17), or intron 9 (patient P18). Two of these mutations—namely, mutations R394Q (patient P15) and 1192delC (patient P16) in exon 9—have not been described previously. The absence of the mutation in both parents was ascertained for three patients (table 1). Sequencing of exons 8 and 9 did not reveal any mutation in patients P19 and P20.

Patients without DMS.—Four patients with urogenital abnormalities and/or WT also were analyzed for the presence of constitutional WT1 mutations. Mutations in exon 3 (patient P21), exon 4 (patient P22), and exon 7 (patients P23 and P24) were identified. Mutations detected in patients P21, P22, and P24 have not been reported previously. The mutations carried by patients P21 and P22 were heterozygous in blood and normal kidney samples but were homozygous in tumor tissue. This was in agreement with the previously detected loss of 11p alleles in the tumor from patient P22 (I. Henry, unpublished data). We also showed that the mutation carried by patient P21 was inherited from her unaffected father (table 1).

Characterization of a New Polymorphism in Exon 1

Sequencing of part of exon 1 allowed the identification of a new polymorphism, with a PIC value of .34. This T→G variation of the sequence, at position −7 with respect to the ATG initiation codon, created a *Bbv*I site, which was used to estimate the frequency of the two alleles, by analysis of the genotypes of 32 unrelated individuals (data not shown). The frequencies were 32% (T) and 68% (G).

Genotype/Phenotype Correlations Using the WT1 Mutation Database

Of the 16 mutations we describe in this paper, 14 were added to the database. The 2 mutations that lie in intron 9 could not be accommodated in the present version of the software. The number of registered germ-line WT1 mutations increased to 60 for patients with nephropathy and to 20 for patients without nephropathy. Because of uncertainty about the clinical diagnosis of patient P24, his mutation was not included in the correlation analyses.

The selection of records corresponding to particular criteria and the computerized analysis of the distribution of mutations are possible by use of the software package associated with this database. The routine "binary comparison" supplies graphical displays, which allowed us to compare the distribution of mutations among the different categories of patients, according to the different clinical features of the DDS triad: nephropathy, genital abnormalities, and WT.

Distribution of mutations, according to the presence or absence of nephropathy.—We compared the distribution of mutations in patients selected according to the criterion "DMS or nephropathy" and patients selected according to the criterion "no nephropathy" (60 patients and 20 patients, respectively), independent of the other symptoms. Note that the description of nephropathy is not always clearly documented in the published papers. Thus, in the first category of patients, we included those with ascertained DMS and those reported as having a nephropathy. Figure 1 clearly emphasizes (1) that the mutations involved almost exclusively exons 8 and 9 in patients with nephropathy (58 of 60 mutations), with a

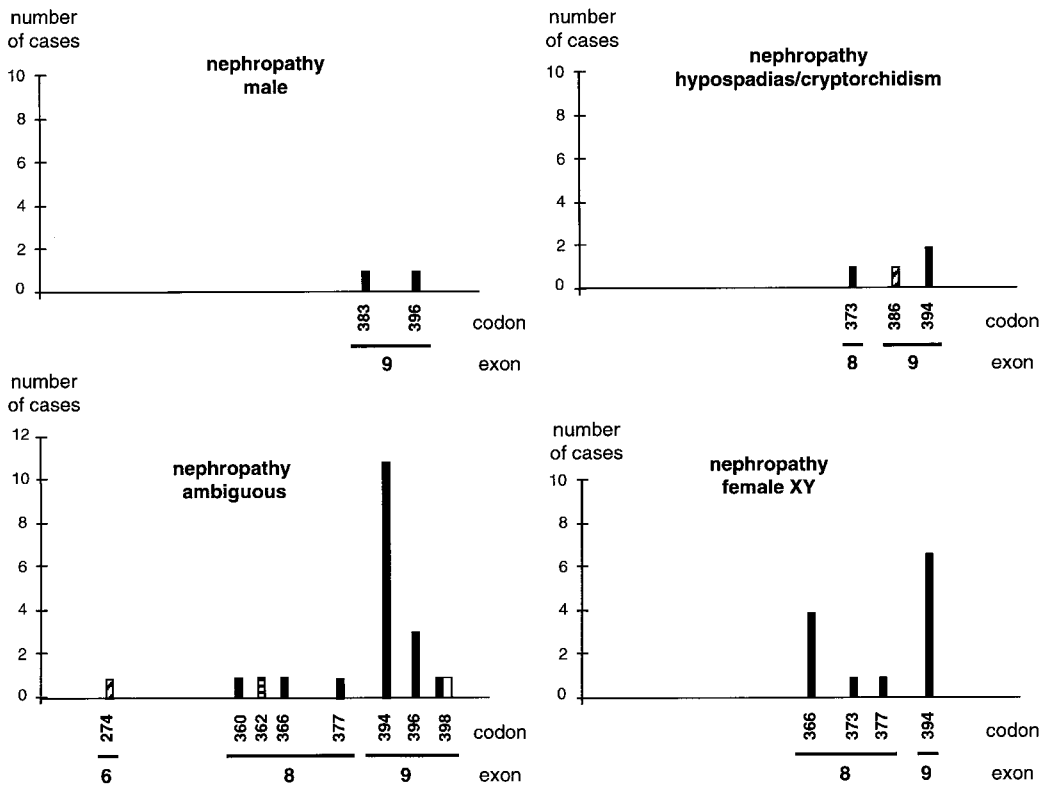

Figure 2 Analysis of the distribution of WT1 mutations among patients with nephropathy, according to external genitalia, by use of the WT1 mutation database and associated software. Records were selected by use of the following categories: (1) DMS or nephropathy and male genitalia (2 cases); (2) DMS or nephropathy and hypospadias/cryptorchidism (4 cases); (3) DMS or nephropathy and ambiguous genitalia (21 cases); and (4) DMS or nephropathy, 46,XY karyotype, and female external genitalia (13 cases). The key for missense, deletion, insertion, and stop mutations is as shown in figure 1.

high percentage of missense mutations (55 of 58 mutations, of which 30 involved codon 394) (the mutations in intron 9 were not taken into account in this analysis, as explained in Patients and Methods), and (2) that a large majority of mutations (16 of 20) involved exons 1–7 in the patients without nephropathy, with a large majority of these mutations (16 of 20) leading to truncated proteins. Among the four missense mutations described in patients without nephropathy, one lies in exon 2, which encodes the dimerization domain, one lies in exon 3, one lies in the alternatively spliced exon 5, and one is a R394W mutation in exon 9.

Distribution of mutations, according to karyotype/external genitalia associations.—The observation of WT1 mutations in patients with IDMS and, thus, without urogenital abnormalities prompted us to analyze the distribution of mutations in patients with nephropathy/DMS, according to karyotype/external genitalia associations. Because of the absence of external genitalia abnormalities in 46,XX DDS patients, we assumed that WT1 mutations do not interfere with normal female external genitalia development. Thus, the role of WT1 mutations in genitalia abnormalities was investigated among 46,XY patients. Interestingly, the relative prevalence of exon 8 versus exon 9 missense mutations appeared to be higher in 46,XY patients with no male differentiation of external genitalia (female phenotype; 6 exon 8 mutations vs. 7 exon 9 mutations) than in all the other categories of patients (ambiguous, hypospadias/cryptorchidism, and male), when the latter were considered as a single group (4 exon 8 mutations vs. 19 exon 9 mutations), although this did not reach statistical significance ($P = .1$) (fig. 2). Accordingly, the two mutations described in patients with nephropathy and male phenotype—namely, patient P2 (this study) and a previously reported patient (SS/12) with DMS and WT (Jadresic et al. 1990; Baird et al. 1992)—lie in exon 9. Note that neither of these two mutations involves the arginine codon at position 394. These data suggested that, for male development, exon 8 mutations could be more deleterious than exon 9 mutations. We thus hypothesized that mutations in exons 8 and 9 could differently disrupt the normal function of the protein, and we compared the roles of the amino acids affected by these mutations.

We considered amino acids either involved in DNA binding (arginine 366 in exon 8; arginine 394 and aspartic acid 396 in exon 9) or critical for zinc-finger structure (cysteines 355 and 360 and histidines 373 and 377, in exon 8; cysteines 385 and 388 and histidines 401 and 405, in exon 9) (fig. 3). Among the 55 missense mutations in exons 8 and 9 in patients with nephropathy, a highly significant ($P < .001$) difference appeared, with 38 mutations affecting arginine 394/aspartic acid 396 versus a single mutation affecting histidine 401, in exon 9, and with 5 mutations affecting arginine 366 versus 8 mutations affecting cysteine or histidine residues, in exon 8 (fig. 3). However, the distribution of exon 8 mutations appeared to be different among the 46,XY female patients, with four mutations affecting arginine 366 versus two mutations affecting histidine residues (373 and 377) (fig. 2). Owing to the small number of mutations, the significance of this observation has to be evaluated.

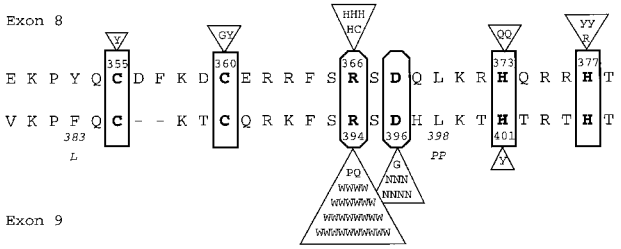

Figure 3 Diagrammatic representation of exon 8 and exon 9 missense mutations, among the 55 patients with nephropathy, included in the database. Amino acids either involved in DNA binding (arginine 366, in exon 8; arginine 394 and aspartic acid 396, in exon 9) or critical for zinc-finger structure (cysteines 355 and 360 and histidines 373 and 377, in exon 8; cysteines 385 and 388 and histidines 401 and 405, in exon 9) are boxed. Amino acids resulting from mutations are outlined by triangles. Mutations at other positions are indicated in italics.

Discussion

To elucidate the role of constitutional WT1 mutations in the different clinical components of DDS, we analyzed a series of 24 patients, 10 of whom presented with IDMS only. We report the identification of 16 mutations, 6 of which have not been reported previously. Four mutations were detected in patients with IDMS and involved exon 8 or exon 9. By computerized analysis of genotype/phenotype correlations, we demonstrated (1) the association between exon 8 and exon 9 mutations and DMS, either in its isolated form (IDMS) or within a context of DDS; (2) a higher frequency of exon 8 mutations among 46,XY patients with no male external genitalia development than among patients with sexual ambiguity or normal male phenotype; and (3) that mutations in exons 8 and 9, encoding zinc fingers 2 and 3, respectively, clearly affect amino acids with different functions.

We describe the first WT1 mutation, in a male child (patient P2) with IDMS, as the only abnormality. This patient had no sexual ambiguity, underwent normal puberty, and had not developed WT by the time of nephrectomy at ages 5 years (left kidney) and 6 years (right kidney). Thus, his clinical features clearly do not correspond to the definition of DDS. The exon 9 mutation F383L, detected in this patient, is a newly described mutation. Since it appeared de novo, it is highly likely to be responsible for the disease. An exon 9 mutation was reported previously in another male patient with DMS, who also developed WT (patient SS in the study by Baird et al. [1992], or patient 12 in the study by Jadresic et al. [1990]). The R396D mutation detected in patient SS/12, which also is a de novo mutation, was different from the mutation we describe here and also was reported in three DDS patients presenting with DMS and ambiguous genitalia, with or without WT. Because WT is not a constant criterion for DDS, our patient P2 and patient SS/12, both with DMS and male phenotype, could belong to the same, as-yet-unrecognized category of DMS patients with WT1 mutations. Thus, mutations in exon 9 may be compatible with normal male external genitalia development.

The other three patients with IDMS and WT1 mutations (P1, P3, and P4) were normal 46,XX females, and two of them had already undergone puberty. Patients P1 and P3 carry mutations that have been described in several DDS patients. The absence of external genitalia abnormalities in 46,XX DDS patients and the lack of information concerning the occurrence of puberty in cases reported in the literature raise the question of the true difference between 46,XX DDS patients and 46,XX IDMS patients. Patient P4 carries a mutation at position +4 of intron 9. Although we did not demonstrate that this mutation precludes alternative splicing, the absence of the mutation in both parents supports its role in nephropathy. Interestingly, identical mutations at position +4 of intron 9 were reported recently in two patients with Frasier syndrome (Barbaux et al. 1997). Frasier syndrome associates nephropathy and pseudohermaphroditism but differs from DDS by a later age at onset of nephropathy, the presence of focal and segmental glomerulosclerosis, the absence of WT, and an increased likelihood of gonadoblastoma. Although the histological and clinical features of the patients described in the study by Barbaux et al. (1997) were clearly those of Frasier syndrome, the renal lesions in our patient P4, examined by the same physician and the same pathologist (P.N. and M.-C.G., respectively) as the patients in the study by Barbaux et al. (1997), are unambiguously those of DMS. Moreover, the early age at onset of nephropathy, at 4 mo, with ESRF at 14 mo, and the normal pubertal development and menstruations, at age 11

years, in this 46,XX girl confirm the diagnosis of IDMS. This demonstrates heterogeneity of the clinical features associated with this intron 9 mutation at position +4.

No mutation was identified in the six other patients with IDMS, either by analysis of exons 8 and 9, in patients P8–P10, or by analysis of the 5′ part of exon 1 and the entirety of exons 2–10, in patients P5–P7. Thus, our data suggest the existence of at least two genetic forms of IDMS: (1) one form could be considered a variant of DDS, involving a mutation in either exon 8 or exon 9, and (2) the other form could be due to either another mechanism of inactivation of WT1, which is different from a mutation in the coding sequence, such as methylation of the CpG-rich promotor region (Baylin 1997), or maybe to the involvement of another gene, which is not involved in genital development.

Most of the mutations we detected in DDS patients were missense changes affecting zinc fingers 2 or 3, and we demonstrated de novo occurrence for three of them. Several mutations have already been reported in other patients. We report the second case (patient P16) of a frameshift mutation in exon 9, leading to a truncated protein in zinc finger 3 (Ogawa et al. 1993). We also identified a new variant mutation at position 394, which converts an arginine to a glutamine (patient P15). We describe a mutation at position +5 of intron 9 in patient P18, whose nephropathy was clearly identified as DMS. Therefore, this mutation is not strictly Frasier syndrome specific, as was proposed by Barbaux et al. (1997). All these mutations involving exon 8 or exon 9 presumably affect the function of regulation of transcription or the ability to interact with splicing factors, by loss of KTS and zinc finger 4 (nonsense mutation in patient P16), by loss of the KTS isoform (intron 9 mutation at position +5 in patient P18), or by alteration of DNA recognition (missense mutations) (Little et al. 1995). Sequencing of exons 8 and 9 did not enable us to identify mutations in patients P19 and P20. For patient P19, the late age at ESRF (11 years 6 mo) suggests a peculiar form of DMS. Patient P20 is a male child with cryptorchidism and DMS, and the clinical diagnosis of DDS versus IDMS thus may be questionable.

The four mutations we detected in patients without nephropathy lie outside exons 8 and 9, and three of them are nonsense mutations producing proteins lacking the zinc-finger domain. Interestingly, the mutation presented by patient P21, who had unilateral WT, was inherited from her asymptomatic father. This is the fourth case of a transmission of a WT1 mutation, and in all four cases the mutation was inherited from the father. An exon 6 mutation has been described in a father with WT and in his son, who has urogenital abnormalities and WT (Pelletier et al. 1991). However, in all the other cases, the transmitting father was asymptomatic, and inheritance of an exon 8 or exon 9 mutation has been reported in children with familial WT or DDS, respectively (Coppes et al. 1992; Kaplinsky et al. 1996). The difference in or the absence of phenotypic expression of these mutations may be relevant to mosaic, polymorphic, and tissue-specific imprinting of WT1 (Jinno et al. 1994; Mitsuya et al. 1997).

Both the heterogeneity of phenotypes associated with WT1 mutations and the difficulties of manual analysis of such complex information (Coppes et al. 1993; Huff 1996; Little and Wells 1997) prompted us to develop a computerized tool (Jeanpierre et al. 1998). This tool allows comparison of the distribution of mutations between different categories of patients from the database, sorted according to selected clinical or molecular criteria. In this article, we report the first analysis of the distribution of WT1 mutations, according to the clinical features of the DDS triad. The most striking comparison is the preponderance of exon 8 and exon 9 missense mutations, in patients with nephropathy, versus mutations leading to truncated proteins lacking the zinc-finger domain, in patients without nephropathy. Mutation R394W is unambiguously the mutation most frequently associated with DMS, in DDS patients. However, none of the 10 IDMS patients whom we studied carry this mutation. A R394W mutation has been reported in a female patient presenting with only unilateral WT but no DMS, at age 7 years (Akasaka et al. 1993). This patient may be at risk for the development of a late form of nephropathy. Interestingly, although exon 9 mutations clearly represent a hot spot in patients with nephropathy, our analysis suggests a difference in the roles of exon 9 and exon 8 mutations, with regard to urogenital abnormalities. We found a higher frequency of exon 8 missense mutations among 46,XY patients with complete absence of male external genitalia development (46%) than among patients with normal or disrupted male genitalia development (17%). Accordingly, the two patients with normal male development both displayed exon 9 mutations. Interestingly, although most missense mutations in the zinc-finger region probably affect DNA binding, they appear to do so through different mechanisms for zinc finger 2 and zinc finger 3. Disruption of zinc-finger folding by exon 8 mutations at cysteine and histidine residues (62% of exon 8 mutations) probably prevents any DNA binding, whereas amino acid changes at positions critical for DNA-sequence recognition (38% of exon 8 mutations and 90% of exon 9 mutations) could create proteins with the potential for binding novel DNA target sequences. Our analysis of the distribution of mutations suggests that exon 8 mutations from this second category could be more deleterious for male differentiation. However, analysis of a greater number of patients is necessary, to reach statistical significance and to understand how these mutations really affect development.

No correlation could be established between the presence of any particular mutation and the occurrence of WT. This is not surprising, because, first, germ-line WT1 mutations confer only a predisposition for development of this tumor. Other events are required for the tumor to develop, and we were unable to investigate the probability of the occurrence of these events. Second, the status of many patients reported in the literature cannot be ascertained, because of incomplete data concerning the occurrence of and age at nephrectomy.

Our study provides new information concerning the heterogeneity of phenotypes associated with WT1 mutations, by demonstrating that an exon 9 mutation is compatible with normal male development. Moreover, computerized analysis of genotype/phenotype correlations suggests that exon 8 and exon 9 mutations carried by patients with DMS might affect genitalia development differently. Analysis of a larger number of patients, who are characterized clinically with great accuracy, is necessary to confirm these observations and would contribute to a better understanding of the WT1-gene function. Moreover, our data suggest the existence of at least two genetic forms of IDMS: a variant form of DDS due to an exon 8 or an exon 9 mutation and a form related to another, as-yet-unidentified mechanism. This implies that WT1 mutations should be searched for in male and female patients with IDMS.

Acknowledgments

We thank Drs. Broux, Dehennault, Jaubert, Landthaler, Leblanc, Nihoul-Fékété, Oriot, Parchoux, Tournade, and Tovar, for providing patients, clinical information, and DNA samples. This work was supported by the Institut National de la Santé et de la Recherche Médicale, the Direction de la Recherche Clinique (Assistance Publique-Hôpitaux de Paris), the Association pour la Recherche sur le Cancer, and the Mutuelle Générale de l'Education Nationale. We thank Myriam Le Strat for help in the preparation of the manuscript.

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