Letters to the Editor

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Mutations of the *TIGR/MYOC* Gene in Primary Open-Angle Glaucoma in Korea

To the Editor:

Glaucoma affects >3.5 million people in North America. Although treatable in the early stages, often it is not diagnosed and treated in time, which results in irreversible blindness. Primary open-angle glaucoma (POAG), the most common form of glaucoma, represents >50% of glaucoma cases in Western countries (Raymond 1997). POAG is an eye disorder characterized by the progressive excavation of optic disks, typical visual-field defects, and optic-nerve damage. Many families with autosomal dominant POAG have been reported (Brezin et al. 1997). The initial finding of a linkage between the juvenile-onset form of POAG (JOAG) and markers at the GLC1A locus on 1q21–31 (Sheffield et al. 1993) was subsequently confirmed in other families (MIM 137750). Recently, Stone et al. (1997) showed that mutations of the *TIGR/MYOC* (trabecular meshwork–induced glucocorticoid-response protein/myocilin) gene (MIM 601652), which maps at the GLC1A locus, were responsible for JOAG, as well as for middle-age–onset POAG. After publication of that report, other investigators have described various mutations in *TIGR/MYOC* in patients with JOAG/POAG (Adam et al. 1997; Suzuki et al. 1997; Mansergh et al. 1998; Michels-Rautenstrauss et al. 1998). Although a few mutations were found in different exons (Alward et al. 1998), most mutations reported to date are clustered in the third exon. This exon is evolutionarily conserved and bears sequence homology with the olfactomedin gene (Yokoe and Anolt 1993). We investigated whether Korean patients with JOAG/POAG have the mutations of the *TIGR/MYOC* gene. In our report we present two patients whose genomes harbor different *TIGR/MYOC* gene mutations.

After obtaining informed consent, we collected peripheral-blood samples from 45 unrelated patients with POAG who visited the Department of Ophthalmology at the Catholic University Medical Center, Korea. The patients were given diagnoses of POAG on the basis of findings from ocular examinations. Patients with POAG were determined to be affected if intraocular pressure (IOP) was >22 mmHg in both eyes and if the cup/disk ratio was >0.3, with characteristic visual-field loss and gonioscopic grade III or IV. Blood samples were also obtained from 106 patients who had visited the Catholic University Medical Center because of diseases other than POAG and who served as controls. Genomic DNA was extracted from each blood sample by means of DNA-isolation kits for mammalian blood (Boehringer Mannheim). The DNA fragments encoding portions of TIGR/MYOC protein were amplified by means of PCR and were analyzed by cold SSCP. The primers used for PCR are shown in table 1. The nucleotide numbers correspond to those in the work of Nguyen et al. (1998). A PCR reaction was performed in a 30-μl volume containing 50 ng of genomic DNA, 0.2 μM each of forward and reverse primers, 0.19 mM of each deoxyribonucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), 50 mM KCl, 1.5 mM $MgCl_2$, and 10 mM Tris-HCl, pH 8.3. The PCR product was denatured and separated on a 20% polyacrylamid Tris-borate EDTA gel (Novex). The DNA fragments were visualized by staining the gel with ethidium bromide solution. The PCR products exhibiting aberrant SSCP patterns were subcloned and sequenced by means of an ALF express DNA sequencer (Pharmacia) with fluorescent dye–primer chemistry. Multiple clones were sequenced to confirm the presence of both normal and mutant clones.

Of the 45 patients who were screened for mutations, 2 were found to carry variants in the *TIGR/MYOC* gene. We were able to recruit one patient for a family study. This family consists of five members (fig. 1*A*). The proband (individual 3; fig. 1*A*) was given a diagnosis of JOAG at age 15 years. At the time of diagnosis, her IOPs were 29 mmHg (right eye [OD]) and 30 mmHg (left eye [OS]), and she exhibited severe visual-field loss and optic-nerve damage. Because the disease progressed aggressively and medical treatment was not effective, surgery was required to control the progress of glaucoma in both eyes. None of the other family members was given a diagnosis of POAG, although individuals 1, 2, and 4 had slightly elevated IOPs compared with individual 5 (table 2), as well as with those in the control group.

Another patient whom we were unable to recruit for a family study received a diagnosis of POAG at age 59

Table 1

Primer Sequences for PCR and Conditions for PCR and SSCP

Region (nt[a])	Sequence: Forward	Sequence: Reverse	Temperature (°C): Annealing, for PCR	Temperature (°C): Running, for SSCP[b]	Product Size (bp)
88 to 281	5′-TGTGCACGTTGCTGCAGC-3′	5′-ATGGATGACTGACATGGCC-3′	56	10	204
1014 to 1202	5′-ATACTGCCTAGGCCACTGG-3′	5′-CAATGTCCGTGTAGCCACC-3′	62	14	189
1296 to 1493	5′CTGGAACTCGAACAAACCTGG-3′	5′-CATGCTGCTGTACTTATAGCG-3′	60	8	198
−83 to 281	5′-TGGCCACCTCTGTCTTCC -3′	5′-ATGGATGACTGACATGGCC-3′	60		384
177 to int1A	5′- AGGAAGGCCAATGACCAG -3′	5′-TAGGAGAAAGGGCAGGGGAGGC -3′	62		593
int1B to 795	5′-AACATAGTCAATCCTTGGGCC -3′	5′- CGGTGTCTCCCTCTCCACT -3′	56		170
796 to 1316	5′-GATGTGGAGGACTAGTTTGG -3′	5′- CCAGGTTTGTTCGAGTTCCAG -3′	56		521
1296 to 3′UTR	5′-CTGGAACTCGAACAAACCTGG-3′	5′-GCTTGGAGGCTTTTCACATC-3′	60		283

[a] Numbers correspond to those in the work of Nguyen et al. (1998).

[b] Three parts of the MYOC coding region were analyzed by SSCP, and the rest were used for sequencing the *MYOC* gene of the proband, individual 3.

years and displayed a moderate phenotype. Her IOPs before medication treatment (betaxolol HCl two times a day and dorzolamide HCl three times a day) were 26 mmHg (OD) and 24 mmHg (OS). Severe loss (OU) of visual field was observed, and cup/disk ratios were 0.8 (OD) and 0.6 (OS). Because her sister and one of her daughters were also given diagnoses of POAG, a genetic basis for its etiology could be suggested.

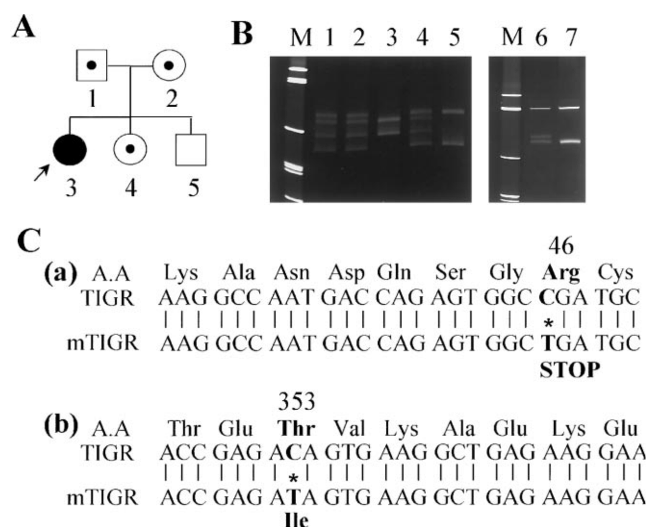

Figure 1 Pedigree of a family, analysis of SSCP, and sequences of regions with mutations in the *TIGR/MYOC* gene. *A,* Pedigree of a family with the C201T mutation. The unblackened symbol denotes a genotypically and phenotypically unaffected individual. The blackened symbol indicates an individual with documented evidence of POAG. A symbol with a dot indicates an obligatory carrier. The arrow indicates the proband. *B,* SSCP analysis of C201T (lanes 1–5) and C1123T (lanes 6 and 7) mutations, described in the text. Lane M, *Hae*III fragments of $\phi \times 174$RF. Lanes 1, 2, 4, and 6, Heterozygotes. Lane 3, Homozygote. Lanes 5 and 7, Normal. *C,* Sequence comparison between the *TIGR/MYOC* gene harboring mutations and the wild type. The altered nucleotides are shown in boldface and are denoted by an asterisk (*). Rows *TIGR,* wild-type sequence. Rows *mTIGR,* sequence from patients with POAG.

Mutations of the *TIGR/MYOC* gene were detected by SSCP analysis and were confirmed by sequence analysis. SSCP followed by sequence analysis of the proband, individual 3, revealed a C→T transition at nucleotide 201 in exon 1. This alteration resulted in a nonsense mutation at codon 46 (arginine→TAG, amber; fig. 1*Ca*). The complete *TIGR/MYOC* coding region of the proband, individual 3, was sequenced to confirm that there was no alteration other than a C201T transition. This mutation was presumed to result in a truncated protein with 45 amino acids. This proband carried only mutated alleles (fig. 1*B*), which was indicative of homozygosity for the *TIGR/MYOC* gene. Further analysis of her family revealed that her father, mother, and sister were heterozygous for *TIGR/MYOC,* apparently without any detectable symptom. The proband's brother, individual 5, had two normal copies and did not have any symptoms of POAG. The homozygote with this mutation is severely affected, whereas heterozygotes do not display any detectable POAG symptom. Thus, this family shows possible autosomal recessive inheritance of JOAG, whereas other families, which have been described elsewhere, show autosomal dominant inheritance. The heterozygotes may develop POAG later in life, because it is known that late-onset POAG shows age-dependent penetrance. Another possibility is that, because of consanguinity, the proband is homozygous at other loci that may modify the glaucoma phenotype. Importantly, there was an individual with the C201T mutation in the control group (1/106), whose POAG status has not been documented. This suggests that the C201T mutation may not be so rare in the Korean population, which supports the notion of autosomal recessive inheritance of JOAG. However, at this point, we cannot exclude the possibility that the proband's parents are genetically re-

Table 2

Clinical Data on Members of a Family with Familial JOAG and Their Genotypes for the *MYOC* Gene, Determined by SSCP and Sequencing

Pedigree[a]	Age (years)	Vertical Cup/ Disk Ratio		Tension (mmHg)		Gonioscopy	Visual Field[b]	Treatment	Genotype[c]
		OD	OS	OD	OS				
1	42	.5	.5	22	24	IV(D40r)	NA (OU)	None	Heterozygous
2	40	.4	.3	20	20	IV(D40r)	NA (OU)	None	Heterozygous
3	15	.4	.5	29	30	IV(D45r)	Nasal step (OU)	Trabe (OU)	Homozygous
4	13	.4	.5	22	22	IV(D40r)	NA (OU)	None	Heterozygous
5	12	.6	.4	17	17	IV(D40r)	NA (OU)	None	Wild type

[a] As in figure 1*A*.
[b] NA = not affected.
[c] As determined by SSCP and sequencing.

lated. Whereas further study is required to determine the recessive inheritance for JOAG/POAG, this family provides an opportunity to elucidate a molecular mechanism for *TIGR/MYOC* in POAG pathogenesis.

The mutation identified in a different family was heterozygous and was confirmed to be a C→T transition at nucleotide 1123 (fig. 1*Cb*). None of the 106 patients in the control group was found to contain the same mutation. The change resulted in the conversion Thr353 Ile (fig. 1*B,* lane 6, and *Cb*). This mutation resided in the olfactomedin-homology region in the third exon yet was different from all the mutations in the *TIGR/MYOC* gene that have been reported to date. Threonine at 353 residue is a putative phosphorylation site by protein kinase C, predicted by the PROSITE Pattern DB search program. Thus, the phosphorylation of TIGR/MYOC may play a role in the regulation of IOP in trabecular-meshwork cells.

The prevalence of C201T and C1123T mutations in the *TIGR/MYOC* gene in Korean patients was estimated by screening 45 unrelated patients with JOAG/POAG. The prevalence of each mutation in the *TIGR/MYOC* gene was 1 (2.2%) of 45 in patients with JOAG/POAG; thus the combined prevalence was 4.4%.

With support from another report (Kee and Ahn 1997), the present study indicates that the mutations in the *TIGR/MYOC* gene are responsible for JOAG/POAG in Korean patients. That report also described a phenotypic homozygote with JOAG linked to GLC1A, and it alluded to the autosomal recessive inheritance of JOAG. The analysis of the function of the different mutant forms of *TIGR/MYOC* in the regulation of IOP will enhance our understanding of POAG pathogenesis.

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Sung-Joo Kim Yoon,[1, 2] Hae-Suk Kim,[1, 2]
Joung-Il Moon,[2, 3] Jung Min Lim,[1, 2]
and Choun-Ki Joo[1, 2]
[1]*Department of Ophthalmology, and* [2]*Research Institutes of Medical Science, Catholic University Medical College, and* [3]*Department of Ophthalmology, St. Mary's Hospital, Seoul*

Electronic-Database Information

The URLs for data in this study are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for POAG [MIM 137750] and *TIGR/MYOC* [MIM 601652])

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Address for correspondence and reprints: Dr. Choun-Ki Joo, Department of Ophthalmology, Catholic University Medical College, 505 Banpo-dong, Seocho-ku, Seoul 137-701, Korea. E-mail: ckjoo@cmc.cuk.ac.kr

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Evidence for the Genetic Heterogeneity of Nephropathic Phenotypes Associated with Denys-Drash and Frasier Syndromes

To the Editor:

The association of constitutional heterozygous mutations of the Wilms tumor 1 (WT1) gene with the majority of cases of both Denys-Drash syndrome (DDS [MIM 194080]) (Denys et al. 1967; Drash et al. 1970) and Frasier syndrome (FS [MIM 136680]) (Frasier et al. 1964) has been well described; however, the characteristic nephropathies connected with these syndromes—that is, diffuse mesangial sclerosis (DMS [MIM 256370]) and focal segmental glomerulosclerosis (FSGS [MIM 603278])—occur more commonly in early life, as disorders confined to the kidney. It is unclear how frequent WT1 gene mutations are in this population.

The WT1 gene encodes a transcription factor critical for the normal development and function of the urogenital tract, reflected by the knockout-mouse homologue of WT1, in which homozygous inactivation of Wt1 causes absent kidneys and malformation of the gonads (Kreidberg et al. 1993). Alternative splicing results in at least 4 different zinc-finger protein isoforms, and the possibility of RNA editing and use of an alternative initiation codon increases this number to 16 (Bruening and Pelletier 1996). A correct ratio of isoforms appears crucial for normal gene function. During renal development, maximum WT1 expression occurs in condensing mesenchyme and during the mesenchymal-epithelial switch. In the mature nephron, WT1 expression is confined to the podocytes, a highly specialized layer of epithelial cells in the glomerulus. Here it may have a role in the maintenance of these cells, thus affecting the integrity of the glomerular filter (Pritchard-Jones et al. 1990). Accumulating evidence supports a regulatory role for WT1 in kidney development, although little is known about either its targets or which genetic cascades are affected by its abnormal function.

A wide variety of WT1 mutations is seen in DDS—a triad of intersex, nephropathy due to DMS, and Wilms tumor—making genotype-phenotype correlation difficult even with the aid of computer programs (Jeanpierre et al. 1998*a*). In contrast, FS is caused by specific intronic point mutations that disrupt the exon 9 alternative splice-donor site, reversing the normal WT1 +/− KTS isoform ratio (Klamt et al. 1998). It is also associated with intersex, but there is no predisposition to Wilms, and the nephropathy typically results from FSGS. These conditions act as human disease models of the effects of WT1 gene mutations and provide further strong evidence of WT1's crucial role in both renal and gonadal development. How WT1 mutations affect glomerular development remains a matter of debate; some mutations may be mediated during the progression of nephrogenesis, perhaps through aberrant interactions of WT1 with genes and proteins important for this process, thereby causing abnormal glomerular differentiation. It is equally possible is that the problem could lie in the terminally differentiated, nondividing podocytes, particularly if silencing of the normal allele were to occur, as in some tissues (e.g., placenta and brain [Jinno et al. 1994]), and if the mutant WT1 protein were incapable of maintaining normal podocyte function. Further study of animal models with targeted gene mutations may shed light on this increasingly complex picture.

Jeanpierre et al. (1998*b*) demonstrated that 4 of their 10 patients with DMS but no other features of DDS had mutations in exons 8 and 9 of WT1; 3 of these 4 patients were female. One mutation, 1147T→C (F383L) in exon 9, was novel. Two other mutations, 1186G→A (D396N) in exon 9 and 1129C→T (H377Y) in exon 8, had been found in previously reported cases of DDS. The fourth

mutation, (+4C→T) in intron 9, had been shown to cause FS (Barbaux et al. 1997; Kikuchi et al. 1998; Klamt et al. 1998). Schumacher et al. (1998) studied a broader spectrum of patients with early-onset nephrotic syndrome, to identify possible WT1 gene mutations; two of four patients who had DMS but lacked other features of either DDS or FS had mutations; one of them had a newly discovered mutation, 1135G→T (G379C) in exon 8, and the other had a mutation, 1180C→T (R394W) in exon 9, that previously had been reported described in a case of DDS; both patients were female and were reported to have other unspecified features consistent with this syndrome.

We tested the hypothesis that WT1 gene mutations occur in cases of DMS and of congenital/early-onset FSGS occurring in the absence of other features of DDS or FS. Our intent was to identify how common mutations of the WT1 gene were in this population and to begin establishing the boundaries of the DDS/FS spectrum of disease.

A series of 30 patients, 22 with DMS and 8 with FSGS, were screened for mutations of WT1 (table 1). The diagnosis of DMS or FSGS was established on the basis of either renal biopsy or postmortem renal histological findings. The majority of surviving patients with DMS were followed-up with yearly renal ultrasounds, to exclude Wilms tumor. Twenty-seven of the 30 patients had documented karyotype analysis. Congenital malformations were investigated by clinical examination and the appropriate investigations. None of the patients with psychomotor abnormalities were given a diagnosis of Galloway-Mowat syndrome. Abnormalities of the internal gonads were examined by pelvic ultrasound, and in some cases, by laparotomy. Patient 14 had an equivocal report with regard to karyotype—and, therefore, possible intersex status—but no other features of DDS. Two patients with DMS (patients 21 and 22) had DDS with known 1180C→T (R394W) mutations in exon 9 and were used as positive controls. Genomic DNA was obtained from blood of 23 patients and from paraffin-section material in 7 patients, by standard phenol-chloroform extraction methods. The two most common DDS mutations located in exon 9 (i.e., 1180C→T [R394W], 60%; and 1186G→A [D396N], 15%) abolish an *Rsr*II site in zinc finger 3 (Little et al. 1993). Heterozygous loss of the site was observed in patients 21 and 22, as expected, whereas patients 1–20 showed normal restriction-enzyme digests. We used SSCP to analyze exons 1–10 after PCR amplification, using oligonucleotides that had also been published by Baird et al. (1992*a*) and radiolabeling them with [^{32}P]. Products were electrophoresed, at 4°C, on both 5% and 10% nondenaturing polyacrylamide gels. In addition, exons 5, 8, and 9 were directly sequenced on an ABI 377 automated sequencer. PCR products were purified either on spin columns (Promega Biotec) or by gel extraction (Qiagen Gel Extraction Kit). Samples (2–5 μl) of the purified product were sequenced by use of a Thermo-Sequenase dye terminator cycle sequencing kit and 5 pmol of the original primer.

No WT1 mutations were detected in either the 20 patients with isolated DMS or the 7 patients with isolated FSGS, although patient 14 demonstrated a newly discovered polymorphism in codon 178 C→T in exon 4, which conserves a threonine, and patient 16 a demonstrated previously reported (Groves et al. 1992) polymorphism, an A→G transition in codon 313 (arginine) in exon 7, which destroys an *Afl*III restriction-enzyme recognition site. Both DDS patients showed the expected (as reported by Baird et al. 1992*b*) 1180C→T mutations in exon 9; in addition, a +5G→A mutation in intron 9 was detected during this study in patient 30, who had classic features of FS (previously reported by Klamt et al. 1998).

In conclusion, when a larger, more generalized population of cases with DMS is examined, mutations of WT1 are both less frequent than initial data have suggested and absent in isolated FSGS. This confirms the genetic heterogeneity of DMS and FSGS, despite the uniform renal histological findings seen throughout this group of conditions. Supportive evidence comes from linkage studies of familial FSGS, which show that the candidate gene for this condition lies on chromosome 1q25-q31 (Fuchshuber et al. 1995). Isolated DMS and FSGS may therefore also result from abnormalities of other glomerular genes, perhaps downstream of WT1 and mimicking the effects of WT1 mutations seen in DDS and FS.

We propose that, if WT1 gene mutations are present in isolated renal DMS or FSGS, this lends strong support to a diagnosis of DDS or FS, and the spectrum of DDS should be broadened to include occasional cases in which the characteristic nephropathy does occur alone. In this situation, WT1 mutations appear more common in phenotypic females, which affirms the less critical role of WT1 in female gonadal development, as has been suggested by experimental data (Nachtigal et al. 1998). This finding also correlates with cases of FS in which individuals with characteristic WT1 mutations in intron 9 and with a 46,XX karyotype develop nephropathy but have no obvious gonadal abnormality (Klamt et al. 1998). Karyotype analysis remains the most important first-line investigation in phenotypic females with isolated renal DMS or FSGS; however, WT1 mutation analysis should also be considered in isolated DMS and FSGS, since the presence of characteristic WT1 gene mutations may be important in the determination of Wilms tumor risk, especially in 46,XX individuals who may not demonstrate any other clues for an underlying diagnosis of DDS.

Table 1

Results and Clinical Status

		Nephropathy									
Case	Karyotype	Onset of Proteinuria	ESRF/ Dialysis?	Nephrectomy?	Transplant?	Syndromal Tumor?	Genital Status (Internal or External)	Other Renal or Extrarenal Defects?	Family History?	*WT1* Gene Mutation?	Age at Last Follow-up
DMS:											
1	46,XX	Day 1	Yes	No	Yes	No	Female	Psychomotor	Yes	No; exons 1–10	10 years
2	46,XX	Day 1	Yes	Yes, right and left	Yes	No	Female	No	No	No; exons 1–10	4 years
3	46,XX	Day 1	Yes	Yes, right and left	No	No	Female	No	Yes	No; exons 1–10	8 years
4	46,XY	Day 1	Yes	No	No	No	Male	No	No	No; exons 1–10	(Died at age $3\frac{1}{2}$ years)
5	46,XY	>1 year	Yes	Yes, right and left	Yes	No	Male	No	No	No; exons 1–10	13 years
6	46,XY	>1 year	Yes	Yes, right and left	Yes	No	Male	No	No	No; exons 1–10	$11\frac{1}{2}$ years
7	46,XY	>1 year	No	No	No	No	Male	No	No	No; exons 1–10	3 years
8	46,XX	Day 1	No	No	No	No	Female	Psychomotor, hypothyroidism	No	No; exons 1–10	(Died at age 7 mo)
9	46,XY	6 mo	Yes	No	Yes	No	Male	Pulmonary stenosis	No	No; exons 1–10	$14\frac{1}{2}$ years
10	46,XX	>1 year	No	No	No	No	Female	Psychomotor	No	No; exons 1–10	(Died at age $3\frac{1}{2}$ years)
11	46,XY	18 mo	Yes	No	No	No	Male	No	No	No; exons 1–10	$5\frac{1}{2}$ years
12	46,XX	Day 1	Yes	No	No	No	Female	Psychomotor	?	No; exons 2–10	(Died at age 2 mo)
13	Unknown	Day1	Yes	No	No	No	Female	No	Yes	No; exons 2–10	(Died at age 7 mo)
14	46,XY	Day1	No	No	No	No	Female	No	No	Exon 4 polymorphism	(Died at age 3 mo)
15	46,XX	Day1	No	No	No	No	Female	Psychomotor	No	No; exons 2–10	(Died at age 10 mo)
16	46,XY	Day 1	No	No	No	No	Male	Cryptorchidism?	No	*Afl*III polymorphism	(Died at age 2 mo)
17	Unknown	Day 1	No	No	No	No	Unknown	No	No	No; exons 2–10	(Died at age 10 wk)
18	Unknown	Day 1	No	No	No	No	Unknown	No	No	No; exons 2–10	(Died at age 8 mo)
19	46,XX	1 year	Yes	No	No	No	Female	Left kidney dysplastic	No	No; exons 5, 8, 9	$2\frac{1}{2}$ years
20	46,XY	9 mo	No	No	No	No	Male	no	No	No; exons 5, 8, 9	14 mo
21	46,XY	>2 years	Yes	Yes, right and left	Yes	Wilms	Female	No	No	Exon 9: 1180C→T (R394W)	13 years
22	46,XY	1 year	Yes	Yes, right and left	Yes	Wilms	Ambiguous	No	No	Exon 9: 1180C→T (R394W)	$11\frac{1}{2}$ years
FSGS:											
23	46,XX	4 mo		No	No	No	Female	No	No	Exons 1–10	7 years
24	46,XX	>1 year	?	?	?	No	Female	No	Yes	Exons 1–10	Lost to follow-up
25	46,XY	>1 year	?	?	?	No	Male	No	Yes	Exons 1–10	Lost to follow-up
26	46,XX	Day 1	No	No	No	No	Female	No	Yes	Exons 1–10	$3\frac{1}{2}$ years
27	46,XY	18 mo	Yes	No	Yes	No	Male	No	Yes	Exons 1–10	13 years
28	46,XY	>1 year	Yes	?	Yes	No	Male	No	No	Exons 1–10	7 years
29	46,XX	1 year	Yes	Yes, right and left	No	No	Female	Spondyloepiphyseal dysplasia	No	Exons 1–10	(Died at age 6 years)
30	46,XY	5 years	8 years	No	Yes	Gonadoblastoma	Female	No	?	Intron 9 +5G→A	16 years

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A. B. KOZIELL,[1] R. GRUNDY,[3] T. M. BARRATT,[2,*] AND P. SCAMBLER[1]

[1]*Molecular Medicine,* [2]*Nephrourology,* [3]*Haematology and Oncology Units, Institute of Child Health, London*

Electronic-Database Information

Online Mendelian Inheritance in Man (OMIM): http://www.ncbi.nim.nih.gov/Omim (for FS [MIM 136680], DDS [MIM 194080], DMS [MIM 256370], and FSGS [MIM 603278])

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Address for correspondence and reprints: Dr. Ania Koziell, Molecular Medicine Unit, Institute of Child Health, 30 Guilford Street, London WC1N 1EH. E-mail: A.Koziell@ich.ucl.ac.uk

*Present affiliation: Department of Oncology, The Birmingham Children's Hospital NHS Trust, Birmingham, England.

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Rett Syndrome in a Boy with a 47,XXY Karyotype

To the Editor:

Rett syndrome (RS [MIM 312750]) is a progressive encephalopathy characterized by severe mental retardation, autism, apraxia, seizures, stereotypical hand movements, and deceleration of head growth. Its prevalence is estimated at 1:10,000–15,000 female births (Hagberg 1995). The majority of cases are sporadic, but rare reports of familial recurrence have been made. In addition, all but 1 of the 10 MZ twins reported in the literature are concordant, whereas all 11 DZ twins reported are

discordant for the disorder (Migeon et al. 1995). Laboratory investigations have not revealed any metabolic abnormalities in affected individuals.

Chromosomal abnormalities and/or association with another syndrome have already been reported in patients with RS: a translocation t(X;22)(p11.22;p11) by Journel et al. (1990), a translocation t(X;3)(p21.3;p25.2) by Zoghbi et al. (1990) and Ellison et al. (1993), a deletion del(3)(3p25.1-p25.2) by Wahlström et al. (1996), and a deletion del(13)(13q12.1-q21.2) by Herder et al. (1996). RS was described in association with fragile X by Alembick et al. (1995) and with Down syndrome by Easthaugh et al. (1996). No concordance for the chromosomal abnormalities has been found, however, since different chromosomes and/or breakpoints were involved in each case. Vorsanova et al. (1996) reported a boy with RS and karyotype 46,XY/47,XXY (the 47,XXY cell line was observed in 6%–12% of the studied lymphocytes).

Here, we describe a patient with RS and a 47,XXY karyotype. The propositus, a male patient born in January 1995, was referred for genetic studies at age 28 mo. His parents are healthy, were aged 30 years (father) and 29 years (mother) at the time of the birth, and are not consanguineous. The child was born at term, after an uneventful pregnancy. His birth weight was 3.330 g (25th–50th percentile), his Apgar indices were 6 (1st minute) and 7 (5th minute), and his birth occipitofrontal head circumference was 32 cm (2.5 percentile). The perinatal period was uneventful. The propositus is the fourth child, and his older sibs—two boys aged 16 and 9 years and one girl aged 13 years—are normal. There is no history of neuropsychiatric diseases in the families of the mother or the father. The propositus showed normal development until age 8 mo. At that time, he sat without support, played normally, and was able to grasp objects and to put food into his mouth. He had also started to say some words comprehensibly.

The family noticed that, at age 11 mo, he had lost purposeful hand movements and language skills. He also began to show regression in social contact. At age 1 year, he began to show stereotypical hand movements, bruxism, and constipation. At age 28 mo, he presented severe global retardation and slightly diffuse hypotonia. He was socially isolated and made few spontaneous movements (other than the stereotypical hand movements). He did not grasp or otherwise show interest in any object or toy. He could vocalize but did not form any words. He reacted to luminous and sonorous stimuli. When standing up with support, he presented axial ataxia. Bruxism and short episodes of apnea were observed during consultation. No focal neurological signs or alteration in cranial nerves were observed. His occipitofrontal head circumference was 45 cm (2.5 percentile), his weight was 12.220 g (35th percentile), and his height was 87 cm (25th percentile).

When the patient was last seen, at age 37 mo, the loss of purposeful hand movements, the manual apraxia, and the slight global hypotonia were persistent. The stereotypy of his hand movements was midline, was constant in vigil, and showed a slightly athetoid component. When walking with support, he presented ataxia/apraxia. He reacted to luminous and sonorous stimuli. The episodes of apnea were more frequent and more sustained. His occipitofrontal head circumference was 46 cm (2.5 percentile), his weight was 15.200 g (35th percentile), and his height was 94 cm (25th percentile). Results of electroretinogram, magnetic resonance imaging of the brain, and electroencephalogram were normal. The results for rubella, syphilis, HIV I and HIV II, cytomegalovirus, herpes, cerebrospinal fluid, and serum amino acid testing were all normal. Toxoplasmosis testing showed that the patient's IgG level was slightly increased. However, acquired neurological disorders resulting from congenital toxoplasmosis infection were ruled out, since the boy was normal from birth until age ~8 mo.

Chromosomal analysis, including GTG banding, was performed on peripheral blood leukocytes as described by Seabright (1971). Karyotype analyses from all 300 banded metaphase preparations showed 47 chromosomes with an extra X chromosome (47,XXY).

To establish the origin of the nondisjunction, we analyzed DNA from the mother and the propositus with eight microsatellite markers from the dystrophin gene—5′DYSI; 5′DYSII; 3′DYSMS; STR 44; STR 45; STR 49; STR 50; and 3′-19n8. DNA from the father was not available. DNA analysis showed that the propositus had an allele that was not present in his mother, indicating, therefore, that the additional sex chromosome was paternal in origin—that is, it resulted from nondisjunction at the paternal first meiotic division.

For X-inactivation analyses, DNA was extracted from peripheral blood from the mother and the propositus, and 1 μg of digested (with *Alu*I and *Cfo*I) and nondigested DNA samples were used as templates for amplification of the androgen receptor (AR) highly polymorphic $(CAG)_n$ repeat, as reported (Allen et al. 1992; Edwards et al. 1992). All samples were run in duplicate in a 5% polyacrylamide gel (19:1 acrylamide:bis-acrylamide). A densitometer (Shimadzu CS-9000) was used to determine the ratio of X inactivation in each sample, and the mean of two readings was considered for each case. Since one allele may amplify more than the other, a correction factor was applied to compensate for unequal amplification of alleles. We did this for the mother and for the son, calculating, first, the ratio between the two alleles of the undigested DNA and correcting the final values for preferential PCR amplification (Pegoraro

et al. 1994). We calculated the degree of X inactivation on the digested DNA by normalizing the sum of allele A plus allele B to 100%, as reported in Sumita et al. (1998). The analysis of the X-chromosome–inactivation pattern in blood DNA showed X-inactivation ratios of 73:27 in the mother and $41X^{P}:59X^{M}$ in the affected son.

To rule out a possible diagnosis of Angelman syndrome (AS), the methylation status of the locus *SNRPN* mapped within the PWS/AS region was assessed by Southern blotting. The probe used was a 0.6-kb *Eco*RI-NotI fragment that contains exon 1 of *SNRPN* (Glenn et al. 1996). Methylation assay for AS was analyzed at the *SNRPN* CpG island and a normal result was obtained, with the presence of the 0.9-kb band from the unmethylated paternal allele and a 4.2-kb band from the methylated maternal allele. This method confirms the diagnosis in ~80% of cases, since in the remaining 20% AS may be due to UBE3A mutations or other unknown mechanisms (Kishino et al. 1997; Matsuura et al. 1997)

The parental origin of additional sex chromosomes was studied by Lorda-Sanchez et al. (1992) in 47 patients with a 47,XXY chromosome constitution. In 23 (49%) cases, the error occurred during the first paternal meiotic division, as observed in the present case. No significant clinical differences were found among patients of distinct parental origin.

To date, RS has been convincingly described only in females. Some cases described as RS syndrome in males have been reported (Coleman 1990; Eeg-Olofsson et al. 1990; Philippart 1990; Topçu et al. 1991; Christen and Hanefeld 1995; Vorsanova et al. 1996). The clinical signs and symptoms, however, were but suggestive, atypical, and/or partial. In the present report, the clinical and laboratory findings do not overlap with any described for Klinefelter syndrome. AS was excluded with 80% certainty, and extensive testing did not disclose any other alternative etiology, such as infantile neuronal ceroid-lipofuscinosis. The clinical findings met the criteria of inclusion and exclusion for the diagnosis of RS (Trevathan and Naidu 1988).

Several authors (Zoghbi et al. 1990; Webb et al. 1993; Camus et al. 1996; Webb and Watkiss 1996; Krepischi et al. 1998) reported that, as a group, RS patients tended to present a higher frequency of moderate skewing (20%–35% or 65%–80%) of X inactivation in lymphocytes, when compared with their mothers and normal controls, and that this skewing, when present, favors, in most cases, preferential inactivation of the paternally inherited X chromosome. On the other hand, it has been suggested that extreme skewed X inactivation could prevent manifestation of the RS phenotype in mutant-gene female carriers, which would be consistent with RS being a male-lethal trait (Schanen and Franke 1998; Xiang et al. 1998). In the present report, analysis of X inactivation in the proband and his mother did not show extreme skewed X inactivation, suggesting that the proband might be the result of a new paternal or maternal germ line mutation event. However, as shown previously, it is not known whether the X-inactivation pattern found in DNA from blood is representative of other tissues and, furthermore, a skewed pattern of X-inactivation in blood is not rare in normal females (Naumova et al. 1996; Sumita et al. 1998). Therefore, although the occurrence of moderate skewing is more frequent in RS patients and extreme skewed X inactivation has been observed in obligate RS carriers (Sirianni et al. 1998), a correlation between X-inactivation skewing and the RS phenotype must be interpreted with caution.

An explanation for the exclusive occurrence of RS in females, without evidence of male lethality, was proposed by Thomas (1966) on the basis of the fact that de novo X-linked mutations occurring exclusively in male germ cells could only be passed on to, and result in, an affected daughter. Under such a hypothesis, the absence of affected males is explained by the fact that sons do not inherit their X chromosomes from their fathers. Since our patient inherited one of his two X chromosomes from his father, his RS phenotype would be consistent with Thomas's hypothesis if the mutated gene was on the paternal X chromosome. On the other hand, RS-affected half sisters with the same mothers have been described (Archidiacono et al. 1991; Sirianni et al. 1998). However, under Thomas's hypothesis, it would be expected, in rare instances, to find families with half sisters with the same father, because of germinal mosaicism. This has already been demonstrated for other disorders such as achondroplasia (Philip et al. 1988) and Duchenne muscular dystrophy (Darras and Francke 1987) but apparently has not been reported for RS.

In a recent report, Sirianni et al. (1998) postulated that the relatively high frequency for RS would be explained by a high mutation rate in either male or female germ lines. In the present case, it was not possible to determine whether the mutation was inherited through paternal or maternal gametes.

With respect to the etiology of RS, several investigators have suggested the possibility of an alteration in the timing of replication of a gene (or genes) on the late X chromosome in RS patients (Riccardi 1986; Martinho et al. 1990; Kormann-Bortolotto 1992; Webb and Watkiss 1996). If this alteration represents the "misbehavior" of a gene (or genes) that should be inactive on the inactivated X chromosome but, when mutated, does not respond to XIST (the product of the X-inactivation–center gene), the consequence would be transient functional disomy at one or more loci. Partial functional disomy as a cause for RS (Webb et al. 1993) and other abnormal phenotypes, such as hypomelanosis of Ito or mental retardation, has already been suggested (Journel

1990; Schmidt and Du Sart 1992; Correa-Cerro et al. 1997; Wolff et al. 1998). If such a mechanism occurred in RS patients, this condition could be the result of functional disomy.

The present report, confirming an RS phenotype in a 47,XXY male, is consistent with the hypothesis that two X chromosomes are required for the manifestation of Rett syndrome.

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JOSÉ SALOMÃO SCHWARTZMAN,[1] MAYANA ZATZ,[2] LUCIANA DOS REIS VASQUEZ,[2] RAQUEL RIBEIRO GOMES,[2] CÉLIA P. KOIFFMANN,[2] CINTIA FRIDMAN,[2] AND PRISCILLA GUIMARÃES OTTO[2]
[1]*Universidade Mackenzie, and* [2]*Departamento de Biologia, Instituto de Biociências, Universidade de São Paulo, São Paulo*

Electronic-Database Information

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Address for correspondence and reprints: Dr. Mayana Zatz, Centro de Estudos do Genoma Humano, Departamento de Biologia, Instituto de Biociências, Universidade de São Paulo, CEP:05508-900, São Paulo, SP, Brazil. E-mail: mayazatz@usp.br
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Combining the Sibling Disequilibrium Test and Transmission/Disequilibrium Test for Multiallelic Markers

To the Editor:
Horvath and Laird (1998) describe the SDT (sibling disequilibrium test) which, like the sibling-association test (Curtis 1997), is a test for association in addition to linkage even when applied to sibships larger than sib pairs. These tests thus differ from the sibling transmission disequilibrium test (S-TDT [Spielman and Ewens 1998]), which is a test for linkage but not for association (unless attention is restricted to sib pairs). The possible advantage that the SDT has over Curtis's test is that it uses all affected sibs in the sibship, although it does not allow for special provision to be made to detect a recessive effect by testing whether there is an excess of affected sibs homozygous for one particular allele. Horvath and Laird demonstrate how the SDT can be applied to a multiallelic marker and how, in the case of a biallelic marker, the SDT and TDT can be combined, but they do not show how the tests can be combined for a multiallelic maker. Curtis described by using logistic regression how his test could be combined with multiallelic TDT data as implemented in the extended TDT (ETDT [Sham and Curtis 1995]), and here we show, using their multivariate sign test, that it is straightforward to apply Horvath and Laird's own approach to combine the multiallelic SDT with multiallelic TDT data.

Horvath and Laird use the "component" sign test (Bickel 1965; Randles 1989) as follows. For N sibships and a marker with m alleles, let s_i^j be 1, 0, or -1, according to whether, in the ith sibship, the frequency of allele j in affected sibs is higher than, equal to, or lower than that in unaffected sibs. Then define $\mathbf{S} = (S^1, S^2, \ldots, S^{m-1})$ where $S^j = \Sigma_{i=1}^{N} s_i^j$ and a matrix $\mathbf{W}$ having elements $W_{jk} = \Sigma_{i=1}^{N} s_i^j s_i^k$. The multiallelic SDT statistic is then $T = \mathbf{S}'\mathbf{W}^{-1}\mathbf{S}$, which is asymptotically χ^2_{m-1} under the null hypothesis of no association or no linkage. In order to extend this approach to include TDT data, we note that we can apply exactly the same formula to a sample of

$N/2$ trios (containing N parents) by using s_i^j to denote, instead, the transmission for the ith parent, being 1 or -1 if the parent has one copy of allele j and, respectively, does or does not transmit it to the affected subject and being 0 if the parent is uninformative for allele j (i.e., has 0 or 2 copies). Then the same statistic, $T = \mathbf{S}'\mathbf{W}^{-1}\mathbf{S}$, provides a non-parametric multiallelic TDT statistic. (This test is mathematically identical to the Stuart [1955] test presented by Sham [1997], and is in fact the score test of the Bradley-Terry model [Bradley and Terry 1952].) Of course it is obvious that we can sum both forms of s_i^j over a mixed sample of sibships and trios in a combined multiallelic SDT and TDT analysis. Formally, if we write $\mathbf{S}_{\mathrm{SDT}}$ and $\mathbf{W}_{\mathrm{SDT}}$ for the totals derived from the sibship data and $\mathbf{S}_{\mathrm{TDT}}$ and $\mathbf{W}_{\mathrm{TDT}}$ for those from the trios then $\mathbf{S}_{\mathrm{BOTH}} = \mathbf{S}_{\mathrm{SDT}} + \mathbf{S}_{\mathrm{TDT}}$, $\mathbf{W}_{\mathrm{BOTH}} = \mathbf{W}_{\mathrm{SDT}} + \mathbf{W}_{\mathrm{TDT}}$ and $T_{\mathrm{BOTH}} = \mathbf{S}'_{\mathrm{BOTH}}\mathbf{W}^{-1}_{\mathrm{BOTH}}\mathbf{S}_{\mathrm{BOTH}}$ is the combined statistic.

In order to use TDT data from families with more than one affected child, we can follow Martin et al. (1997) and can define s_i^j for the ith parent as being 1 if the parent is heterozygous for allele j and transmits this allele to more than half the affected children, -1 if the allele is transmitted to fewer than half the affected children, and 0 if the allele is transmitted to exactly half the affected children or if the parent is uninformative for this allele. (When there is only one affected child, this scoring scheme is equivalent to that given above.) TDT data can be used if only one parent is genotyped, provided that affected children homozygous for the marker are disregarded (Curtis and Sham 1995). When all these procedures are combined, the summations of the appropriate s_i^j can be performed over families consisting of discordant sibships and consisting of one or two parents having one or more affected children. The overall statistic $T_{\mathrm{ALL}} = \mathbf{S}'_{\mathrm{ALL}}\mathbf{W}^{-1}_{\mathrm{ALL}}\mathbf{S}_{\mathrm{ALL}}$ provides a test, for association with linkage, that makes appropriate use of all the available information from these different family types and that is asymptotically χ^2_{m-1}.

We propose that further efforts could proceed in three directions. First, the work of Horvath and Laird that considers the relative power of SDT and TDT could be extended in order to determine which is preferable to apply to a family suitable for either. This would depend on the transmission model of the disease and on the numbers of parents, affected siblings, and unaffected siblings who were genotyped. Second, a comparison of the performance of the above test versus those of tests utilizing logistic regression would be of interest. Third, the appropriateness of the asymptotic distribution could be investigated, since, for markers having large numbers of alleles, it might be that a Monte Carlo approach to assessment of significance could be desirable.

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David Curtis,[1] Michael B. Miller,[3] and Pak C. Sham[2]

[1]*Academic Department of Psychological Medicine, St. Bartholomew's and Royal London School of Medicine and Dentistry,* [2]*Department of Psychology, University of Missouri and* [3]*Department of Psychological Medicine, Institute of Psychiatry, London*

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Address for correspondence and reprints: Dr. David Curtis, Department of Adult Psychiatry, 3d Floor, Outpatient Building, Royal London Hospital, Whitechapel, London E1 1BB, United Kingdom. E-mail: dcurtis@hgmp.mrc.ac.uk
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