Letters to the Editor

J Med Genet 2000;**37**:698–701

Constitutional *WT1* mutations correlate with clinical features in children with progressive nephropathy

EDITOR—The *WT1* tumour suppressor gene encodes a transcriptional factor containing four zinc fingers.[1 2] This gene has two alternative splicing regions, one consisting of 17 amino acids which are encoded by the whole of exon 5 and the other comprising three amino acids (lysine, threonine, and serine (KTS)) situated between the third and fourth zinc fingers encoded by the 3' end of exon 9. Four isoforms of the gene thus occur depending on the presence or absence of these regions.[3] These isoforms are present in a fixed proportion in tissues where they are expressed. *WT1* is expressed from the condensing mesenchyme to mature podocytes in fetal kidneys. The other sites are genital ridges and fetal gonads. Therefore, this gene is thought to play an important role in the development of the kidneys and gonads.[4 5] Functional impairment of this gene is considered to give rise to urogenital abnormalities and Wilms tumours. Denys-Drash syndrome and Frasier syndrome, both of which are characterised by nephropathy with genital abnormalities, have been recognised as disorders related to *WT1* mutations.

Denys-Drash syndrome consists of the triad of progressive nephropathy characterised by diffuse mesangial sclerosis (DMS), genital abnormalities, and Wilms tumour.[6] The incomplete form consists of nephropathy with genital abnormalities or Wilms tumour. In virtually all patients with Denys-Drash syndrome, point mutations are detected in the zinc finger domain encoded by exons 7 to 10 of the *WT1* gene.[7] The mutations noted in Denys-Drash syndrome patients are frequently missense changes in exons 8 and 9 that encode the second and third zinc fingers.

Frasier syndrome is a clinical entity proposed by Moorthy *et al*[8] to be related to but distinguished from Denys-Drash syndrome. Frasier syndrome is characterised by a slowly progressing nephropathy, male pseudohermaphroditism, and no Wilms tumour. As for histological findings of the kidneys, focal segmental glomerular sclerosis (FSGS) or focal glomerular sclerosis (FGS) is often observed in cases of Frasier syndrome, whereas DMS is noted in Denys-Drash syndrome patients. Recent reports have shown that Frasier syndrome arises from heterozygous mutation at the intron 9 splicing donor site of the *WT1* gene.[9–11] This intron 9 mutation leads to impairment of exon 9 alternative splicing with a consequent decrease in the +KTS isoform, thereby eventually causing a quantitative +KTS/−KTS isoform imbalance.

Recently, the presence of constitutional *WT1* mutations has been documented in some patients with nephropathy alone, such as isolated DMS (IDMS).[12 13] IDMS presents the same clinical features of nephropathy as those seen in Denys-Drash syndrome, but is not accompanied by any other anomalies or Wilms tumours.[14] The mutations were mostly located in exons 8 and 9 in these IDMS patients, and were thus similar to the mutations seen in Denys-Drash syndrome.

As noted above, some progressive nephropathies in children are thought to be related to *WT1* mutations. However, the clinical features of *WT1* related nephropathy have not been fully elucidated at the molecular level, especially in patients with FSGS not accompanied by other abnormalities. Analysis of the *WT1* gene in such progressive nephropathy is of value in clarifying the role of *WT1* in urogenital organ development. In this study, we analysed constitutional *WT1* mutations in patients with progressive nephropathies, such as FSGS, FGS, and DMS, which may be related to functional impairment of *WT1*, to investigate the correlation between the type of *WT1* mutation and the phenotype of nephropathy.

The clinical features of the 42 patients with progressive nephropathy are summarised in table 1. Thirty four patients were analysed in the present study, eight of whom (patients 1-3 and 10-14) were reported in our previous study.[9]

Of the 42 patients studied, 16 had nephropathy accompanied by genital abnormalities or Wilms tumours. Nine patients including a set of identical twins (patients 8 and 9) had been clinically diagnosed with incomplete Denys-Drash syndrome. In all the Denys-Drash syndrome patients, except one (No 5) who lacked detailed clinical progress data, the nephropathy had progressed rapidly to renal failure by the age of 2 years. Six patients (Nos 2, 4, and 6-9) with a 46,XY karyotype had diverse genital anomalies ranging from hypospadias and cryptorchidism to complete female external genitalia. Three patients (Nos 1, 3, and 5) developed a Wilms tumour. In seven patients including another set of identical twins (Nos 10 and 11), Frasier syndrome was strongly suspected based on the following clinical features. The course of nephropathy was slowly progressive; in four patients (Nos 12 and 14-16), the nephropathy did not require dialysis until more than 6 years after the onset and in the other three (Nos 10, 11, and 13) nephropathy had not yet led to renal failure. All patients had female external genitalia despite a 46,XY karyotype. None showed evidence of a Wilms tumour.

Of the remaining 26 patients, none was noted to have genital anomalies or Wilms tumours. In seven patients with IDMS, the nephropathy had developed at or before 2 years of age and progressed rapidly to renal failure in all but one patient (No 21). The clinical picture was diverse in 19 patients with FSGS. Age at the onset of nephropathy varied widely, ranging from 0 months to 15 years, and the age at which the condition progressed to end stage renal failure also varied considerably, 1 to 17 years. There were no signs of renal failure in three patients (Nos 30, 34, and 42). In two patients (Nos 32 and 41), detailed clinical progress data pertaining to the nephropathy were not available.

DNA extraction from leucocytes was carried out by the SDS-proteinase K method as previously described[15] or using the GFX genomic blood DNA purification kit (Amersham-Pharmacia Biotech). Amplification of exons 7 to 10 was performed using primers designed in the pre- and post-exon introns. The primers used included the following: for exon 7, 7D-S (5'-GACCTACGTGAATGTTCACATG-3') and 7C-A (5'-CTTAGCAGTGTGAGAGCCTG-3'); for exon 8, 8-S (5'-AGATCCCCTTTTCCAGTATC-3') and 8C'-A (5'-CAACAACAAAGAGAATCA-3'); for exon 9, 9C-S (5'-AAGTCAGCCTTGTGGGCCTC-3') and 9C-A (5'-TTTCCAATCCCCTCTCATCAC-3'); and for exon 10, 10C-S (5'-CACTCGGGCCTTGATAGTTG-3') and 10C-A (5'-GTCAGACTTGAAAGCAGTTC-3').[9] The

cycling protocol consisted of incubation at 94°C for three minutes, followed by 30 cycles of 94°C for one minute, 55°C (exons 8 and 9)/58°C (exons 7 and 10) for one minute, 72°C for one minute, and a final extension time of 72°C for five minutes. The resultant PCR products were purified through Microspin S-400 HR columns (Amersham-Pharmacia Biotech). The sequence reactions were carried out using either fluorescently labelled forward or reverse primers, a Thermo Sequenase fluorescently labelled primer cycle sequencing kit (Amersham-Pharmacia Biotech), and ALF DNA sequencer II (Amersham-Pharmacia Biotech). Whenever the sequence of the PCR product was ambiguous or mutated, the sequence reaction was repeated using the contradirected primer. The fluorescently labelled primers used were as follows: exon 7 forward: 7-S (5'-AGCCTCCCTTCCTCTTACTC-3'), and reverse: 7-A (5'-GTTTGCCCAAGACTGGACAG-3'); exon 8 forward: 8-S (5'-AGATCCCCTTTTCCAGTATC-3'), and reverse: 8C-A (5'-AAATCAACCCTAGCCCAAGG-3'); exon 9 forward: 9B-S (5'-TAGGGCCGAGGCTAGACCTTC TCT-3'), and reverse: 9C-A (5'-TTTCCAATCCCT CTCATCAC-3'); and exon 10 forward: 10B-S (5'-TGTGCCTGTCTCTTTGTTGC-3'), and reverse: 10B-A (5'-TGCTGCCTGGGACACTGAAC-3').[9]

Of the 42 patients studied, 18 were found to have constitutional mutations in the *WT1* gene (table 1). These mutations were heterozygous in all but one patient (No 1), in whom the mutation was homozygous.[15]

WT1 mutations were found in all 16 patients with genital abnormalities or Wilms tumour, the mutations being exonic in seven patients and intronic in nine patients. Of seven patients with exonic mutations, who had been clinically diagnosed as having Denys-Drash syndrome, six (Nos 1-3 and 5-7) carried missense mutations within exon 8 or 9. The conversions of the amino acids cysteine 355, 385, and 388, critical for the zinc finger structure, were found in patients 1, 2, and 5, respectively. The mutations in patients 3, 6, and 7 were located in the amino acids arginine 366, asparagine 396, and arginine 394, respectively, which are essential for DNA binding. These mutations have frequently been seen in patients with Denys-Drash syndrome.[16] In the other patient (No 4) with exonic mutations, there was a missense mutation in exon 7 which has not been reported previously. In this case, transversion of the nucleotide T 1025 to G leading to replacement by arginine of the amino acid 342 methionine adjacent to the zinc coordinating histidine was identified. Of the nine patients with intronic mutations, the identical twins (Nos 8 and 9), who had been clinically considered to have Denys-Drash syndrome and seven patients with Frasier syndrome (Nos 10-16), had point mutations at the intron 9 splicing donor site +2 (two cases), +4 (five cases), and +5 (two cases). These mutations, detected in patients in the present

Table 1 Clinical features and WT1 mutations in patients

Patient	*Age*	*Nephropathy onset*	*Renal failure*	*Histology*	*Karyotype*	*Genital status external & internal genitalia*	*Wilms tumour*	*WT1 mutation (exon/intron)*		*DNA*	*Amino acids*
Nephropathy with genital abnormalities or Wilms tumours											
Clinical Denys-Drash syndrome											
1*	1 y	1 y	1 y	FGS	46,XX	Female	+	Exon 8	1064	G→A	^{355}Cys → ^{355}Thr
2*	22 y	Unclear	2 y	FGS	46,XY	Female, streak gonad	−	Exon 9	1154	T→C	^{385}Cys → ^{385}Arg
3*	5 y	Unclear	1 mth	FGS	46,XX	Female	+	Exon 8	1097	G→A	^{366}Arg → ^{366}His
4	7 y	Unclear	1 y	ESRD	46,XY	Female	−	Exon 7	1025	T→G	^{342}Met → ^{342}Arg
5	5 y	<5 y	Not followed	Not known	NE	Female	+	Exon 9	1163	G→T	^{388}Cys → ^{388}Phe
6	1 y	1 y 2 mth	1 y 2 mth	DMS	46,XY	Hypospadias, cryptorchidism	−	Exon 9	1186	G→A	^{396}Asp → ^{396}Asn
7	9 y	Unclear	11 mth	DMS	46,XY	Hypospadias, cryptorchidism	−	Exon 9	1180	C→T	^{394}Arg → ^{394}Trp
8†	18 y	Unclear	7 mth	Dysplasia	46,XY	Female	−	Intron 9	+4	c→t	Disruption of splicing
9†	18 y	7 mth	7 mth	Dysplasia	46,XY	Female	−	Intron 9	+4	c→t	Disruption of splicing
Clinical Frasier syndrome											
10*‡	18 y	3 y	No	FGS	46,XY	Female, streak gonad	−	Intron 9	+4	c→t	Disruption of splicing
11*‡	18 y	3 y	No	FGS	46,XY	Female, streak gonad	−	Intron 9	+4	c→t	Disruption of splicing
12*	19 y	5 y	16 y	FGS	46,XY	Female, streak gonad	−	Intron 9	+4	c→t	Disruption of splicing
13*	3 y	3 y	No	Not known	46,XY	Female, streak gonad	−	Intron 9	+5	g→t	Disruption of splicing
14*	26 y	6 y	23 y	FGS	46,XY	Female, streak gonad	−	Intron 9	+2	t→c	Disruption of splicing
15	19 y	6 y	12 y	FSGS	46,XY	Female	−	Intron 9	+5	g→a	Disruption of splicing
16	18 y	2 y	8 y	FSGS	46,XY	Female	−	Intron 9	+2	t→c	Disruption of splicing
Nephropathy without genital abnormalities and Wilms tumours											
17	3 y	2 y	2 y	DMS	46,XY	Male	−	Exon 7	935	G→A	^{312}Arg → ^{312}Gln
18	2 y	1 y	1 y	DMS	46,XY	Male	−	−			
19	7 y	2 y	2 y	DMS	46,XY	Male	−	−			
20	18 y	0 y	1 y	DMS	46,XY	Male	−	−			
21	3 y	2 y 8 mth	No	DMS	46,XY	Male	−	−			
22	6 y	2 mth	2 mth	DMS	46,XY	Male	−	−			
23	Not known	8 d	1 y 2 mth	DMS	46,XX	Female	−	−			
24	18 y	2 y	4 y	FSGS	46,XX	Female, puberty(+)	−	Intron 9	+4	c→t	Disruption of splicing
25	8 y	1 y 9 mth	3 y	FSGS	NE	Female	−	−			
26	13 y	7 y	8 y	FSGS	46,XX	Female	−	−			
27	15 y	2 y 8 mth	7 y	FSGS	NE	Male	−	−			
28	19 y	1 w	5 y 2 mth	FSGS	46,XX	Female	−	−			
29	30 y	Unclear	11 y	FSGS	46,XX	Female	−	−			
30	24 y	7 y	No	FSGS	NE	Male	−	−			
31	11 y	4 y 6 mth	7 y	FSGS	NE	Not known	−	−			
32	Not known	15 y	Not followed	FSGS	NE	Male	−	−			
33	9 y	2 y 10 mth	3 y 11 mth	FSGS	46,XY	Male	−	−			
34	Not known	13 y	No	FSGS	NE	Male	−	−			
35	8 y	1 y 6 mth	1 y 7 mth	FSGS	NE	Male	−	−			
36	4 y	1 y 7 mth	2 y 10 mth	FSGS	NE	Male	−	−			
37	16 y	3 y 2 mth	4 y 4 mth	FSGS	NE	Male	−	−			
38	19 y	3 y 2 mth	17 y	FSGS	NE	Male	−	−			
39	9 y	3 y 1 mth	7 y 6 mth	FSGS	NE	Male	−	−			
40	19 y	5 y 5 mth	7 y	FSGS	NE	Male	−	−			
41	12 y	6 y 2 mth	Not followed	FSGS	NE	Male	−	−			
42	5 y	3 y	No	FSGS	NE	Not known	−	−			

*Patients 1–3 and 10–14 have been reported before.[9] †8 and 9, and ‡10 and 11 are identical twins. FGS, focal glomerular sclelrosis; ESRD, end stage renal disease; DMS, diffuse mesangial sclerosis; FSGS, focal segmental glomerular sclerosis; NE, not examined.

study (Nos 15 and 16), were consistent with those which have been reported in patients with Frasier syndrome.[9–11]

Of the 26 patients with nephropathy alone, only two (No 17 with IDMS and No 24 with FSGS) were noted to have *WT1* point mutations. The *WT1* mutation detected in patient 17 has been reported in cases without nephropathy, Wilms tumours with genital anomalies, or sporadic Wilms tumours. The amino acid 312 arginine was replaced by glutamine as a result of alteration of the nucleotide T 935 to A, and this mutation occurred upstream from the zinc finger domain. In patient 24, who had FSGS, a *WT1* mutation situated at position +4 of the intron 9 splicing donor site was detected; this mutation was the same as those reported in Frasier syndrome patients.[9–11]

The published constitutional *WT1* mutations in patients with nephropathy are thought to be classifiable into two groups: one is mutations occurring within the zinc finger domain, especially within exon 8 or 9, most of which are missense mutations, and the other is point mutations at the intron 9 splicing donor site.[16] In the present analyses in patients with nephropathy as well as our previous studies,[9] we identified *WT1* mutations in 18 such cases, that is, missense mutations at exons 7 to 9 in eight cases and mutations at the intron 9 splicing donor site in 10 cases (table 1). Depending on whether they had the exonic or intronic mutations, differences in clinical features were noted among these patients.

The eight patients with exonic mutations clinically had either Denys-Drash syndrome or IDMS. In six patients with Denys-Drash syndrome (Nos 1-3 and 5-7), *WT1* mutations were missense mutations within the second or the third zinc finger encoded by exon 8 or 9, respectively, whereas in one Denys-Drash syndrome patient (No 4), a missense mutation was detected in a rarely involved site, the first zinc finger encoded by exon 7. In patient 4, a point mutation with replacement of 342 methionine by arginine was detected. This replacement of a hydrophobic amino acid by a basic amino acid may disrupt the conformation of the first zinc finger. This mutant protein would potentially affect *WT1* gene functions, like the mutant proteins resulting from injury to the second or the third zinc finger that are often noted in Denys-Drash syndrome and IDMS patients.

The clinical features of patients with the exonic mutations studied may be summarised as follows. Nephropathy progressed rapidly to renal failure in all but one patient (No 5) whose clinical course was uncertain. Genital abnormalities were diverse, ranging from little if any impairment to 46,XY female. A Wilms tumour developed in three of the eight patients; the potential risk of Wilms tumour development in patients with exonic *WT1* mutations was thus reconfirmed.

The missense mutation observed in patient 17 was situated in exon 7 but upstream from the zinc finger domain. In published nephropathy cases, the mutation upstream from the zinc finger domain was rarely detected.[12 16] This mutation may exert an effect on *WT1* gene functions different from that of the mutations located within the zinc finger domain. To understand the mechanism of nephropathy development, further studies are needed to clarify the function of the resulting mutant protein.

Among 10 patients with intron 9 splicing donor site mutations, the clinical features of seven patients (Nos 10-16) were consistent with Frasier syndrome. Patient 24 actually had features concordant with nephropathy in Frasier syndrome. The patient developed nephropathy at the age of 2 that had progressed to renal failure by the age of 4. Renal biopsies showed findings characteristic of FSGS. The patient's karyotype was 46,XX, however. The conventional disease concept of Frasier syndrome has not been applicable to this patient since cases with a 46,XX karyotype were not described in the definition of Frasier syndrome proposed by Moorthy *et al.*[8] In this regard, we consider this case with a 46,XX karyotype to represent a variant form of Frasier syndrome based on both the clinical features of nephropathy and the types of *WT1* mutations. This patient, No 24, had normal female external genitalia and presented secondary female sex characteristics. Besides this patient, three other cases with a 46,XX karyotype accompanied by intron 9 splicing donor site mutations have been reported.[11 12 17] All these patients had nephropathy and normal sex differentiation, as did our case 24. They can be considered to have "genotypic female Frasier syndrome". It would be reasonable to assume that the intron 9 splicing donor site mutation does not interfere with feminisation in patients with a 46,XX karyotype, whereas this mutation strongly interferes with masculinisation since patients with a 46,XY karyotype who have intron 9 mutations invariably have female external genitalia.[9–11]

As mentioned above, the intron 9 mutation leads to impairment of exon 9 alternative splicing, thereby eventually causing a quantitative +KTS/−KTS isoform imbalance. Many studies have suggested functional differences among the isoforms, with or without KTS. These isoforms have different DNA binding properties[18]; the −KTS isoform has greater binding affinity for growth related genes.[19] In addition, two of these isoforms have been shown to vary in subnuclear localisation. The +KTS isoform appears to be involved in post-transcriptional RNA processing in association with splicing factors, while the −KTS isoform is situated in the transcriptional factor domain.[20 21] In the patients with Frasier syndrome, in whom masculinisation is impaired, the amount of the +KTS isoform diminishes relative to the −KTS isoform owing to the intron 9 mutation. Therefore, it appears probable that the +KTS isoform plays a significant role in masculinisation. The clinical features of patients with a 46,XX karyotype nevertheless indicate that the +KTS isoform probably has little significance in feminisation.

The clinical features of the patients with intron 9 mutations discussed so far are summarised as follows. The patients exhibited slowly progressive nephropathy, as reported for Frasier syndrome, as compared to those with exonic mutations. Whereas all 46,XY cases presented female external genitalia, normal sex differentiation was evident in the 46,XX cases. There was no evidence of Wilms tumour in the patients with intron 9 mutations analysed in this study. The incidence of Wilms tumour is considered to be markedly lower in patients with intron 9 mutations than in those with exonic mutations, since Wilms tumour had occurred in only one of the 27 cases analysed in this and previous studies.[9–12 17 22–25]

In the two patients with an intron 9 mutation (Nos 8 and 9), who were identical twins, the clinical manifestations also overlapped in part with those of Frasier syndrome, that is, 46,XY female external genitalia without the development of Wilms tumour. However, nephropathy progressed to renal failure as early as 7 months after onset. Because of the rapidity with which nephropathy progressed, the disease state was clinically considered to represent Denys-Drash syndrome rather than Frasier syndrome. Histological findings were those of dysplastic kidney and were not characteristic of either Denys-Drash or Frasier syndrome. An IDMS case with an intron 9 splicing donor site mutation with early onset and rapid progression of nephropathy was reported (patient P4 in the study by Jeanpierre *et al*[12]). The twin cases described here, and the case documented by Jeanpierre *et al*,[12] had a nephropathy course consistent with the clinical features of Denys-Drash syndrome. It follows that the nephropathy associated with *WT1* intron 9

mutations is more heterogeneous than was previously thought. The rapid progress of nephropathy in these cases might be the consequence, in part, of a possible influence of additional genes related to development of the kidneys.

In conclusion, this review of the clinical features of nephropathy in patients with *WT1* gene mutations indicates that differences exist in the progression of nephropathy, the degree of genital abnormalities, and the incidence of Wilms tumours between two types of mutations, that is, exonic and intronic mutations, although in a few cases (patients 8 and 9) features occasionally overlap. Thus, detecting and clarifying types of *WT1* mutations is considered to be useful for prognostic estimation of the clinical course in children with progressive nephropathy.

The authors are grateful to Dr K Yamamoto (Osaka Medical Center and Research Institute for Maternal and Child Health) and Dr O Motoyama (Toho University School of Medicine) for providing materials. This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas (09254258), a Grant-in-Aid for Scientific Research (A) (10307004) from the Ministry of Education, Science, Sports and Culture to JH, the Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare to JH, and Keio University Grant-in-Aid for Encouragement of Young Medical Scientists to AT.

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J Med Genet 2000;**37**:701–704

Interstitial deletion of chromosome 17 (del(17)(q22q23.3)) confirms a link with oesophageal atresia

EDITOR—There have been five previously described cases with a de novo interstitial deletion within the distal long arm of chromosome 17.[1–5] We describe a sixth case, 46,XY,del(17)(q22q23.3)de novo. Three patients, including the proband in this report, presented with tracheo-oesophageal fistula (TOF)/oesophageal atresia, highlighting a potential genetic locus for this significant congenital anomaly.

The patient was born at 32 weeks' gestation by Caesarean section for fetal distress and intrauterine growth retardation. The liquor volume was low. He was the first child of a 20 year old mother and 22 year old father who were non-consanguineous and healthy. The birth weight was 1420 g (9th centile) and head circumference was 27 cm (2nd centile). Apgar scores were 7 at one minute and 9 at five minutes. The patient was noted to be dysmorphic at birth and oesophageal atresia was diagnosed at 2 days of age (fig 1). The patient had a small fontanelle, a sloping forehead with wrinkled skin, a round facial appearance, hypertelorism, small eyes with upward slanting palpebral fissures, a broad nasal tip with a short philtrum, a downturned mouth, and thin lips. His palate and ears were normal. He had small nails, proximally placed thumbs, and a deep crease between his first and second toes. He had knee contractures and a fixed flexion deformity of his hips. There was no palpable hip instability and ultrasound confirmed this.

Echocardiography showed a ventricular septal defect with an overriding aorta, pulmonary valve stenosis, and a left to right shunt. Cranial ultrasound was normal, as was renal ultrasound. At surgery oesophageal atresia was