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.

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0002-9297/98/6201-0029$02.00

Am. J. Hum. Genet. 62:196–202, 1998

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

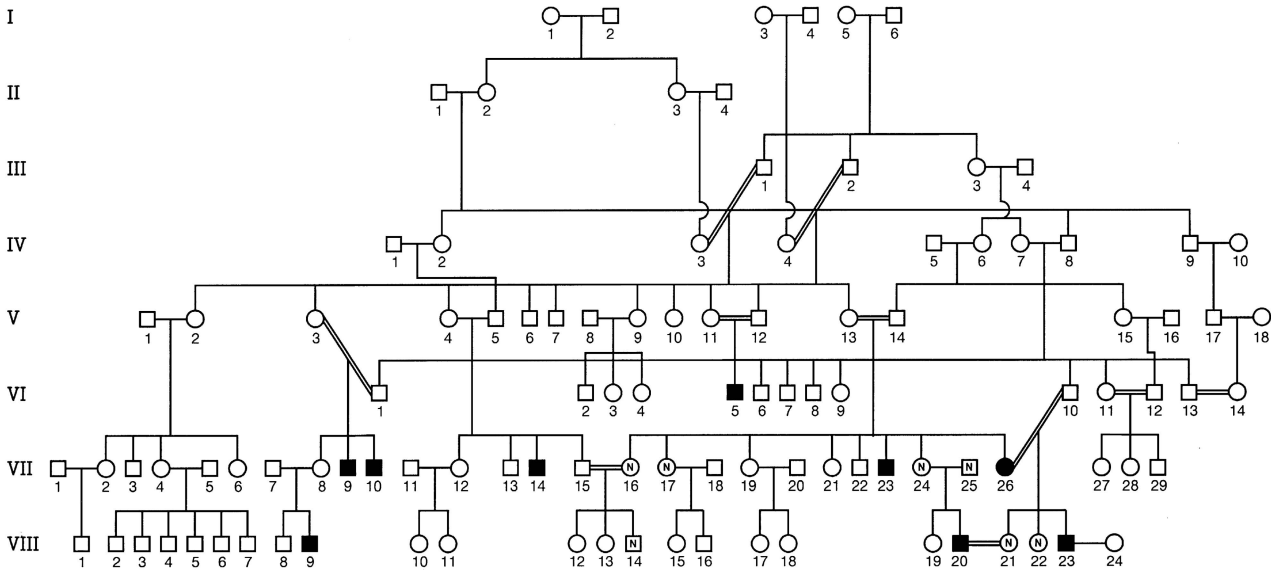

Figure 1 Matrilineal pedigree of 14482 LHON family. The blackened symbols denote those individuals who are affected with optic neuropathy, whereas the unblackened symbols denote those of either normal or unknown ophthalmological status. An "N"a symbol indicates that the individual has been examined by 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.

Acknowledgments

We thank Dr. Edwin Stone (University of Iowa) for his comments on this work and for his assistance with the screening studies of optic neuropathy patients. This research was funded by National Eye Institute grant RO1 EY10758 and by a John Sealy Memorial Endowment Fund grant (both to N.H.).

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Am. J. Hum. Genet. 62:202–204, 1998

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