Letters to the Editor

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No Convincing Evidence of Linkage for Restless Legs Syndrome on Chromosome 9p

To the Editor:

We would like to comment on the Chen et al. (2004) article that addresses the identification of a putative susceptibility locus for restless legs syndrome (RLS) on chromosome 9p. They analyzed 15 large families containing 134 individuals affected with RLS, and families were selected via probands with at least one first-degree relative with RLS. Chen et al. (2004) performed familial aggregation analysis and linkage analysis to identify significant RLS susceptibility loci. Their results suggest that there is strong familial aggregation and a high heritability of the RLS trait in their cohort. They claimed that both model-free and model-based linkage analysis showed a significant signal for a locus in the 9p24-22 chromosome region.

We have several concerns regarding the statistical methodology used by Chen et al. (2004). First, we are concerned about the estimates of heritability and familial correlation. Chen et al. (2004) included a family in their study if the proband had at least one affected first-degree relative. Without any ascertainment correction, they estimated heritability as 0.60 and found evidence of strong familial aggregation. Their failure to correct for ascertainment results in biased and, therefore, meaningless estimates of aggregation and heritability. Their study employed a multiple-ascertainment scheme and not a single-ascertainment scheme, so there is bias in the estimation of the sibling relative-risk ratio, as discussed elsewhere by several authors (Guo 1998, 2002; Olson and Cordell 2000; Zou and Zhao 2004).

Chen et al. (2004) appear to have markedly overstated the significance of their results, most likely because they failed to recognize that nonparametric linkage (NPL) scores are on a different scale than LOD scores. For example, Chen et al. (2004) describe four of their regions, with NPL scores in the range 2.03–2.68, as meeting the criteria for "suggestive evidence" of linkage. However, Lander and Kruglyak (1995) defined "suggestive evidence" as a LOD score >2.2, which is equivalent to an NPL score >3.18 (table 1); none of the four regions described by Chen et al. (2004) satisfy this criterion. Similarly, Chen et al. (2004) claim that their study provided "significant evidence of linkage" for an RLS locus on chromosome 9p24-22, on the basis of an NPL score of 3.22 and an empirical *P* value of .009. However, according to the criteria of Lander and Kruglyak (1995), one should have an NPL score >4.08 and a *P* value <.000022 before claiming to have significant evidence of linkage. Although, to some extent, this is quibbling about terminology, precise use of terminology is important, since the terminology may influence how other people interpret the results, which perhaps could lead to unmerited costly follow-up experiments. For example, the Online Mendelian Inheritance in Man database (see entry for RLS [MIM 102300]) already describes the results of Chen et al. (2004) as "significant": "Model-free linkage analysis identified 1 novel *significant* RLS susceptibility locus on 9p24-p22 with a multipoint nonparametric linkage (NPL) score of 3.22" (emphasis added).

Table 1

Comparison of NPL and LOD Scores on the Basis of Criteria from Lander and Kruglyak (1995)

Level	*P*	LOD	NPL
"Potentially interesting"[a]	.023	.87	2.00
Suggestive	.00074	2.2	3.18
Significant	.000022	3.6	4.08
Highly significant	.0000003	5.4	4.99

[a] As defined by Chen et al. (2004, p. 881).

Not only is the initial evidence for linkage on 9p found by Chen et al. (2004) much weaker than was implied, the follow-up confirmatory analyses are inadequate and potentially biased. Without defining their selection criteria, Chen et al. (2004) have attempted to "validate" their initial linkage signals by further analyzing only 2 extended families, selected from their 15 large multiplex families. If these two families were selected because of initial positive linkage signals, such an approach would certainly bias the results in favor of linkage (see the discussion by Ott [1991]). Instead of following up in a subset of selected families, it is better to analyze all the families as a whole under a model that allows for linkage heterogeneity (Ott 1991). However, Chen et al. (2004)

Table 2

Two-Point LOD Scores at Marker *D9S274* for Pedigree 40004 under a Variety of Disease Models

Model	MZ Twins Included	Marker-Allele Frequencies	K[a]	q[b]	f_0, f_1, f_2[c]	LOD Score at θ = .00	.05	.10	.20	.30	.40
A	Both	.5, .5	.002	.001	.00, .95, .95	1.24	1.09	.94	.64	.33	.09
B	One	.5, .5	.002	.001	.00, .95, .95	.94	.82	.70	.46	.23	.06
C	One	.15, .54, .13, .06, .06, .04, .04	.002	.001	.00, .95, .95	1.30	1.17	1.03	.75	.44	.17
D	One	.06, .54, .13, .15, .06, .04, .04	.002	.001	.00, .95, .95	1.42	1.28	1.14	.85	.53	.21
E	One	.54, .13, .06, .06, .04, .04, .15	.002	.001	.00, .95, .95	.29	.20	.13	.05	−.01	−.04
F	One	.54, .13, .06, .06, .04, .04, .15	.049	.026	.00, .95, .95	.25	.18	.12	.05	.00	−.03
G	One	.54, .13, .06, .06, .04, .04, .15	.052	.001	.05, .95, .95	.24	.17	.13	.06	.01	−.03

[a] K is the population prevalence.
[b] q is the frequency of disease allele "2."
[c] f_0, f_1, and f_2 are the penetrances of the 1/1, 1/2, and 2/2 disease genotypes, respectively.

did no such analysis that would allow for linkage heterogeneity in the families affected with RLS.

We are also concerned about several other possible methodological errors in this article by Chen et al. (2004). Since they computed their NPL statistics by using the program GeneHunter (Kruglyak et al. 1996; Kong and Cox 1997), it is not clear what happened to the data from their very large multiplex families, since GeneHunter cannot handle such large pedigrees. Also, it is not clear what phenocopy rate was used in the parametric linkage analysis. If we assume that the authors used a phenocopy rate of 0, then their genetic model (i.e., autosomal dominant model with a penetrance of 0.95 and a disease-allele frequency of 0.001) implies a low prevalence of 0.2% that is not consistent with the observed prevalence of 5%–12%. If the disease-allele frequency is increased to 0.026 or if the phenocopy rate is set to 0.05, the prevalence becomes consistent with the observed value. Also, the use of a smaller disease-allele frequency than that observed in the pedigree could give false evidence for linkage.

We have several other concerns regarding how the LOD scores were computed by Chen et al. (2004). First, the authors have estimated marker-allele frequencies by using a discredited algorithm that has been shown to often lead to false evidence of linkage. For a subset of their markers, the authors used equal allele frequencies ($1/n$, where n is the number of alleles [note that this "n" should be the number of alleles in the population, not the number in the individual pedigree]). Use of equal-allele-frequency estimates in the presence of untyped individuals may lead to strong false-positive evidence for linkage, as noted by Ott (1992). It is especially puzzling that Chen et al. (2004) improperly estimated marker-allele frequencies for a subset of their markers, whereas they did use best-possible estimates (e.g., those based on maximum likelihood that properly take relationships into account) for the rest of their markers. Second, many of the linkage-analysis programs, such as GeneHunter (Kruglyak et al. 1996) and LINKAGE (Lathrop et al. 1984, 1985), do not recognize MZ twins but instead incorrectly treat them as full siblings. To obtain correct results when using such programs, the analyst should include only one member of each twin pair in the input files; if both members are included improperly, this will lead to an inflation of the LOD score.

To examine these issues, we computed two-point LOD scores, using LINKAGE, for pedigree 40004 (as given in fig. 3 of the article by Chen et al. [2004]) with marker *D9S274* and found that the LOD scores depend rather strongly on assumptions about the allele frequencies and disease-model parameters. Our results (table 2) matched those of Chen et al. (2004) only when we used model A, which implies that Chen et al. incorrectly included both of the MZ twins and used only two equifrequent marker alleles in their analysis. The assumption that there are only two alleles is incorrect, because Chen et al. (2004) observed five alleles in their own data, and external sources indicate that *D9S274* has at least seven alleles (GDB Human Genome Database [accession number GDB:245741]).

We now explore the effects of various assumptions on the LOD scores. First, we computed the LOD scores by taking two equifrequent markers and only one member of the MZ twin pair (model B) and found that the LOD score decreased for all values of θ, compared with the scores obtained under the model used by Chen et al. (2004) (model A). So, as expected, the removal of one of the MZ twins decreases the LOD score. Second, we explored the effect of using the more realistic marker-allele frequencies taken from GDB. We computed the LOD scores for a number of different models (C–E), permuting the allele frequencies from GDB and taking only one member of the MZ twin pair. We permuted the allele frequencies because we do not know how the allele labels used by Chen et al. (2004) correspond to those used by GDB. The LOD score varies quite a bit across models C–E; for example, at $\theta = 0.05$, the LOD

scores range from 0.20 to 1.28. Third, we assessed the sensitivity of the LOD scores to the disease model when the parameters are adjusted to make the predicted prevalence consistent with the observed prevalence. Model F ($q = 0.026$), which differs from model E ($q = 0.001$) only in the value of disease-allele frequency q, has slightly lower LOD scores at small values of θ than model E. Model G, which differs from model E only in the phenocopy rate (model G $f_0 = 0.05$), also has smaller LOD scores at small values of θ than model E. Thus, increasing the disease-allele frequency or the phenocopy rate to bring the population prevalence K up to ~5% lowered the LOD score slightly.

Chen et al. (2004) claim to have identified a "significant" RLS susceptibility locus on chromosome 9p24-22. Their results might not actually be significant, as a result of the many methodological errors we have described above. The use of equal marker-allele frequencies, overinterpretation of the significance of the NPL scores, and "validation" analysis in only 2 of the 15 families are some of the potential problems that lead us to believe that there is no convincing evidence for an RLS locus on chromosome 9p. Chen et al. (2004) should model linkage heterogeneity and should use the proper marker-allele frequencies, penetrances, and disease-allele frequency when they reanalyze their data.

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Electronic-Database Information

The URLs for data presented herein are as follows:

GDB Human Genome Database, http://gdbwww.gdb.org/ (for *D9S274* alleles [accession number GDB:245741])
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for RLS)

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Reply to Ray and Weeks: Linkage for Restless Legs Syndrome on Chromosome 9p Is Significant

To the Editor:

Ray and Weeks (2005 [in this issue]) discussed some concerns about the statistical methodology of our genomewide linkage-scan study that identified a novel susceptibility locus for restless legs syndrome (RLS) on chromosome 9p24-22 (Chen et al. 2004). Before we systematically address their concerns, it is important to note that the 9p24-22 RLS locus has been replicated in a large family from Germany, with a significant LOD score found by an independent research group (J. Winkelmann, personal communication). This independent replication in a different study population has strongly validated our initial linkage finding.

The Genetic Component of RLS: We are aware of the issue of a bias in estimation of familial correlations and heritability in a population enrolled for genetic stud-