Letters to the Editor

Am. J. Hum. Genet. 62:189–192, 1998

A De Novo Mutation (Gln2Stop) at the 5′ End of the SRY Gene Leads to Sex Reversal with Partial Ovarian Function

To the Editor:

In mammals, the development of the embryonic gonads into either testes or ovaries determines gender. Sex reversal in XY females results from the failure of the indifferent gonad to develop into a testis. There are an unknown number of genetic loci in the testis-determining pathway, but there is ample evidence that the Y-chromosome gene that is essential for testicular development is the testis-determining gene, SRY (Goodfellow and Lovell-Badge 1993). Deletions of and inactivating mutations of SRY are among the known causes of XY sex reversal. Of XY females with pure gonadal dysgenesis, 10%–15% have been found to have mutations in the SRY gene (Hawkins et al. 1992*b;* Affara et al. 1993; Hawkins 1994). To date, 22 XY females with SRY mutations have been reported, and all but two of the mutations have been in the highly conserved high-mobility–group portion of the gene (i.e., the "HMG box"). Of the two mutations not in the HMG box, one was a deletion 5′ to the coding region of the gene (McElreavy et al. 1992*a*), and the other was a point mutation 3′ to the HMG box (Tajima et al. 1994). Patients with SRY deletions or mutations are, in general, normal females with complete gonadal dysgenesis. We describe a female patient with premature ovarian failure, an XY karyotype, and a presumably inactivating mutation of her SRY gene. We believe that this is the first reported case of an XY female whose sex reversal is due to an SRY mutation and in whom gonadal dysgenesis is incomplete. In addition, this patient's SRY gene mutation is itself unusual, in that it creates a stop in the second codon of the gene. A third unusual feature of this case is that tubules suggestive of early testicular development are seen in the ovary.

The patient is a 28-year-old West Indian woman who presented to one of us (L.S.), at the age of 28 years, with the primary complaint of infertility. She reported menarche and normal breast development at age 13–14 years and had had regular monthly menses until age 17 years, when she electively began oral contraceptives, which she continued until age 25 years. She reports that irregular menses (every 28–45 d) resumed with the discontinuation of the oral-contraception and that for the 2 subsequent years she attempted to get pregnant. Prior to our evaluation, she had been treated with clomiphene citrate, for presumed anovulation. When she presented to us, she was still having irregular menses. She appeared normally feminized, and her physical exam, including gynecological exam, was entirely normal, except for the fact that she was 193 cm tall. Specifically, she was not hirsute and had no stigmata of Turner syndrome. Breasts and pubic hair were Tanner stage 4. The following hormone levels were found: progesterone, 0.5 ng/ml; FSH, 44 mUI/ml; LH, 36 mUI/ml; prolactin, 12.8 ng/ml; testosterone, 0.25 ng/ml; and estradiol 2, 40 pg/ml. These values all suggested nonfunctional ovaries. A chromosome analysis showed a 46,XY karyotype. At laparoscopy, the gonads appeared to be white fibrous streaks and were removed without difficulty. The excised tissue was formalin fixed and routinely processed.

As noted, the patient's history, physical exam, and laboratory analysis were all suggestive of early ovarian failure. Because of her oral-contraception regimen, we cannot be sure about when her ovarian failure occurred; however, the patient's history of menses for 3 years after the cessation of an oral-contraception regimen is convincing. Standard G-banded chromosome analysis of peripheral lymphocytes showed a 46,XY karyotype in all 50 cells examined. Subsequently, fibroblasts cultured from a skin biopsy gave the same result. Two-color interphase FISH analysis of 100 nuclei by means of X and Y centromere probes (obtained from Oncor) failed to detect any cells with either two X chromosomes or without a Y chromosome. The excised gonad from the right side consisted entirely of fibroadipose tissue. The left gonad contained a small amount of ovarian stromalike tissue. No follicles were seen; however, a cluster of tubular structures was present. One of the tubules was ciliated, reminiscent of epididymis (fig. 1), and it was concluded that the structures were suggestive of embryonic male type. Fibroblastlike cells cultured from both gonadal-tissue samples also had a pure XY karyotype.

Because of the possibility of an SRY gene mutation, the entire coding region of the SRY gene was amplified from peripheral lymphocyte DNA, by means of primers

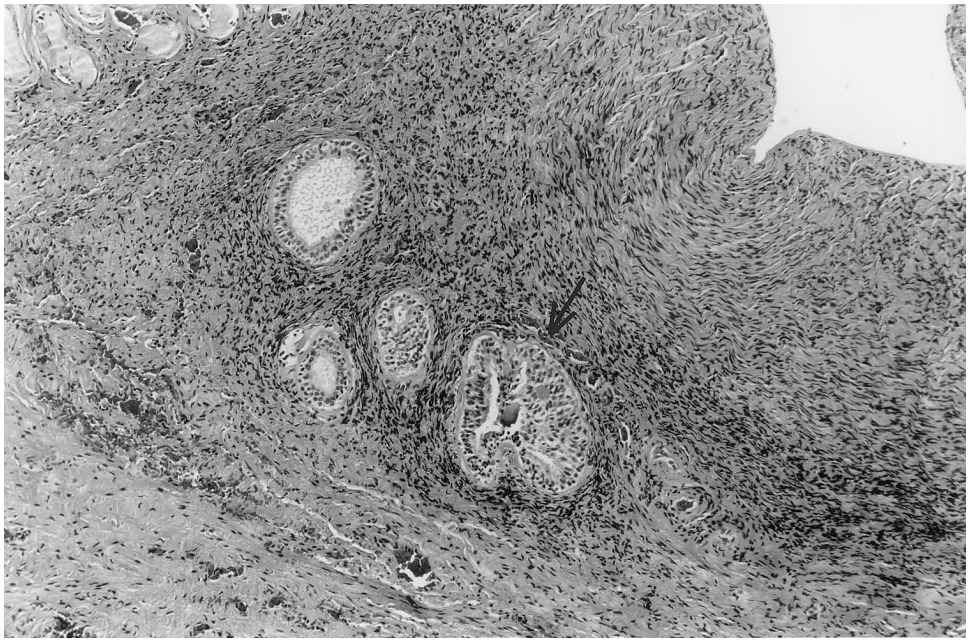

Figure 1 H- and E-stained section of the ovary, showing a ciliated tubule reminiscent of epididymis.

XES10 and XES11, as described elsewhere (Hawkins et al. 1992*a*), and were cloned into a plasmid. Two independent cloned PCR products showed the same C→T transition in the second codon of the gene. This was the only consistent deviation from the published sequence. Subsequent direct sequencing of pooled PCR products showed the same C→T transition (fig. 2). This C→T transition is predicted to create a stop codon (Gln2stop) at the second codon of the gene. The SRY gene was amplified and sequenced from both ovaries, and the same C→T mutation was found to be present bilaterally. Thus, there was no evidence of mosaicism for the mutation. The patient's father's SRY gene was sequenced and was shown to be entirely normal. In addition, paternity was proved by the normal segregation of polymorphic PCR-based markers (data not shown).

A stop mutation in the second codon of a gene is unusual, and it is unclear how a eukaryotic ribosome would respond to a stop placed immediately after the initiating methionine. Either initiation would never proceed to elongation, resulting in complete absence of protein product, or a low level of readthrough would occur with a reduced level of an otherwise normal protein. In order to investigate whether this mutant stop completely destroyed protein synthesis, hemagglutinin (HA)-tagged constructs of both the normal and mutant SRY genes were prepared. These constructs were then cloned into a eukaryotic expression vector so that the HA-tagged SRY gene was placed downstream from the cytomegalovirus promoter/enhancer. Normal and mutant constructs were transiently transfected into the BOSC 23 cell line by calcium phosphate coprecipitation, as described by Pear et al. (1993). SRY-HA proteins from lysates of transfected BOSC 23 cells were analyzed by immunoblotting by means of methods described elsewhere (Munsterberg et al. 1995). A representative result is shown in figure 3; the higher-molecular-weight band in the left-hand lane corresponds to the protein product

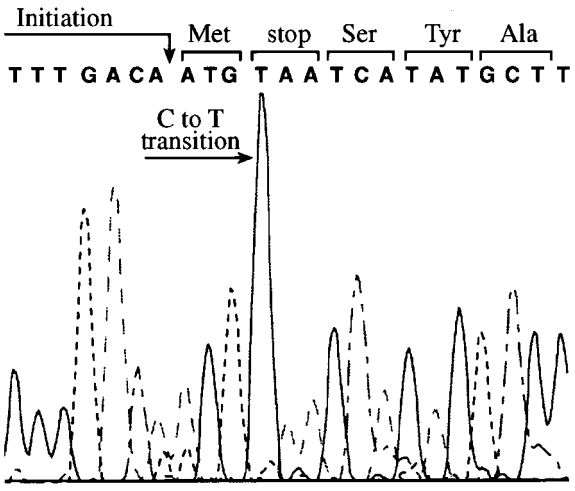

Figure 2 Electropherogram showing mutant SRY sequence. Sequencing was done directly from the PCR product, by use of the forward primer.

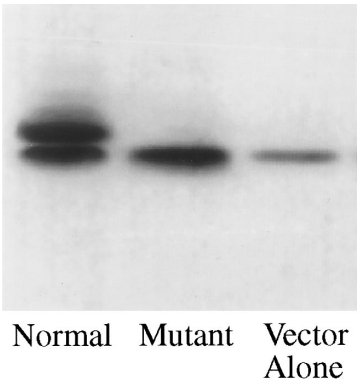

Figure 3 Western blot analysis of transiently transfected HA-tagged SRY constructs. The upper band in the left-hand lane arises from the transfected SRY construct, the lower band, common to all lanes, is due to nonspecific binding of the anti-HA antibody.

from a normal SRY gene, whereas no such band is detectable with the mutant SRY allele (middle lane). The constant band is due to nonspecific binding of the anti–HA-tag antibody. Although only one representative example is shown, this experiment was repeated a total of four times, with identical results. We conclude from this analysis that, if the mutant protein is expressed, it must be at a level below the sensitivity of our system, which we estimate to be ~1%.

Among patients with sex reversal and SRY-gene mutations, this woman is quite atypical, since she had normal menarche, normal secondary-sexual characteristics, and >3 years of at least partial ovarian function after the age of 25 years, when she discontinued an oral-contraception regimen. A total of ⩾22 SRY-gene mutations have been reported by several authors (Hawkins et al. 1992*a;* Jager et al. 1992; McElreavey et al. 1992*a;* McElreavy et al. 1992*b;* Affara et al. 1993; Zeng et al. 1993; Iida et al. 1994; Poulat et al. 1994; Tajima et al. 1994; Schmitt-Ney et al. 1995). In all of the reported cases, pure gonadal dysgenesis was present. This patient has a single-base-change mutation of her SRY gene, which is likely to have occurred either during spermatogenesis or very early postzygotically. This mutation creates a stop codon immediately following the initiating methionine and apparently inactivated her SRY gene. It is unusual, since all other known mutations of the SRY gene are either in the HMG-box region of the gene or 3′ to that region. It has been generally accepted that the reason that most of the reported mutations in the SRY gene are in the HMG box is that these are the mutations that disrupt the function of the gene and that other mutations are likely to be silent. However, premature termination upstream from the HMG box would also destroy function and would not be predicted to be silent. We considered the possibility that the unusual phenotype (i.e., partially functional gonads) in this case might be related to the unusual mutation. For instance, could a low level of SRY protein lead to the survival of germ cells? To this end, we sought to assess the possibility that this mutation is "leaky." Our results suggest that it is not.

Another possibility is that the unusual mutation in this patient has nothing to do with her unusual phenotype and that, with respect to the gonad, XY individuals with an SRY mutation are analogous to 45,X (Turner syndrome) patients. In 45,X, the presence of only one X chromosome is apparently not an absolute barrier to ovarian function, since ~5% of these girls will have some ovarian function (Palmer and Reichmann 1979; Hall et al. 1982). It has been hypothesized that this variation in the Turner-syndrome phenotype is due to a variable rate of decay of the oocytes (Hall et al. 1982; Page et al. 1990). Only 22 patients with XY karyotypes and SRY mutations have been described. If, as in 45,X, there is significant variability in gonadal function, then 22 patients is not enough from which to draw firm conclusions. As the number of such individuals increases, it may become apparent that a small percentage have partial ovarian function. This concept is supported by the observation that, in mice, the presence of only one X chromosome is associated with fertility and only a mild reproductive disadvantage (Epstein 1986). In addition, a mouse with a heritable mutation in the testis-determining gene has been described (Lovell-Badge and Robertson 1990). XY mice with this mutation are fertile females, although fertility is reduced, and their ovaries fail early, a picture quite similar to that of the patient whom we present.

In summary, we conclude that the patient whom we present is sex reversed because of a point mutation in her SRY gene. Further, we conclude that the presence of a cytogenetically normal Y chromosome is not an absolute barrier to ovarian function in humans. In addition, this and other reported patients prove that mutations outside the HMG box of the SRY gene do occur and should be looked for in the setting of sex reversal. Although it is unlikely that many women with premature ovarian failure will have a Y chromosome, it is still desirable to karyotype all such women.

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Stephen Brown,[1] C. C. Yu,[2] Patricia Lanzano,[1] Debra Heller,[3] L. Thomas,[4] Dorothy Warburton,[5] Jan Kitajewski,[1] and Laurel Stadtmauer[1]

[1]*Department of Obstetrics and Gynecology,* [2]*Department of Pathology, and* [3]*Department of Genetics and Development and Department of Pediatrics, Columbia University,* [4]*Presbyterian Hospital in the City of New York, and* [5]*Department of Pathology, Harlem Hospital, New York*

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Address for correspondence and reprints: Dr. Stephen Brown, Department of Obstetrics/Gynecology, PH 16, Columbia University, 630 West 168th Street, New York, NY 10032. E-mail: brown@cuccfa.ccc.columbia.edu

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Founder Effect, Seen in the British Population, of the 172 Peripherin/*RDS* Mutation—and Further Refinement of Genetic Positioning of the Peripherin/*RDS* Gene

To the Editor:

Peripherin/retinal degeneration slow (RDS) is a membrane-associated glycoprotein found in the outer segments of retinal rod and cone photoreceptor cells. It is thought to play a role in membrane structural stabilization, in conjunction with retinal outer segment membrane protein 1 (ROM1).

Mutations in the *RDS* gene give rise to retinal degenerations with a wide phenotypic spectrum. The majority of mutations result in macular dystrophies (reviewed in Keen and Inglehearn 1996). Specific mutations in the *RDS* gene may lead to a wide inter- and intrafamilial variability of phenotype, as seen in one family with retinitis pigmentosa, pattern dystrophy, and fundus flavimaculatus, in three different members with a deletion at codon 153/154. (Weleber et al. 1993)

Mutation analysis by heteroduplex and direct sequencing of PCR-amplified coding exons of the *RDS* gene was performed in 300 British patients with dominantly inherited macular dystrophies; 7.3% of this group had peripherin/*RDS* mutations, segregating with disease. One particular mutation accounted for 11 of

Table 1

Peripherin/RDS Mutations Carried by 10 Families That Do Not Have a 172 Mutation

Mutation Number	Mutation	Phenotype
1	220Arg→Trp	Pattern dystrophy
2	213Cys→Arg	Pattern dystrophy
3	234ins (1 bp)	Pattern dystrophy
4	258Tyr→stop	Pattern dystrophy[a]
5	210Pro→Arg	Pattern dystrophy
6	224ins (37 bp)	Adult vitelliform
7	140ins (1 bp)	Pattern dystrophy[b]
8	219Pro→Arg	Macular dystrophy
9	221Arg→Gln	Pattern dystrophy
10	87del (8 bp)	Pattern dystrophy

[a] Previously reported by Wells et al. (1993).
[b] Previously reported by Keen et al. (1994).

the 22 mutations found in the macular-dystrophy group. This change, identified as a C→T change at codon 172, results in an Arg→Trp change (hereafter designated "172Arg→Trp"). This amino acid is located in the second intradiskal loop of the protein. This loop is thought to be the most important for the functioning of the protein, stabilizing the photoreceptor discs through homophilic or heterophilic interactions across the intradiskal space, and associating covalently with ROM1.

The 172Arg→Trp mutation was found in 11 families (1 of these families has previously been reported by Wells et al. 1993). One additional family was found to have an 172Arg→Gln mutation segregating with disease (this family also has previously been reported by Wells et al. [1993]). The mutations carried by the other 10 families are given in table 1.

Mutations at codon 172 have previously been reported by Wroblewski et al. (1994) and Reig et al. (1995); Wroblewski et al. described three families, two with 172Arg→Trp mutations and one with a 172Arg→Gln mutation, all giving rise to macular dystrophy, and Reig et al. described one Spanish family with a 172Arg→Trp mutation causing central areolar choroidal dystrophy. Two of these latter three families are included in the present study. Phenotypic studies of patients with mutations at codon 172 have been performed by Nakazawa et al. (1995), Wada et al. (1995), and Piguet et al. (1996). All of these groups studied single families and noted that the patients showed a characteristic autosomal dominant macular phenotype. This would suggest that, in addition to the 172Arg→Trp mutation being due to a founder effect in the British population, mutations at codon 172 are not uncommon in causing macular dystrophy.

We investigated whether this preponderance of the mutation at codon 172 was the result of a founder effect or was due to a mutational hotspot in the gene. Haplotype analysis of these 12 families by means of six microsatellite repeat markers, distributed over a 20cM interval around the *RDS* locus, showed remarkable conservation of alleles between the families. Affected individuals share at least five of the alleles. The family with a 172Arg→Gln mutation was included as a control in the analysis (table 2). The frequencies of the alleles that constitute the disease haplotype in the British population are given in table 3. Both their low frequency and the absence of this haplotype, in its entirety, in any of our 50 controls (taken from the same population as that containing the macular-dystrophy families) would therefore support the conclusion that this mutation is due to a founder effect. All 11 families are therefore ancestrally related, with an initial mutation event occurring many generations ago.

Table 2

Ancestral Disease Haplotype Shared by 11 Families That Have the 172Arg→Trp Mutation in Peripherin/*RDS,* Compared with Ancestral Disease Haplotype of a Family That Has the 172Arg→Gln Mutation

	Haplotype in Families with 172Arg→Trp Mutation											Haplotype in Family with 172Arg→Gln Mutation
Marker	Family 1	Family 2	Family 3	Family 4	Family 5	Family 6	Family 7	Family 8	Family 9	Family 10	Family 11	
D6S258	3	3	3	3	3	6	6	6	6	2	2	4
D6S276	2	6	6	7	7	7	7	7	7	7	5	5
D6S291	1	1	1	1	1	1	1	1	1	1	1	2
D6S271	5	5	5	5	5	5	5	5	5	5	5	1
D6S282	3	3	3	3	3	3	3	3	3	3	3	3
D6S459	2	2	2	2	2	2	2	2	2	2	2	2
D6S294	5	5	5	5	4	4	5	5	5	5	5	3

Table 3

Allele Frequencies of Disease Haplotype in 50 Ethnically Matched Controls from the British Caucasian Population

Marker	Allele of Disease Haplotype	Frequency in Control Population
D6S258	6	.21
D6S276	7	.22
D6S291	1	.2
D6S271	5	.18
D6S282	3	.12
D6S459	2	.11
D6S294	5	.11

In addition, recombination events telomeric and centromeric to the *RDS* gene were observed in two separate individuals, both with the 172Arg→Trp mutation; therefore, these two individuals do not share the complete disease haplotype with the other individuals in these 11 families. This has enabled us to genetically localize the *RDS* gene to a 1.2-cM region between D6S1582 and D6S271 (table 4 and fig. 1).

We can therefore conclude that the most commonly occurring peripherin/*RDS* mutation in the British population is the 172Arg→Trp mutation, and this is consistent with the hypothesis of a founder effect. Prior to identification of the 172Arg→Trp mutation, these 11 families had been referred separately with different diagnoses, including cone dystrophy, macular dystrophy, and central areolar choroidal dystrophy. After review of the clinical data, it was clear that these families shared a common phenotype (S. M. Downes, unpublished data). All had significant loss of central vision, with a distinctive retinal appearance. This characteristic phenotype seen in these 11 families should alert the clinician

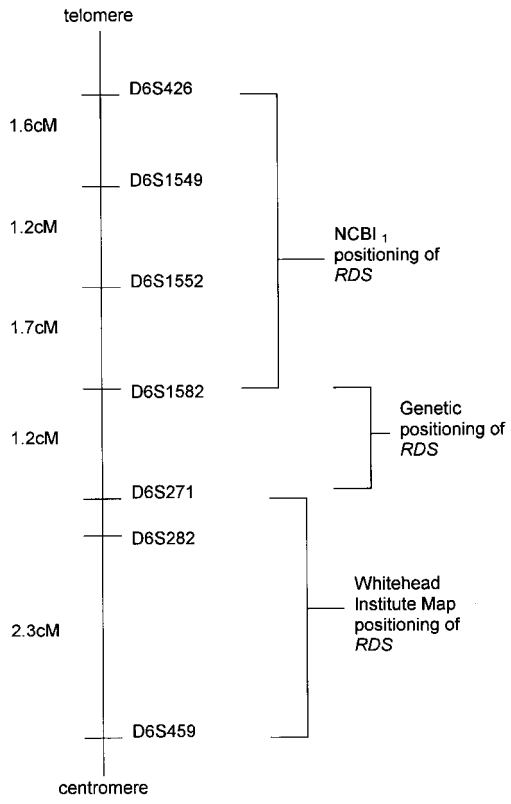

Figure 1 Genetic positioning of *RDS* on chromosome 6p, based on the recombinations seen in the families in the present study. (Information from the National Center for Biotechnology, National Institutes of Health)

Table 4

Recombination Events Enabling Genetic Positioning of *RDS*

Marker	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7
D6S258	6	6	6	6	6	3	3
D6S276	7	7	7	7	7	7	6
D6S291	1	1	1	1	1	1	2
D6S1552	3	3	3	3	3	3	1
D6S1582	3	3	3	3	3	3	4
RDS							
D6S271	4	5	5	5	5	5	5
D6S282	2	3	3	3	3	3	3
D6S459	2	2	2	2	2	2	2
D6S294	4	4	4	5	5	5	5

NOTE.—Patients 1 and 2 are child and parent, respectively; patient 2 shows a recombination event centromeric to the *RDS* gene, whereas patient 7 shows a telomeric crossover. By means of the disease haplotype of the extended ancestral families, the *RDS* gene has been positioned between D6S1582 and D6S271, a distance of 1.2 cM (CEPH data).

to the possibility of a peripherin/RDS mutation at codon 172.

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Annette M. Payne,[1] Susan M. Downes,[1,2] David A. R. Bessant,[1,2] Alan C. Bird,[1,2] and Shomi S. Bhattacharya[1]

[1]*Department of Molecular Genetics, Institute of Ophthalmology, and* [2]*Moorfields Eye Hospital, London*

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Address for correspondence and reprints: Dr. Annette M. Payne, Department of Molecular Genetics, Institute of Ophthalmology, 11-43 Bath Street, London, United Kingdom. E-mail: apayne@hgmp.mrc.ac.uk

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Cystic Fibrosis Transmembrane-Conductance Regulator Mutations among African Americans

To the Editor:

Cystic fibrosis (CF) is less common in African Americans than in Caucasians of northern European descent, with an estimated incidence of 1/15,300 (Hamosh et al., in press), although the severity of the disease is comparable across racial lines. Macek et al. (1997) recently reported in the *Journal* the identification of several CF transmembrane conductance regulator (CFTR) mutations of noteworthy prevalence in blacks. This information will help clinical laboratories to improve the sensitivity of CF mutation testing for African American patients.

We have identified a CFTR mutation in exon 7 in two unrelated individuals of African American descent who were not included in the study by Macek et al. The mutation, ΔF311, results in the loss of a phenylalanine residue in the fifth transmembrane domain of the CFTR protein. One of our patients is a 4-year-old African American female who presented, at age 7 mo, with hypochloremic metabolic alkalosis and dehydration. She was subsequently found to have sweat chloride values on two occasions of 75 and 83 mEq/liter. Her lung disease is mild, with only slight peribronchial thickening on chest x-ray, and she had *Staphylococcus aureus* in her sputum at age 3 years. She is considered pancreatic sufficient, on the basis of qualitative fecal fat analysis. Without pancreatic-enzyme supplementation, she has maintained a normal growth pattern, with height and weight at the 50th percentile. Mutation testing determined her genotype to be ΔF508/ΔF311. The ΔF311 allele was first detected by the appearance of a distinct heteroduplex pattern when PCR product encompassing exon 7 was electrophoresed on 10% polyacrylamide. The mutation was identified as ΔF311 by dideoxy sequencing. No additional ΔF311 alleles have been found after a screening of a further 271 patient samples (~8.5% African American) at the University of North Carolina in Chapel Hill.

The second patient was referred for genetic testing because of abnormal fetal ultrasound findings. The patient was a 25-year-old (G2 P0 SAB1) African American. An ultrasound performed at 17 wk gestation identified a fetus with a Dandy-Walker malformation and an echogenic bowel. Follow-up ultrasound at 18.2 wk gestation confirmed the CNS abnormalities and a grade II echogenic bowel. The patient was counseled with regard to the numerous causes of Dandy-Walker malformations, as well as with regard to the causes of echogenic bowel, including CF. The fetal karyotype was normal, but maternal and fetal CF testing identified a heteroduplex pattern identical to the ΔF311 heterozygote pos-

itive control (Mutation Detection Enhancement gel system; FMC BioProducts). Patient DNA mixed with equal amounts of ΔF311 control DNA showed the same heteroduplex pattern as did either the patient DNA sample or the ΔF311 heterozygote DNA sample alone, suggesting that these abnormal alleles were identical. DNA sequencing using the ABI 377 nucleic acid sequencer subsequently confirmed this sequence change to be the ΔF311 mutation in heterozygous form.

Maternal cell contamination was ruled out by MCT-118 genotyping. The father of the fetus was not available for testing, and no other CF mutation or abnormal heteroduplex pattern was detected in the fetal sample. Because of the presence of the Dandy-Walker malformation, and prior to the CF results being provided to the patient, the patient elected to terminate the pregnancy. An autopsy was not performed, and fetal tissue was not available for confirmation of the amniocentesis results.

ΔF311 was first reported in a 2-year-old boy with a positive albumin-meconium test at birth and with repeatedly elevated sweat tests by age 4 mo (Meitinger et al. 1993). His other mutation is ΔF508. Prophylactic treatment with both pancreatic enzymes and mucolytic agents to deter lung disease has prevented the onset of either pulmonary or pancreatic symptoms in his first 6 years. The authors of that study did not identify any other individuals with this mutation, after screening an additional 205 CF chromosomes by SSCP (T. Meitinger, personal communication). This patient is of Bavarian Caucasian descent, and his pancreatic disease is distinct from that of the patient seen at the University of North Carolina in Chapel Hill (UNC), obscuring any correlation between ΔF311 and a particular phenotype. Apparent clinical dissimilarities among these three patients might be attributable to undefined aspects of either the genetic background or the environment, but low numbers prevent the drawing of conclusions along racial or other lines. Interestingly, the two individuals whom we describe, as well as the index case, each harbor a distinct ΔF311-associated haplotype (1 1 2, 1 2 2, and 2 1 2) defined by the flanking markers, XV2c-KM19-J3.11, suggesting that this mutation has occurred more than once. The multiple origins of ΔF311 suggest that it might be found on additional chromosomes, but this would not be limited to African American patients.

ΔF311 has thus been identified in two individuals of African American ancestry. In this racial group, this mutation appears to be more common than any CFTR mutation except DF508, compared with other alleles also identified in Caucasians. Among the 23 African American CF patients genotyped at UNC, the inclusion of ΔF311 increased total mutation-detection rates by ~2%. On the basis of the criteria established by Macek et al., we feel that molecular diagnostic laboratories should consider the inclusion of ΔF311 in the development of CF mutation-testing panels tailored to African Americans.

K. J. Friedman,[1] M. W. Leigh,[2] P. Czarnecki,[3] and G. L. Feldman[3]

[1]*Curriculum in Genetics and* [2]*Department of Pediatrics, University of North Carolina, Chapel Hill; and* [3]*Department of Medical Genetics, Henry Ford Hospital, Detroit*

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Address for correspondence and reprints: Dr. Kenneth J. Friedman, Curriculum in Genetics, CB #7525, University of North Carolina, Chapel Hill, NC 27599. E-mail: bluemold@med.unc.edu

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mtDNA Mutations That Cause Optic Neuropathy: How Do We Know?

To the Editor:

Leber hereditary optic neuropathy (LHON) is an inherited form of bilateral optic atrophy in which the primary etiological factor is a mutation in the mitochondrial genome (mtDNA) (reviewed in Johns 1994; Riordan-Eva et al. 1995; Nikoskelainen et al. 1996; Howell 1997). Wallace et al. (1988) were the first group to identify a LHON mutation, when they showed that a high proportion of LHON families carried a mutation, at nucleotide 11778, that results in the substitution of histidine for the highly conserved arginine at amino acid position 340 of the ND4 subunit of complex I (NADH–ubiquinone oxidoreductase). The 11778 mutation is found in 50%–70% of all LHON pedigrees (e.g., see Mackey et al. 1996). Since the study by Wallace et al. (1988), hundreds of LHON patients from around

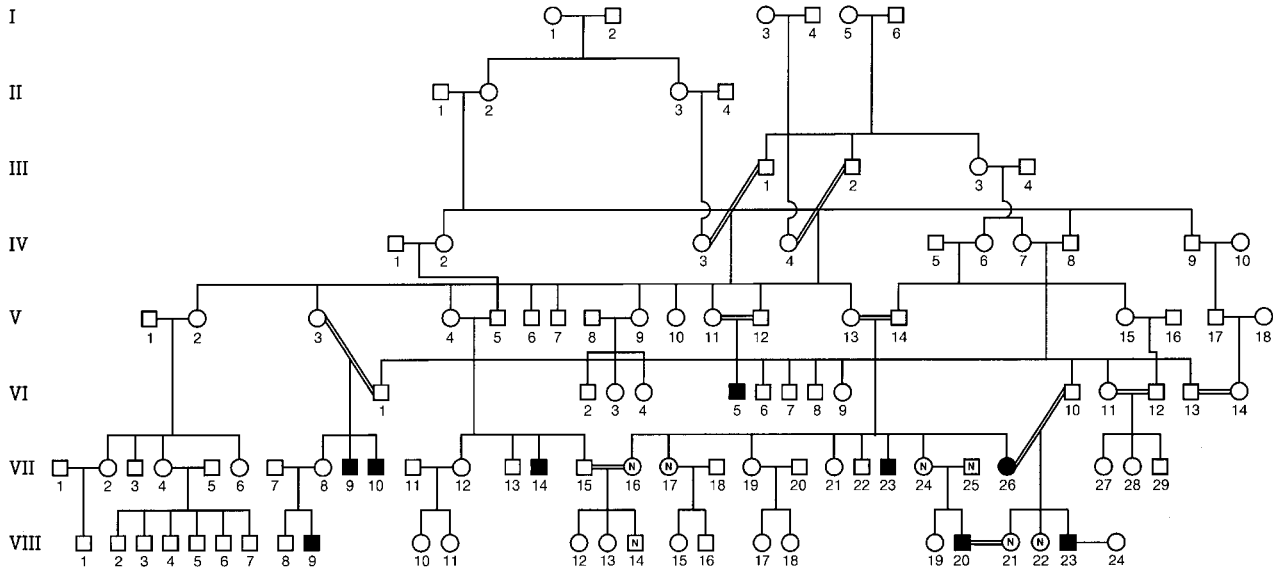

Figure 1 Matrilineal pedigree of 14482 LHON family. The blackened symbols denote those individuals who are affected with optic neuropathy, whereas the unblackened symbols denote those of either normal or unknown ophthalmological status. An "N"a symbol indicates that the individual has been examined by one of the authors (D.A.M.) and has been found to be devoid of any signs of optic neuropathy. Note the high frequency of consanguineous marriages in this family (indicated by double lines). Accurate genealogical information for this complicated pedigree was difficult to obtain, especially for the earlier generations.

the world have been analyzed, to identify other LHON mtDNA mutations. There is now a consensus that transitions at nucleotides 3460 and 14484 (ND1/A52T and ND6/M64V, respectively) are also pathogenic LHON mutations (reviewed in Brown and Wallace 1994; Johns 1994; Howell 1997). These three LHON mutations account for >95% of the multigeneration LHON pedigrees of northern European descent (Mackey et al. 1996), and each of these mutations has arisen multiple times within the human population (Brown et al. 1995; Howell et al. 1995).

Beyond this broad agreement about the 3460, 11778, and 14484 mutations, it is not yet clear how many other mtDNA mutations have an etiological or pathogenic role in LHON. Some investigators maintain that there are numerous mutations associated with LHON and that these can have primary, secondary, or intermediate levels of pathogenicity (e.g., Brown and Wallace [1994] list a total of 16 mutations). The identification of LHON mutations has been controversial, in large part because there is no single "proof," genetic or biochemical, that has emerged (see the discussion in the work of Howell [1994*a*, 1994*b*, 1997]). Uncertainty accompanies, in particular, the analysis of singleton patients or small families with a LHON-like optic atrophy, because maternal inheritance—the unambiguous genetic characteristic of LHON—is not present. Furthermore, the human mitochondrial genome has a high rate of mutation (e.g., see Howell et al. 1996), and pathogenic mutations—particularly those that are rare and/or that have not been described previously—are difficult to identify, because they are "buried" within a background of benign polymorphisms.

In this report, we present evidence for a rare mutation in the mitochondrial ND6 gene of a Turkish matrilineal pedigree in which several family members are affected with bilateral optic atrophy. At the age of 18 years, family member VIII-20 (fig. 1), who is the index case, experienced a loss of vision in his right eye (oculus dexter [OD]), over the course of a few weeks in 1991. Within 2 wk of onset, the left eye (oculus sinister [OS]) also became affected, and LHON was diagnosed, in Turkey, at that time. This individual and his wife (who is also his maternal first cousin; VIII-21 in fig. 1) subsequently moved to Australia where, on examination in 1996, his visual acuities were 6/60 OD and 3/60 OS. He had moderate centrocecal scotomata and marked temporal pallor of the optic disks. His wife's visual acuity and ophthalmological examination were normal. The index case's maternal first cousin (VIII-23), who is also his wife's brother, experienced a loss of vision over a period of 4 mo in 1992, at the age of 22 years. On examination in 1997, his visual acuities were 2/60 OD and 1/60 OS. He had moderate to severe centrocecal scotomata and generalized pallor of the disks.

The index case's mother-in-law (also a maternal relative; VII-26 in fig. 1) reported normal vision, with no recollection of any vision problems, but examination

revealed that she also had ophthalmological abnormalities. Her visual acuities were 6/9 OD and 6/6 OS, but her visual fields showed marked scotomata, and fundoscopic examination revealed bilateral optic atrophy with pseudocupping of the disks. The remainder of the family lives in Turkey, and further clinical information is difficult to obtain. However, six male family members have lost central vision, but they have reportedly recovered vision to a significant extent, because they are now capable of driving automobiles. None of those individuals has been examined by an ophthalmologist. It has recently been possible to examine other family members, during a visit to Australia: the index case's mother (VII-24), his father (VII-25), his wife's father (VI-10), his sister (VIII-19), two of his aunts (VII-16 and VII-17), and his cousin (VIII-14) have normal vision with no signs of optic neuropathy. The optic neuropathy in this family is thus fully consonant with LHON, and it differs from other bilateral optic atrophies, such as juvenile-onset autosomal dominant optic atrophy (e.g., see Brown et al. 1997; Johnson et al. 1997). In addition, the genealogical results are not compatible with autosomal dominant optic neuropathy, because the index case's mother (VII-24) and both of the latter's parents had normal vision. The optic neuropathy shows apparent maternal transmission, but there have been several consanguineous marriages within the pedigree (not all are shown in fig. 1), and there was the additional possibility of autosomal recessive optic atrophy. However, several of the visually affected family members (including the index case) had fathers who were not related to the matrilineal pedigree and who came from outside the village, a circumstance that argues against an autosomal recessive etiology.

Nucleotide sequencing revealed that the mtDNA of this family did not carry one of the three previously identified LHON mutations—at nucleotides 3460, 11778, or 14484—or any of the other possible LHON mutations (Brown and Wallace 1994). However, all eight maternal relatives who were assayed carry, at nucleotide 14482, a C:G transversion that results in the substitution of isoleucine for the methionine at amino acid residue 64 of the ND6 subunit of complex I, the same amino acid residue that is altered by the 14484 primary LHON mutation. Subsequently, we sequenced this region of the mitochondrial ND6 gene from ~200 normal controls, non-LHON controls, optic atrophy patients with no identified LHON mutation, and LHON family members; none of them carried the 14482 mutation (data not shown). In addition, a further 250 unrelated patients, who had a LHON-like bilateral optic neuropathy but who lacked one of the three previously identified pathogenic LHON mutations, were screened by denaturing-gradient gel electrophoresis. None of these patients carried the 14482 mutation. These data therefore suggest that the 14482 mutation has arisen rarely within the human population.

The second stage of the genetic analysis involved determination of the nucleotide sequence of the entire mitochondrial genome from one member of this LHON family (VIII-21). To ensure the accuracy of the nucleotide sequence, several fragments of the mtDNA of her affected husband/maternal first cousin (VIII-20) were sequenced, and agreement was obtained in all instances. A total of 25 sequence changes relative to the Cambridge Reference Sequence (CRS) were determined: 20 transitions, 4 transversions, and a 1-bp deletion (table 1). Six sequence changes were found in the 12S and 16S rRNA genes, and one mutation was found in a tRNA gene. Of the 18 mutations in protein-encoding genes, 5 produced amino acid changes (including that at nucleotide 14482), whereas 13 were phenotypically silent. Therefore, if one includes all mutations that produce amino acid changes *and* those in rRNA and tRNA genes, there are a total of 12 candidate mutations that could be pathogenic. Although no mutations in tRNA or rRNA genes have been identified in previous analyses of LHON patients, we did not eliminate them at this stage, in order not to bias the ultimate conclusions of the analysis. The basic question, therefore, is the following: Is the 14482 mutation the primary etiological factor that causes the optic neuropathy in this matrilineal pedigree, or is it caused by one or more of the other sequence changes that are carried by the mtDNA?

Several of these candidate mutations could be effectively eliminated, because a search of the MITOMAP database (Wallace et al. 1995; Kogelnick et al. 1996) revealed that they have been detected previously in population screening studies (see table 1). To confirm and extend the results of database searching, comparative sequence analyses of mtDNAs that are phylogenetically related to the index lineage were also performed (Jun et al. 1994; Brown et al. 1995; Howell et al. 1995; Hutchin and Cortopassi 1997). As part of the analysis of the 14482 LHON family, we determined the nucleotide sequence of the 1.1-kb noncoding control region, or D-loop (table 1). A search of our D-loop sequence database (derived from ~140 non-LHON and LHON pedigrees; N. Howell, unpublished data) yielded five sequences that were closely related; three of the DNA samples were from normal controls, and two were from unrelated 11778 LHON families. An inspection of the sequence of the 14482 mtDNA D-loop and of the D-loops in the phylogenetically related sequences indicates that these mitochondrial genomes are members of European haplogroup I (Torroni et al. 1994, 1996). The occurrence of shared CRS changes confirms this relationship. Torroni et al. (1994, 1996) found haplogroup I–specific restriction-site changes at map positions 1715, 4529, 8249, 10028, 10394, and 16389, whereas our sequenc-

Table 1

mtDNA CRS Changes in the LHON Pedigree

Nucleotide Position[a]	Gene	Nucleotide Change[a]	Amino Acid Change[b]
1438[c]	12S	A:G	NA
1531	12S	C:T	NA
1719[c]	16S	G:A	NA
2173	16S	C:G	NA
2706[c]	16S	A:G	NA
3106/3107[cd]	16S	C:del	NA
3447[c]	ND1	A:G	Q47Q
4529[c]	ND2	A:T	T20T
6734	COX1	G:A	M277M
8251[c]	COX2	G:A	G222G
8260	COX2	T:C	F225F
8616	ATP6	G:T	L30L
9966[c]	COX3	G:A	V254I
10034[c]	tRNAglu	T:C	NA
10238[c]	ND3	T:C	I60I
10398[c]	ND3	A:G	T114A
10609	ND4L	T:C	M47T
11719[c]	ND4	G:A	G320G
12501[c]	ND5	G:A	M55M
12705[c]	ND5	C:T	I123I
12864	ND5	T:C	R176R
13780[c]	ND5	A:G	I482V
14482[e]	ND6	C:G	M64I
15043[c]	CTYB	G:A	G99G
15589	CTYB	C:T	L281L

[a] Based on the L-strand of the Cambridge Reference Sequence (Anderson et al. 1981). Previous analyses have shown that the CRS contains errors or rare polymorphisms at nucleotides 263, 750, 3423, 4769, 4985, 7028, 8860, 9559, 11335, 13702, 14199, 14272, 14365, 14368, and 15326 (e.g., see Howell et al. 1992; Jun et al. 1994). These sequences changes were also found in this mtDNA. In addition to the sequence changes in the coding region, the following changes were found in the D-loop: 73/A:G, 199/T:C, 204/T:C, 250/T:C, 16129/G:A, 16172/T:C, 16223/C:T, 16311/T:C, 16391/G:A, and 16519/T:C. In addition, the C_6 repeat that begins at nucleotide 568 expands unstably to a length of 9–11 residues in members of the 14482 pedigree.

[b] The first letter is the amino acid residue encoded by the reference sequence, whereas the second is the predicted residue encoded by the mtDNA of the LHON pedigree. Note that most nucleotide changes are phenotypically silent. The intervening number is the position within the amino acid sequence of the relevant gene. NA = not applicable.

[c] Also observed within the population, as determined by a search of the MITOMAP database (Wallace et al. 1995; Kogelnick et al. 1996).

[d] The CRS has a C-C doublet at nucleotides 3106 and 3107, whereas the 14482 lineage has a deletion of one of these base pairs.

[e] Creates a new *Sau*3A restriction site. Nucleotide sequencing indicates that the members of this matrilineal pedigree are homoplasmic for the transversion but that this newly created site is relatively resistant to *Sau*3A cleavage (data not shown). As a result, it appears erroneously that these individuals are heteroplasmic, with 10%–20% of the 14482 wild-type allele, under standard restriction-endonuclease digestion conditions.

ing analyses revealed substitutions at nucleotides 1719, 4529, 8251, 10034, 10398, and 16391, respectively, in the mtDNA of the 14482 LHON family (table 1).

Of the 18 sequence changes in the 14482 lineage that have been analyzed, 4 are carried only by this lineage, including the transversion at nucleotide 14482, whereas 14 are carried by the other haplogroup I lineages (table 2) and/or have been identified previously as polymorphisms within the population (table 1). However, the substitution mutations at nucleotides 10609 (ND4L/M47T) and 14482 (ND6/M64I), the silent polymorphism at nucleotide 8260, and the rRNA sequence change at nucleotide 1531 occur only in the 14482 lineage.

The ophthalmological presentation and its maternal inheritance are strongly indicative of LHON in this matrilineal pedigree. Although the analysis of this family was complicated by several factors, including the rarity of the 14482 mutation within the population, it can be concluded that the transversion at nucleotide 14482 is a rare LHON mutation, which is the primary etiological cause of the ophthalmological disorder in this matrilineal pedigree. We have substantial confidence in this conclusion, for the following reasons, although we distinguish "strong evidence" from "proof."

1. The 14482 mutation (M64I) alters the same amino acid residue that is affected by the well-established 14484 LHON mutation (M64V). Among the 3460, 11778, and 14484 primary LHON mutations, it is the 14484 mutation that is associated with a high frequency of vision recovery (e.g., see Mackey and Howell 1992; Johns 1994). The anecdotal evidence is particularly striking, therefore, in that the 14482 family also presents an optic atrophy in which there is recovery of vision.

2. The 14482 mutation affects an amino acid residue within an apparent "hotspot" for optic neuropathy, the mitochondrial ND6 gene. In addition to the 14484 primary LHON mutation, which is the second most prevalent LHON mutation (e.g., see Johns 1994; Howell et al. 1996), Leo-Kottler et al. (1996) have recently identified a German LHON family that harbors a mutation at nucleotide 14498 of the ND6 gene (Y59C). Furthermore, Shoffner et al. (1995) have found that a mutation at nucleotide 14459 causes LHON plus dystonia (A72V). Similarly, De Vries et al. (1996) have analyzed a large Dutch family in which the LHON-like optic neuropathy is associated with a spastic dystonia (and other neurological abnormalities). They found two candidate mutations, one in the ND4 gene (nucleotide 11696; V396I) and one in the ND6 gene (nucleotide 14596; I26M); either or both mutations may be the primary etiological factor(s). Most recently, Wissinger et al. (1997) have identified a singleton case of optic neuropathy who carries an ND6 mutation at nucleotide 14568 (G36S). The present results thus add support for an emerging "picture" of ND6 mutations, in which they tend to produce subtle amino acid substitutions at sites (probably within a hydrophobic or transmembrane domain) that are only moderately conserved during evo-

Table 2

Comparative Analysis of the 14482 Index-Case Mitochondrial Coding Region Sequence and Phylogenetically Related mtDNA Lineages

		Status of Individual[a]					
Nucleotide	Site	0544	0049	0057	0073	0117	0136
14482	ND6 / M64I	+	−	−	−	−	−
1438	12S	+	+	NT	+	+	+
1531	12S	+	−	NT	−	−	−
1719	16S	+	+	NT	+	+	+
2173	16S	+	+	NT	+	+	+
2706	16S	+	+	NT	+	+	+
3106 / 3107	16S	+	+	NT	+	+	+
3447	ND1 / Q47Q	+	−	−	−	−	−
4529[b]	ND2/T20T	+	+	NT	NT	+	NT
8251[b]	COX2 / G222G	+	+	+	+	+	+
8260	COX2 / F225F	+	−	−	−	−	−
9966	COX3 / V254I	+	−	−	−	−	−
10034[b]	tRNAglu	+	+	+	+	+	+
10238	ND3 / I60I	+	+	+	+	+	+
10609	ND4 / M47T	+	−	−	−	−	−
11719	ND4 / G320G	+	+	+	+	+	+
12705	ND5 / I123I	+	+	+	+	+	+
13780	ND5 / I482V	+	+	+	+	+	+

[a] A plus sign (+) indicates that the sequence change is present, whereas a minus sign (−) indicates that it is absent (i.e., it is the CRS). NT = not tested. 0544 is a member of 14482 lineage, whereas 0049, 0057, and 0136 are normal controls. 0073 and 0117 are independent 11778 LHON lineages.

[b] The presence of this sequence change had been suggested earlier by restriction-site assays of haplogroup I individuals (Torroni et al. 1994, 1996). However, our results indicate that the restriction-site data of Torroni et al. (1994, 1996) are erroneous in one instance: haplogroup I mtDNAs gain an *Alu*I site at position 10032 (in their system for designation of restriction sites), rather than at position 10028.

lution (e.g., see table 1 of Leo-Kottler et al. 1996). This pattern suggests that some aspect of complex I function is exquisitely sensitive to the structure of this putative domain and that the structure is tightly constrained by the interactions of multiple amino acid residues.

3. The 14482 mutation affects a subunit of complex I, as do the three previously established primary mutations. However, that logic also supports a possible etiological role for the ND4 mutation at nucleotide 10609. There is less precedent for an etiological role of the rRNA mutation at nucleotide 1531, because there is no evidence that rRNA mutations are associated with LHON, although a mitochondrial 12S rRNA mutation at nucleotide 1555 causes nonsyndromic deafness (e.g., see Hutchin and Cortopassi 1997, and references therein).

There is a burgeoning interest in the possible role that mtDNA mutations may play in etiologically more-complex disorders, in which a clear-cut pattern of maternal inheritance is lacking. For example, there has been considerable effort expended to determine whether a mutation at nucleotide 4336 of the mitochondrial tRNAgln gene is preferentially associated with Alzheimer disease (AD) patients, relative to normal controls (Shoffner et al. 1993; Hutchin and Cortopassi 1995). The most recent data argue against an etiological role (Tysoe et al. 1996; also see Hutchin and Cortopassi 1997). However, the general question will persist, especially in light of the reports that there is a preferential *maternal* transmission of AD (Edland et al. 1996), a result compatible with a mitochondrial genetic contribution. The results presented here underscore the difficulties that can be encountered in the investigation of the etiological role of rare mtDNA mutations.

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Neil Howell,[1] Christy Bogolin,[1] Robyn Jamieson,[2] Daniel R. Marenda,[3] and David A. Mackey[4]

[1]*Department of Radiation Oncology, Department of Human Biological Chemistry and Genetics, The University of Texas Medical Branch, Galveston;* [2]*Department of Clinical Genetics, New Children's Hospital, Sydney;* [3]*Department of Ophthalmology, University of Iowa, Iowa City; and* [4]*Departments of Ophthalmology and Paediatrics, The University of Melbourne, Melbourne, and The Menzies Centre, University of Tasmania, Hobart, Australia*

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Address for correspondence and reprints: Dr. Neil Howell, Biology Division 0656, Department of Radiation Oncology, The University of Texas Medical Branch, Galveston TX 77555-0656. E-mail: nhowell@utmb.edu

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Power, Mode of Inheritance, and Type I Error in Lod Scores and Affecteds-Only Methods: Reply to Kruglyak

To the Editor:

We had previously written a letter examining some of the issues involved in comparing LOD scores versus affecteds-only and other "nonparametric" methods (Greenberg et al. 1996). We had two motivations for that letter. The more important reason was that many of our colleagues have reported difficulties in getting linkage studies funded—or in getting linkage findings published–when LOD scores are used to analyze data. A related impetus for our letter was that there appears to be widespread ignorance of an extensive literature, some of which was cited in our letter, supporting the use of LOD scores. We believe this lack of awareness accounts for the belief of many peer reviewers, of both grant proposals and manuscripts, that LOD scores represent an analysis method inferior to or less powerful than the affecteds-only methods. We tried to address these issues in our letter, because this incorrect belief not only has the negative consequences alluded to above but also runs counter to the practice of good science. We also hoped that our letter would stimulate open discussion of the mathematical issues involved. In this respect we were glad to see a further commentary on our letter, by Kruglyak (1997; also see Farrall [1997] and our response [Greenberg et al. 1997]). However, we feel that it is necessary to focus on some of the points made by Kruglyak.

We respond to the three major points raised by Dr. Kruglyak, which concern (1) the use and meaning of the terms "nonparametric" and "model free"; (2) LOD scores and power; and (3) the role of the true mode of inheritance in LOD scores and in "model-free" methods.

1. "*Nonparametric*" *and* "*model free.*"—In his comments, Kruglyak (1997, p. 255) gives a strict statistical definition of "nonparametric" or "model-free" tests as being those which "are *valid* [italics his] regardless of the true (unknown) genetic parameters, in the standard sense that they give the correct false-positive rate." He then reiterates that this property applies to LOD-score analyses, *under the wrong model* ("wrod" scores [Hodge and Elston 1994]), just as much as to affected-sib-pair (ASP), affected-pedigree-member, or nonparametric-linkage analyses. The fact that, regardless of whether the assumed model is correct, all of these methods, including LOD scores, satisfy the standard statistical definition of a nonparametric test is apparently not widely understood, although it was formally proved by Williamson and Amos (1990). (Of course, this guarantee of statistical validity holds only for a *single* LOD score or wrod analysis, just as it holds only for a *single* affecteds-only analysis. If an investigator wants to perform two or more linkage analyses, whether LOD score or affecteds-only, allowance must be made for multiple tests. Elsewhere, we have quantified some of this requirement [Hodge et al. 1997].) However, "nonparametric" is currently used by most writers to mean "does not explicitly state a genetic model" (but see Elston [1997]). This usage is so ingrained that, subsequently in his letter, Kruglyak himself uses "nonparametric" in this "common" way (Kruglyak 1997). Thus, this is not merely an issue of terminology. It is important because the current usage of "nonparametric test" hides the fact that the nominal probability of type I error is asymptotically correct in *all* of the analytic methods under discussion, including LOD scores under the wrong model.

2. *LOD scores and power.*—In his letter, Kruglyak (1997, p. 255) concludes that "the interesting issue in the design of such [alternative linkage] methods is how to achieve a minimal loss of power while retaining robustness to a maximal range of alternatives." We strongly agree. However, he seems to imply—although he does not explicitly state—that, in this respect, LOD scores fare worse than other methods. He says that, when they use LOD scores, researchers who "guess wrong" about the genetic model can "lose big." We, too, were concerned about this danger, and that concern provided the impetus for the research cited in our original letter, research that showed that this was not a danger. Kruglyak (1997, p. 255) also says that investigators can "fish over all possible models and pay the statistical price." However, it is not necessary to fish over all possible models (again, the reasoning and citations are in our original letter), and our recent work has shown that comprehensive coverage of models can be had at a modest price in terms of type I error (Hodge et al. 1997).

3. *Role of the true mode of inheritance.*—Here is where the terms used in current parlance—"nonparametric" and "model free"—have proved to be somewhat misleading. Some colleagues with whom we have spoken have concluded incorrectly that all statistical properties of these methods are independent of the

true mode of inheritance of the trait being investigated. On the contrary, although *test size* (probability of type I error, discussed above) of "nonparametric" methods does not depend on the true mode of inheritance (just as it does not do so for LOD scores), their *power* does depend on the true mode of inheritance (just as it does for LOD scores). But the similarity between "model-free" tests and LOD-score analysis goes deeper than that.

Kruglyak (1997, p. 255) points out that, although a "nonparametric" test is equivalent to a LOD-score analysis, "this *does not* mean that a nonparametric test *assumes* [italics his] this choice of parameters." This is, of course, correct, but the fact remains that the two equivalent tests, although not making the same assumptions, can be seen to be statistically identical. In Knapp et al.'s (1994) example, the "mean test" uses ASP data and does not assume a mode of inheritance, whereas a recessive LOD-score analysis of the same data explicitly assumes a fully penetrant recessive model. The mean test has a rejection region *identical* to that of the recessive LOD-score analysis. This means that, if the two tests are set to the same significance levels, they will necessarily also achieve *exactly* the same power, whatever the underlying true model. If the true model is dominant, for example, both analysis methods will suffer the same loss of power, compared with what would have occurred if the true model had been recessive (for some concrete examples of the magnitude of this effect for ASPs, see the report by Hodge [in press]). The important point is that it is misleading to imply that the mean test, or any "nonparametric" test, is somehow "purer" than the corresponding LOD-score analysis, as though its power did not depend on the underlying true model. One can argue about whether the mean test *implicitly* assumes a recessive model or not, but it seems to us that this is a matter of semantics or philosophy.

Whittemore (1996) has shown how this example from Knapp et al. can be generalized. Other "nonparametric" approaches also have statistical properties identical to those of the maximum-likelihood method under specific assumptions. Again, although these methods do not assume a mode of inheritance, one could, in theory, determine which set or sets of mode-of-inheritance assumptions each "nonparametric" analysis method corresponds to and then use one of those sets of mode-of-inheritance assumptions in a LOD-score analysis, to achieve statistically identical results. Thus, using a model-free test can be statistically equivalent to using a LOD-score analysis assuming a wrong genetic model, except that, in the "nonparametric" case, the corresponding genetic models are unknown and are not amenable to adjustment by the investigator.

The existing evidence suggests that the range of genetic models *at a single locus* is robustly spanned by the dominant and recessive models. Extensive work on two-locus multiplicative models (e.g., see Vieland et al. 1992, 1993; Goldin and Weeks 1993; Dizier et al. 1996; Durner et al. 1997) has shown that it is not the mode of inheritance of the disease as a "whole" that needs to be specified in a linkage search; rather, the mode of inheritance at the disease locus *linked to the marker* is critical. (Also, we have now investigated the same issue for "intermediate" and two-locus additive models [Abreu et al. 1997].) If a disease allele exists at all, it must act either alone or together with its sister allele on the homologous chromosome. With relatively little loss of power, effects at other loci can probably be subsumed in other parameters, just as the effect of a second unlinked, epistatic locus can be taken into account of by assuming that there is simple reduced penetrance (Vieland et al. 1992, 1993). If that is indeed the case, then assuming that there is one dominant model and one recessive model will actually cover most possibilities rather well. In this case, "complex" genetic disease can be viewed as being determined by a series of genetic contributions, each of which may be independently detected, and the main issue becomes the relative contribution of a given locus to both the disease and its detectability, not the fact that the disease is "complex."

As we said in our original letter, which approach is "best" will depend on the data available and on other factors particular to the trait and population being studied. We think that it is wrong to condemn a study because the investigators have chosen one analysis method over another, if that method had a reasonable chance of success. What sometimes escapes notice in discussions of analysis methods is that the greater difficulty in studying the genetic contribution to human disease lies in the problems of collecting data good enough for the methods to yield anything at all. Heterogeneity, misdiagnosis, and poorly defined phenotype represent more-serious obstacles to finding disease genes than do the statistical methods available for analysis.

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David A. Greenberg,[1] Susan E. Hodge,[2] Veronica J. Vieland,[3] and M. Anne Spence[4]

[1]*Departments of Psychiatry and Biomathematics, Mount Sinai Medical Center, and* [2]*New York State Psychiatric Institute and Columbia University, New York;* [3]*Departments of Preventive Medicine & Environmental Health and Psychiatry, University of Iowa College of Medicine, Iowa City; and* [4]*Department of Pediatrics, University of California, Irvine Medical Center, Orange*

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Address for correspondence and reprints: Dr. David A. Greenberg, Box 1229, Mount Sinai Medical Center, New York, NY 10029. E-mail: dag@shallot.salad.mssm.edu

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Efficient Strategies for Genome Scanning with Affected Sib Pairs

To the Editor:

Holmans and Craddock (1997) present the results of their investigations into the performance of different approaches designed to reduce the number of genotypings required to detect linkage, using a sample of affected sib pairs and their parents; but their method of evaluation is fundamentally flawed, and hence their results do not provide useful information. Two techniques are applied—sample splitting and grid tightening—to produce a two-stage test. In the first stage a reduced number of subjects and/or markers are genotyped, and only regions reaching a certain LOD-score criterion in stage 1 are followed up, in stage 2, by genotyping of all subjects at all markers. Holmans and Craddock present their results in terms of both the power of the procedure to detect linkage and the number of genotypings required. However, in many cases, increasing the number of subjects genotyped in the first stage actually reduces power. Intuitively, it is clear that the detection of linkage rests on being able to identify regions likely to contain linked markers in the first stage and then to follow them up adequately in the second stage. Yet, Holmans and Craddock's results seem to show that a more thorough search in the first stage leads to a decrease in the probability that linkage will be detected, sometimes to a substantial degree (e.g., from .62 to .52 or from .61 to .48). Furthermore, in 3 of their 18 scenarios they recommend a threshold that is higher for the first stage than for the second. This means that one could find a LOD score >3 in the first stage, which one would have to discard and not follow up, even though, if the same LOD score were to be found in the second stage, it would be taken to imply linkage.

The explanation for these paradoxical findings lies with the test strategy that Holmans and Craddock have used. What they propose as a two-stage test for linkage is to choose in advance a LOD score that must be achieved in stage 2 and then to choose as the stage 1 criterion that LOD score that will produce an overall type I error rate of .05. For example, the stage 2 criterion may be set to 3, and then simulations are performed, with the specified data set and scanning procedure, to discover that LOD score that, if used for the stage 1 criterion, will produce a genomewide probability of .05 for an unlinked locus to get through to stage 2 and produce a LOD of 3. As a test for linkage, this is perhaps valid in a narrow sense, but even intuitively it might be expected to perform badly, since it lacks any intrinsic

appeal. Here, the stage 2 LOD score has no real meaning but just serves to act as a benchmark that must be attained. Values of 3.0, 3.3, and 3.6 are used, but no justification is given for choosing them; nor could there be one. It would be perfectly reasonable to find the power of a procedure to attain a certain LOD score or to attain a statistic having a certain *P* value (type I–error probability). What makes no sense is to aim to attain a certain LOD score in the second stage but then to achieve a specified type I–error probability overall by manipulating the threshold applicable for the first stage. The more natural approach, which I am sure would yield completely different results with regard to power and efficiency, would be to fix the threshold for the first stage (probably at .5–1.0) and, in the second stage, to aim either for some predetermined LOD score or for a LOD producing a certain overall *P* value.

The effects of Holmans and Craddock's approach are clear to see. The stage 1 criterion has to be made high enough so that only a small number of unlinked regions will achieve it and hence go on to produce false-positive results in stage 2. The more subjects and markers that are typed in the first stage, the more likely it is that high LOD scores will be thrown up by chance, and hence the higher the stage 1 criterion must be set. The higher this criterion is, the harder it may be for a truly linked locus to achieve it, and so such loci may be more frequently discarded. Thus, doing more genotyping in the first stage generally leads to a reduction in power, despite involving an increase in the total amount of genotyping required.

The first scenario that Holmans and Craddock present illustrates this clearly. A wide, 20-cM grid is used for stage 1, narrowing to 10 cM in stage 2, and the LOD score to be taken to indicate linkage, after stage 2, is chosen to be 3. When only 100 of the 200 sib pairs are typed, the threshold to move from stage 1 to stage 2 is set to a modest and sensible .89, and the overall power is .62. However, when all 200 pairs are typed, the stage 1 threshold has to be raised to 2.14, so many true linkages are missed, and the power falls to .57. Using 100 pairs together with their parents needs a threshold of 1.57 and yields a power of .52. Finally, initially using all 200 pairs and their parents apparently demands a stage 1 threshold of 3.1 and has a power of only .54. This would mean that, if one got a LOD of 3.05 with the initial 20-cM grid scan, one would not follow up this finding, even though it would count as a positive result if it were to be found in stage 2.

Given that genotyping is becoming ever cheaper and easier, given that linkage can easily be missed in sib-pair samples, and given that performing a genome scan but missing a disease locus is highly undesirable, my own personal view is that the initial scan should probably be fairly thorough, using all available subjects and a relatively narrow marker grid.

DAVID CURTIS
Department of Psychological Medicine
St. Bartholomew's and Royal London School of Medicine and Dentistry
London

Reference

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Address for correspondence and reprints: Dr. David Curtis, Department of Psychological Medicine, St. Bartholomew's and Royal London School of Medicine and Dentistry, 3d Floor Alexandra Wing, Turner Street, London E1 2AD. E-mail: dcurtis@hgmp.mrc.ac.uk; URL: www.gene.ucl.ac.uk/~dcurtis

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EDITOR'S NOTE.—This letter is a truncated version of the letter submitted by Dr. Curtis.

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Reply to Curtis

To the Editor:

Curtis (1998 [in this issue]) has raised some criticisms regarding our paper on efficient strategies for genome screening for linkage (Holmans and Craddock 1997). We reply to them as follows:

Curtis has said that our decision to fix the stage 2 criterion "lacks any intrinsic appeal" and "makes no sense." However, we would like to point out that judgements regarding the significance of a linkage study are generally based on the final LOD score obtained. Therefore, to facilitate comparison between the various strategies, it is desirable that a given stage 2 LOD score should correspond to the same significance level in all the strategies, as far as possible. This can most easily be done by fixing the stage 2 criterion and varying the stage 1 criterion, to obtain the desired type I error probability. In practice, one would not regard such criteria as benchmarks of "significant" versus "nonsignificant" linkage—their purpose is to ensure a fair comparison of the power of the various strategies and as a guide to which LOD scores correspond at *P* value of $\leqslant .05$.

We chose 3.6 as one of our criteria since this was recommended by Lander and Kruglyak (1995) as corresponding to a genomewide *P* value of .05 and is in widespread use. The criterion of 3.0 was chosen as the traditional criterion for significant linkage. The criterion 3.3 was adopted when it became clear that 3.6 was too stringent for the strategies to give a *P* value of .05. It is clear from our results that higher criteria would make

it impossible for a type I error *P* value of .05 to be obtained, whereas lower criteria would reduce efficiency by requiring an excessively high stage 1 criterion.

As noted by Curtis, it is also possible to fix the stage 1 criterion and to vary the stage 2 criterion, although we do not see why this approach should be regarded as "more natural.". What is more important, we disagree with Curtis's comment (made without any justification) that such an approach "would yield completely different results", provided that the stage 2 criteria were chosen to produce the same overall *P* value. Our reasoning is as follows: Strategies using narrow grids in stage 1 will produce more false-positive results to be followed up in stage 2, because of the greater number of loci being tested. Therefore, a higher stage 2 criterion will be necessary, thereby reducing power. In addition, the degree of dependence between the stage 1 test and the stage 2 test is increased for strategies in which a large proportion of the total sample is typed in stage 1, because of the similarity in the data analyzed in the two stages. This means that loci giving false-positive results in stage 1 are more likely also to give high LOD scores in stage 2. Again, a higher stage 2 criterion will be needed. It is therefore quite possible that strategies involving a large amount of genotyping in stage 1 may not increase power, as we found in our simulations.

Following Curtis's suggestion, we investigated the power of the various strategies when, as suggested, the stage 1 criterion was fixed and the stage 2 criterion was varied to give a type I error *P* value of .05. A stage 1 criterion of .9 was used—this is similar to the stage 1 criteria of the best-performing tests in our original paper and also is within the range suggested by Curtis. The results are displayed in table 1. Fixing the stage 1 criterion certainly improves the performance of the strategies in which the whole sample (200 pairs + parents) is typed in stage 1, compared with the results displayed in our original paper. However, it can still be seen that typing the whole sample in stage 1 gives, at best, a minimal power increase over that of strategies in which only the affected pairs are typed, while requiring considerably more genotyping. Use of a tight (10-cM) grid in stage 1 gives no more power than the use of a wide (20-cM) grid, and it requires a large increase in genotyping. The main conclusions of our original paper—that is, that both sample-splitting and grid-tightening increase efficiency and that a relatively wide initial grid is preferable to a narrow one—therefore stand.

Of course, if the stage 2 criterion is "predetermined," as suggested by Curtis, then strategies involving large amounts of genotyping in stage 1 will give the highest "power"—that is, the highest number of LOD scores exceeding the criterion. However, this would be meaningless, since such strategies would also have the highest number of false positives.

Table 1

Performance of Two-Stage Strategies

Grid and Stage 1 Sample [a]	Stage 2 LOD Criterion [b]	Power [c]	No. (SD) of Genotypings
20 cM/10 cM:			
100 Pairs	2.96	.622	47,359 (400)
200 Pairs	3.15	.709	78,166 (333)
100 Pairs + parents	3.10	.665	78,493 (350)
200 Pairs + parents	3.15	.722	140,134 (265)
20 cM/5 cM:			
100 Pairs	3.10	.652	59,180 (705)
200 Pairs	3.25	.782	90,829 (619)
100 Pairs + parents	3.19	.717	91,209 (654)
200 Pairs + parents	3.41	.778	154,671 (562)
10 cM/5 cM:			
100 Pairs	3.19	.733	94,174 (627)
200 Pairs	3.41	.783	156,274 (527)
100 Pairs + parents	3.19	.785	155,800 (564)
200 Pairs + parents	3.41	.786	278,908 (423)

[a] Grid-tightening strategies are denoted as the intermarker interval in stage 1 (to the left of the slash) followed by that in stage 2 (to the right of the slash). The stage 1 LOD-score criterion is .9; $\lambda_s = 2$.

[b] Fixed to make genomewide type I error probability ~.05.

[c] SD $\leqslant$.01.

By means of the reasoning mentioned above, the explanation for the seemingly counterintuitive results noted by Curtis becomes apparent. Strategies in which a large number of loci are typed in stage 1 require a higher stage 1 test criterion, to restrict the number of false positives being tested in stage 2 to the correct level. Similarly, strategies using a high proportion of the sample in stage 1 require an increased stage 1 test criterion, to offset the increased dependence of the stage 1 and stage 2 tests. We can therefore see that strategies that utilize a "more thorough search in the first stage" require a higher stage 1 criterion, for a given type I error probability. This is why the power of such strategies may be reduced, despite the increase in the amount of genotyping, as Curtis himself notes. The numerical example that Curtis gives illustrates the point perfectly.

As Curtis notes, in the extreme case, in which the entire sample is genotyped in stage 1, the required stage 1 criterion may actually exceed the stage 2 criterion. However, we are not recommending such strategies; we are merely quoting the stage 1 criteria that make these strategies as efficient as possible, given the stage 2 criterion. These strategies perform very poorly, requiring a large amount of genotyping and giving only low power. In fact, the implausibly high stage 1 criteria should act as a warning not to use these strategies—in the Discussion section of our original paper (Holmans and Crad-

dock 1997), we note that the best-performing strategies have stage 1 criteria of ~1.

It is true that we have not considered multipoint analysis, and this would be an interesting area for further work. Given that our conclusions hold up under two-locus analysis (Holmans and Craddock 1997), we would be hopeful that they would also be true under multilocus analysis.

In conclusion, although there is certainly scope for further work—particularly that involving multilocus analysis—we disagree strongly with Curtis's statements that our work is "fundamentally flawed" and that our results "do not provide useful information." The results presented here show that the alternative approach advocated by Curtis would result in conclusions similar to ours, despite his assertions to the contrary.

PETER HOLMANS AND NICK CRADDOCK*
Division of Psychological Medicine
University of Wales College of Medicine
Heath Park
Cardiff

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Address for correspondence and reprints: Dr. Peter Holmans, Division of Psychological Medicine, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN, Wales. E-mail: wpcpah@cardiff.ac.uk

* Present affiliation: Department of Psychiatry, University of Birmingham, Birmingham, United Kingdom.

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