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Genomewide Linkage Scan Identifies a Novel Susceptibility Locus for Restless Legs Syndrome on Chromosome 9p

Shenghan Chen,[1,3,*] William G. Ondo,[4,*] Shaoqi Rao,[1,3] Lin Li,[1,3] Qiuyun Chen,[2] and Qing Wang[1,3]

[1]Center for Molecular Genetics, Department of Molecular Cardiology, Lerner Research Institute, and Center for Cardiovascular Genetics and [2]Cole Eye Institute, The Cleveland Clinic Foundation, and [3]Department of Molecular Medicine, Cleveland Clinic Lerner College of Medicine of Case Western Reserve University, Cleveland; and [4]Department of Neurology, Baylor College of Medicine, Houston

Restless legs syndrome (RLS) is a common neurological disorder that affects 5%–12% of all whites. To genetically dissect this complex disease, we characterized 15 large and extended multiplex pedigrees, consisting of 453 subjects (134 affected with RLS). A familial aggregation analysis was performed, and SAGE FCOR was used to quantify the total genetic contribution in these families. A weighted average correlation of 0.17 between first-degree relatives was obtained, and heritability was estimated to be 0.60 for all types of relative pairs, indicating that RLS is a highly heritable trait in this ascertained cohort. A genomewide linkage scan, which involved >400 10-cM–spaced markers and spanned the entire human genome, was then performed for 144 individuals in the cohort. Model-free linkage analysis identified one novel significant RLS-susceptibility locus on chromosome 9p24-22 with a multipoint nonparametric linkage (NPL) score of 3.22. Suggestive evidence of linkage was found on chromosome 3q26.31 (NPL score 2.03), chromosome 4q31.21 (NPL score 2.28), chromosome 5p13.3 (NPL score 2.68), and chromosome 6p22.3 (NPL score 2.06). Model-based linkage analysis, with the assumption of an autosomal-dominant mode of inheritance, validated the 9p24-22 linkage to RLS in two families (two-point LOD score of 3.77; multipoint LOD score of 3.91). Further fine mapping confirmed the linkage result and defined this novel RLS disease locus to a critical interval. This study establishes RLS as a highly heritable trait, identifies a novel genetic locus for RLS, and will facilitate further cloning and identification of the genes for RLS.

Introduction

Restless legs syndrome (RLS [MIM 102300]) is a common sensorimotor disorder that affects 5%–12% of white populations (Phillips et al. 2000; Rothdach et al. 2000; Ulfberg et al. 2001*a*, 2001*b*). Asian and African populations appear to be less affected (Kageyama et al. 2000; Tan and Ondo 2000). In 1995, the International Restless Legs Syndrome Study Group (IRLSSG) described a set of minimal inclusion criteria for RLS, consisting of four primary features (Walters 1995): (1) an urge to move the extremities, often because of uncomfortable sensations (paresthesia/dysesthesia); (2) motor restlessness; (3) worsening of symptoms with rest and at least partial relief during movement; and (4) worsening of symptoms in the evening or night. A more recent National Institutes of Health (NIH) consensus statement provided a modified version of the criteria: (1) an urge to move the limbs with or without paresthesia; (2) worsening of symptoms at rest; (3) at least transient or partial relief of symptoms with movement; and (4) symptoms worsening during the evening or night (Allen et al. 2003*a*, 2003*b*; Walters et al. 2003). The RLS diagnosis is based on these entry criteria exclusively (Allen et al. 2003*b*), although periodic limb movements while asleep (PLMS) (Montplaisir et al. 1997), a normal neurological examination, improvement with dopaminergic medications, and a family history of RLS all support the diagnosis. Presentation of symptoms in adolescence or even infancy is not rare (Picchietti et al. 1998), although many pediatric patients probably manifest the same condition as adult RLS without meeting the same accepted diagnostic criteria (Allen et al. 2003*b*).

The etiology of RLS is not known, but recent CNS pathology studies demonstrate reduced intracellular, and possibly extracellular, iron stores (Connor et al. 2003). The actual symptoms of RLS, however, improve with dopaminergic medications, which implicates a dopaminergic system in the pathogenesis of RLS (Ondo and Jankovic 1996). The exact interaction between reduced iron and dopaminergic dysfunction is currently under investigation (Earley et al. 2000; Allen and Earley 2001; Allen et al. 2001).

A family history of RLS is reported by ~65% of RLS patients, suggesting the involvement of genetic factors

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Address for correspondence and reprints: Dr. Qing Wang, Center for Molecular Genetics, Lerner Research Institute/ND40, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195. E-mail: wangq2@ccf.org

* The first two authors contributed equally to this article.

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Table 1

Summary Statistics of RLS Families

ITEM	STATISTIC
No. of pedigrees:	
All	15
With 2 generations	1
With 3 generations	5
With 4 generations	7
With 5 generations	2
Mean size of pedigrees	30.2 ± 23.8 (min = 7, max = 90)
No. of pairs:	
Parent-offspring	634
Sib-sib:	399
Sister-sister	94
Brother-brother	121
Brother-sister	184
Grandparent-grandchild	492
Half-sib	12
No. of subjects:	
All	453
Affected	134
Founder	136
Non-founder	317
Male:female ratio	227:226 (50.1%:49.9%)

in the development of RLS (Montplaisir et al. 1997; Lazzarini et al. 1999; Rothdach et al. 2000; Allen et al. 2002; Winkelmann et al. 2002). It is probable that females are affected somewhat more than males (Rothdach et al. 2000). The finding that MZ twins are highly concordant for the presence of RLS also supports the hypothesis that genetic factors contribute to the pathogenesis of RLS (Ondo et al. 2000). Segregation analysis with RLS families argued for a single major gene acting in an autosomal-dominant manner with a multifactorial component (Winkelmann et al. 2002). Nevertheless, as with other common diseases, RLS may have a polygenic basis, possibly with mixed contributions of multiple major genes, modifier genes, and complex interactions of genes with genes and of genes with environmental factors. Thus, in this study, we attempted to genetically analyze RLS as a complex trait. We recruited 15 multiplex RLS families with 453 subjects (134 affected with RLS) and did a genomewide scan to identify novel susceptibility loci for RLS.

Material and Methods

Ascertainment of Multiplex RLS Families

A total of 15 large and extended multiplex RLS families, with a total of 453 subjects, including 134 individuals affected with RLS, were recruited in North America for this study. The largest family consisted of 90 members, and the smallest family had 7 members. All probands were recruited from the patients seen at the Baylor College of Medicine Movement Disorders Clinic (W.G.O.). Families were selected if the proband had at least one first-degree relative who was also affected with RLS. We attempted to contact all living genetic relatives within these families, and then a majority of them (~75%) were enrolled in this study. Because recruitment began in 1996, RLS diagnosis was based on the 1995 IRLSSG criteria (Walters 1995). The phenotype was not determined on the basis of symptom severity. All subjects completed written questionnaires (Hening and Allen 2003) and, subsequently, were interviewed by a neurologist with particular expertise in RLS (W.G.O.). These interviews took place at two family-reunions, at the Baylor College of Medicine, or by phone. The interviewer was not blinded to the family status of the study subject. Physical examination is not part of the diagnostic criteria for RLS, but it was performed on subjects who were interviewed in person. To determine the secondary causes of RLS, interviewed subjects were asked whether they had a history of kidney disease, a history of anemia, a history of damage to the nerves, and so forth, except in the case of probands, who all had normal serum ferritin levels. Two probands had minor neuropathy, but we concluded that it was not related to the RLS. Subjects who experienced RLS only during pregnancy were not phenotyped as positive for RLS. Despite that children may have a different presentation of symptoms than the presentation seen in adults, children were only phenotyped as having RLS if they met the inclusion criteria for adults. Although patients were not reinterviewed after publication of the 2003 NIH criteria (Allen et al. 2003*a*, 2003*b*; Walters et al. 2003), we believe that applying these criteria would not change the phenotype in any case. Informed consent was ob-

Table 2

Correlation Coefficients (*r*) of RLS among Various Relative Pairs

RELATIONSHIP	NO. OF PAIRS	*r*
1st-degree relatives:	918	.171**
Parent-offspring:	567	.096** ± .033
Father-son	135	.195* ± .088
Mother-son	134	−.182* ± .110
Father-daughter	148	.232** ± .087
Mother-daughter	150	.132 ± .103
Siblings:	351	.291** ± .096
Brother-brother	106	.480** ± .161
Brother-sister	166	.161 ± .132
Sister-sister	79	.392** ± .122
2nd-degree relatives:	1205	.039
Grandparent-grandchild	439	−.032 ± .051
Avuncular	766	.079 ± .089
3rd-degree relatives:	1243	.090**
Great-grandparent	184	−.015 ± .078
First cousin	604	.091 ± .088
Great-avuncular	455	.131 ± .098
4th-degree relatives:	1412	.0640*
First cousin once removed	920	.067 ± .073
Second cousin	492	.058 ± .084
Unrelated spouse	117	−.412** ± .085

NOTE.—*$P < .05$; **$P < .01$

Table 3

Summary of Chromosomal Regions with an NPL Score >2.0

Chromosome and Marker	Location	Map Position (cM)	NPL Score	
			Multipoint	Two-point
3:				
D3S2427	3q26.31	188.0	2.03	.75
4:				
ATT015	...	26.2	1.76	2.09
D4S1644	4q31.21	143.4	2.28	.98
D4S1625	4q31.21	146.1	2.19	1.40
D4S2417	4q34.3	182.0	2.16	1.00
5:				
D5S2505	5p15.32	14.3	2.03	1.07
D5S2845	5p14.3	36.0	2.61	2.25
D5S1470	5p13.3	45.0	2.68	1.68
6:				
D6S2439	6p22.3	42.0	2.06	1.16
9:				
D9S286	9p24.1	17.9	3.22	3.41
GATA187D09	9p23	22.0	2.87	.60

Note.—NPL scores were computed using GENEHUNTER. Allele frequencies for the markers were estimated as ML, by the use of SAGE FREQ and the genotyping data generated in this study.

tained from participants in accordance with standards established by local institutional review boards. The summary statistics for the 15 multiplex pedigrees are shown in table 1.

Genotyping

We performed genomewide genotyping for 144 study subjects from the 15 multiplex RLS families, including both RLS patients and unaffected family members, on the basis of DNA-sample availability. Genomic DNA was prepared from whole blood using the DNA Isolation Kit for Mammalian Blood (Roche Diagnostic). Initial genotyping was performed by the NHLBI Mammalian Genotyping Service, directed by Dr. J. Weber, with 404 ~10-cM–spaced polymorphic markers (microsatellite markers and SNPs) spanning the human genome, with a maximum gap of 17 cM (Weber and Broman 2001). Additional genotyping with microsatellite markers was done in our laboratory, as described elsewhere (Wang et al. 1995, 1996, 2003; Chen et al. 1998).

Data Preparation

The process of data collection was monitored and supervised by experts in statistical genetics and human genetics, for suitability of genetic analysis. The RLS disease phenotype information was updated on a regular basis and was entered into our database for genetic analysis of RLS. Prior to the genetic analysis, several rounds of data cleaning and assurance were performed on the data set. Obvious locus-order errors and genotyping errors that commonly occur with large-scale genotyping were corrected (locus-order errors were detected by the Marshfield NHLBI Genotyping Service). Allele frequencies for the markers in the cohort were estimated according to maximum likelihood (ML), with the use of the SAGE program FREQ (SAGE 2003) and the genotyping data generated in this study. Pedigree relationship was tested using the SAGE RELTEST, which employs a Markov-process model of allele-sharing along the chromosome and uses genome-scan data to classify pairs of pedigree members according to their true relationship (Olson 1999; SAGE 2003). After correcting relationships, SAGE MARKERINFO (SAGE 2003) was used to detect any Mendelian-inheritance inconsistency for each marker, and, if detected, then the inconsistent genotyping data for the marker were removed manually. Inheritance inconsistency was detected in one male, and the individual was excluded from linkage analysis.

Familial Aggregation Analysis

Familial correlations were estimated using SAGE FCOR (SAGE 2003) to quantify the genetic contributions in the ascertained multiplex families. FCOR calculates multivariate familial correlations and their asymptotic SEs, for all pair-types available in the RLS pedigrees, on the basis of the equivalent number of independent pairs that, theoretically, could have been used to obtain the same SE for a given correlation (SAGE 2003). The program estimates familial correlations for both subtypes and main types (groups of subtypes), together with the corresponding asymptotic SEs derived from the variance-covariance matrices of the estimated correlations (SAGE 2003). Correlations for relative pairs at different levels (the first-degree relative pairs to the fourth-degree relative pairs, etc.) were averaged over all the types and weighted uniformly (each pair with inversely proportional to the number of such pairs in the pedigrees) (SAGE 2003). These large extended families provide sufficient information to decompose genetic components from RLS phenotypic variations. Reliable estimates for various types of relative pairs can be obtained. As shown in table 1, the recruited pedigrees consist of 1 pedigree with 2 generations, 5 with 3 generations, 7 with 4 generations, and 2 with 5 generations, which provide a combined maximum of 634 parent-offspring pairs, 399 sibling-sibling pairs, and 492 grandparent-grandchild pairs. Technically, familial aggregation analysis is a more detailed version of the mixed linear model approach, in that each type of relative pair is estimated separately instead of modeling them as a function of a few parameters in a single covariance matrix. Historically, familial aggregation analysis has been the most popular method for determining genetic causes in disease manifestation. This method, in essence, is to

estimate the correlations between various biological relatives and then assume that they can be explained parsimoniously by an additive genetic contribution and a common household contribution, without having to make other assumptions of the mixed linear model.

Familial Risk Ratios Estimation

Familial risk ratios (λ_R) were estimated, as described elsewhere (Risch 1990). In brief, λ_R is the risk of type-*R* relatives of affected individuals of being affected themselves, divided by the population-prevalence frequency (*K*). If the frequency of affected pairs with relationship *R* is denoted by K_2, then $\lambda_R = K_2/K^2$ (Risch 1990).

Linkage Analysis

Nonparametric linkage (NPL) analysis.— Affected relative pair (ARP) analysis was done by use of the NPL analysis implemented in GENEHUNTER (Kruglyak et al. 1996). Like other ARP methods, the NPL statistic measures allele sharing among the affected individuals within a pedigree (Kruglyak et al. 1996). The scoring function statistic was used to evaluate, simultaneously, allele sharing among all those affected in a nuclear family, in contrast to pairwise comparison. Both two-point and multipoint NPL analyses were performed. This scoring function was asymptotically distributed as the *Z* statistic.

Model-based linkage analysis.— Two-point linkage analysis between the underlying disease locus and each marker was performed using LINKAGE version 5.2 (Lathrop et al. 1985). Multipoint LOD scores were computed using SimWalk2 (Lange and Sobel 1991), with input files automatically made by Mega2 version 2.5 (Mukhopadhyay et al. 1999). An autosomal-dominant inheritance mode was assumed for the putative disease locus, and penetrance was set at 0.95 on the basis of observations of the high frequencies of affected persons in at-risk sibships within pedigrees. The frequency of the disease allele was set to 0.001. The allele frequencies of markers were $1/n$, where n is the number of alleles observed.

Table 4

Pairwise LOD Scores between RLS and Chromosome 9p24-22 Markers Obtained by Model-Based Linkage Analysis in Two Extended RLS Kindreds

	Recombination Fraction (Θ)					
Marker and Kindred	.00	.05	.10	.20	.30	.40
D9S1779:						
40004	.00	.00	.00	.00	.00	.00
40015	−1.19	.61	.75	.70	.49	.22
Total	−1.19	.61	.75	.70	.49	.22
D9S1871:						
40004	.29	.27	.25	.20	.14	.07
40015	1.99	1.83	1.65	1.29	.89	.47
Total	2.28	2.10	1.90	1.49	1.03	.54
D9S2169:						
40004	.89	.82	.75	.60	.43	.23
40015	1.91	1.75	1.58	1.21	.83	.41
Total	2.80	2.57	2.33	1.81	1.26	.64
D9S286:						
40004	1.79	1.63	1.47	1.11	.72	.32
40015	1.98	1.81	1.64	1.28	.89	.47
Total	3.77	3.44	3.11	2.39	1.61	.79
D9S168:						
40004	.00	.00	.00	.00	.00	.00
40015	1.84	1.68	1.51	1.16	.79	.39
Total	1.84	1.68	1.51	1.16	.79	.39
D9S268:						
40004	.58	.47	.36	.14	−.03	−.08
40015	1.54	1.40	1.26	.96	.64	.31
Total	2.12	1.87	1.62	1.10	.61	.23
D9S274:						
40004	1.24	1.09	.94	.64	.33	.09
40015	1.98	1.81	1.64	1.28	.89	.47
Total	3.24	2.90	2.58	1.92	1.22	1.56
D9S1839:						
40004	.00	.00	.00	.00	.00	.00
40015	.77	.68	.58	.38	.20	.05
Total	.77	.68	.58	.38	.20	.05
D9S162:						
40004	−1.50	−1.70	.02	.11	.07	.00
40015	2.02	1.85	1.67	1.30	.90	.47
Total	.52	.15	1.69	1.41	.97	.47
D9S1121:						
40004	−1.50	−1.48	−1.29	−.80	−.47	−.23
40015	.20	.17	.14	.08	.03	.01
Total	−1.30	−1.31	−1.15	−.72	−.44	−.22

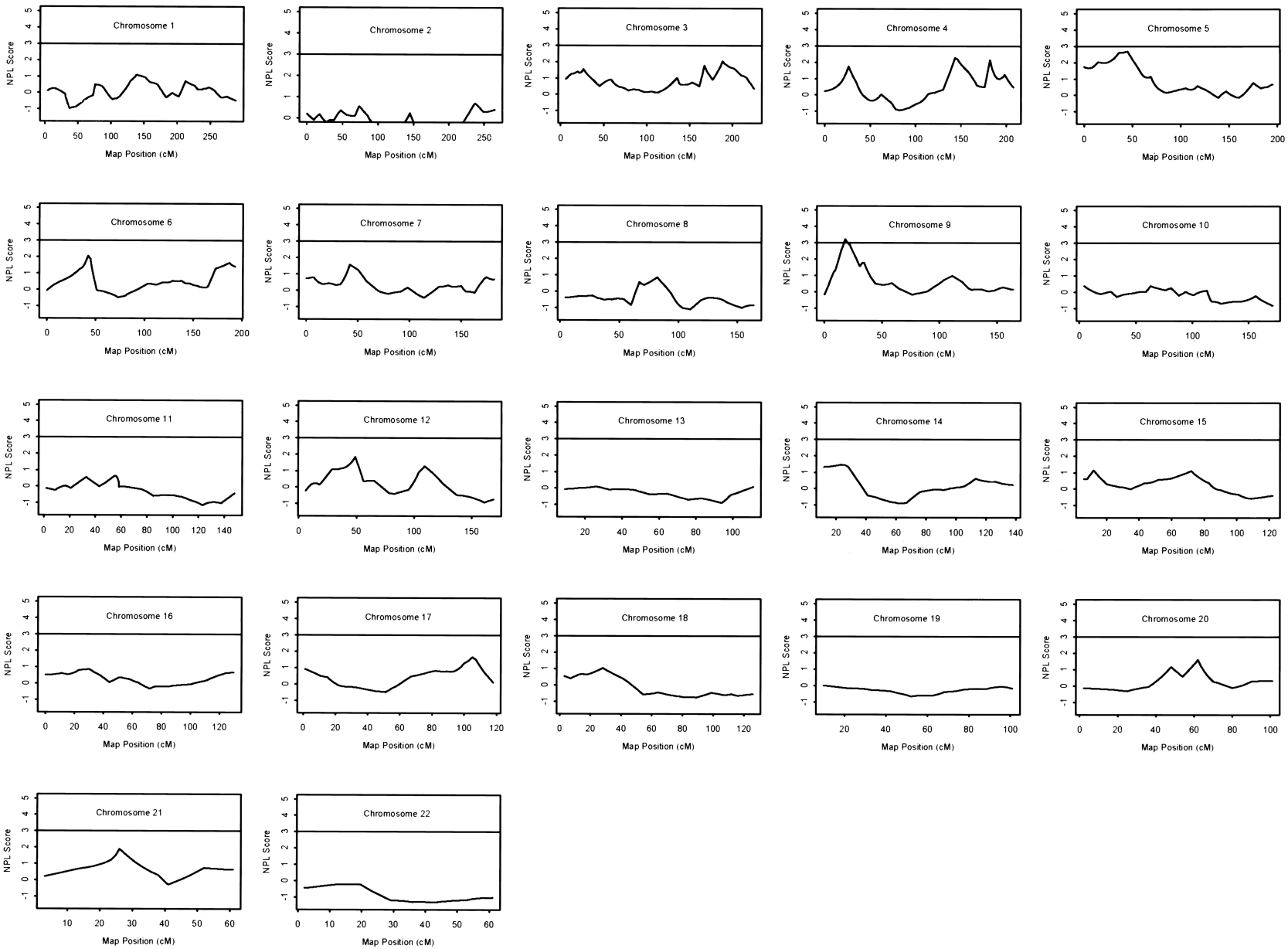
Note.—LOD scores were computed with the assumption of 95% penetrance and a gene frequency of 0.001. The allele frequencies of markers were $1/n$, where n is the number of alleles observed.

Results

Familial Aggregation Analysis

Familial aggregation analysis (table 2) yielded correlation coefficients (*r*) for pedigree relatives at four levels of degrees, consisting of 14 major relative-types. The correlations for sibling relationship are intraclass correlations, and those for other relationships are interclass correlations. Statistical tests, against the null hypothesis of zero correlation, were conducted by a *t* test using the asymptotic SE estimates supplied by SAGE FCOR (SAGE 2003).

For the first-degree relative pairs (918 pairs), $r = 0.17$; for the second-degree relative pairs (1,205 pairs), $r = 0.04$; for the third-degree relative pairs (1,243 pairs), $r = 0.09$; and, for the fourth-degree relative pairs (1,412 pairs), $r = 0.06$ (table 2). These results suggest a strong familial aggregation of RLS in this ascertained cohort. The correlation estimates between the same-gender pairs of individuals were higher than those between individuals of the opposite sex, with brother-brother pairs having the highest correlation ($r = 0.48$), followed


Chromosome 1
Chromosome 2
Chromosome 3
Chromosome 4
Chromosome 5
Chromosome 6
Chromosome 7
Chromosome 8
Chromosome 9
Chromosome 10
Chromosome 11
Chromosome 12
Chromosome 13
Chromosome 14
Chromosome 15
Chromosome 16
Chromosome 17
Chromosome 18
Chromosome 19
Chromosome 20
Chromosome 21
Chromosome 22
NPL Score
Map Position (cM)

by sister-sister pairs ($r = 0.39$), and sister-brother pairs having the lowest correlation ($r = 0.19$), which may suggest a sex-linked effect for the disorder. There is a considerable negative correlation between spouses ($r = -0.41$), which may reflect the lower risk of RLS among the spouses who married into the family (5%–12%) than in the ascertained families (29.6%). The derived heritability, estimated by combining all information from all types of relative pairs, was 0.60, which indicates that RLS is a highly heritable trait in this ascertained cohort.

We also estimated familiar relative risk ratios for the first-degree relative pairs using Risch's model (1990). For a conservative estimate, a high-population prevalence rate of 12% was used in the calculation. The absolute risks, in terms of the concordance rate of affected status between the pairs, were 23% for parent-offsprings, whose $\lambda_R = 10.25$, and 15% for siblings, whose $\lambda_R = 16.23$. These results are in agreement with FCOR correlations-analysis and suggest that there is a strong familial aggregation in the RLS families studied.

Model-Free Linkage Analysis

Genomewide NPL analysis of our RLS cohort was conducted using GENEHUNTER. The chromosomal regions identified as "potentially interesting" with a peak NPL score of >2.0 (Kruglyak et al. 1996) are listed in table 3 and shown in figure 1. Ten markers on five different chromosomes (chromosomes 3, 4, 5, 6, 9) generated multipoint NPL scores >2.0 (table 3; fig. 1). The highest NPL scores, 3.22 and 2.87, were obtained for two markers, D9S286 and GATA187D09, that are separated by 4.1 cM on chromosome 9p24-22. The linkage to another marker, GATA27A11 (D9S925) which is 10.1 cM to GATA187D09, remains positive for linkage, with an NPL score of 1.69. Then, a permutation test with up to 10,000 permutations was performed using SimWalk2 (Lange and Sobel 1991). A pointwise empirical *P* value of .009 was obtained for this 9p24-22 RLS locus.

Model-Based Linkage Analysis

To validate the results from NPL analysis, we analyzed chromosomal regions with NPL scores >2.0 by use of model-based linkage analysis. The results from two extended RLS families, 40004 and 40015, confirmed the existence of the chromosome 9p24-22 locus that was identified by model-free linkage analysis (table 4). The results of two-point linkage analysis with selected markers at the chromosome 9p24-22 locus are shown in table 4. Combined two-point LOD scores of 3.77 and 3.24, at a recombination fraction of zero, were obtained for two nearby markers, D9S286 and D9S274, respectively, on the assumption of an autosomal-dominant mode of inheritance (table 4).

Fine mapping was performed with additional markers D9S1779, D9S1871, D9S2169, D9S168, D9S268, D9S1839, D9S162, and D9S1121 at the chromosome 9p24-22 RLS locus. Multipoint LOD scores for the region were obtained using SimWalk2 (Lange and Sobel 1991) for random walk analysis of multiple marker information, and the resulting scores are shown in figure 2. The peak multipoint LOD score of 3.9 was obtained from marker D9S2169 to D9S286 (fig. 2).

Haplotype transmission-pattern analysis further validated the mapping of a novel RLS locus to chromosome 9p24-22 (figs. 3 and 5). In kindred 40004, all affected individuals—but none of the normal individuals—carried a common haplotype, 2_2_3_5_2_2_1_1 for the eight contiguous markers D9S1779, D9S1871, D9S2169, D9S286, D9S168, D9S268, D9S274 and D9S1839 (fig. 3). The common haplotype shared by all affected individuals in kindred 40015—but not by any normal individuals—was 3_2_3_3_2_2_3_10 for the eight contiguous markers D9S1871, D9S2169, D9S286, D9S168, D9S268, D9S274, D9S1839, and D9S162.

Two obligate recombination events, one in kindred 40004 (individual III-1; fig. 3) and the other in kindred 40015 (individual II-1; fig. 4), defined the critical 9p24-22 RLS disease gene within a region spanned by markers D9S1779 and D9S162 (fig. 5).

Discussion

This study represents the first model-free linkage analysis designed to genetically dissect the complex disease RLS and to identify genetic loci causing susceptibility to RLS. Our study provided significant evidence of linkage for a novel disease-susceptibility RLS locus on chromosome 9p24-22. Model-free multipoint linkage analysis revealed an NPL score of 3.22 at marker D9S286. The permutation tests by SimWalk2 revealed an empirical pointwise *P* value of .009. Later, model-based linkage analysis with the assumption of an autosomal-dominant mode of inheritance resulted in a multipoint LOD score of 3.91 at D9S286 in two extended families. These results validate the identification of the significant linkage to RLS on chromosome 9p24-22.

Figure 1 Genomewide NPL scan for RLS-susceptibility loci. A total of 404 microsatellite markers spanning the entire human genome were genotyped in 144 individuals from multiplex RLS families. The vertical *Y*-axis of each plot denotes NPL scores generated by GENEHUNTER. The *X*-axis represents marker map positions in cM from the telomere of the p arm of each chromosome. The horizontal solid line in each plot corresponds to an NPL score of 3.0.

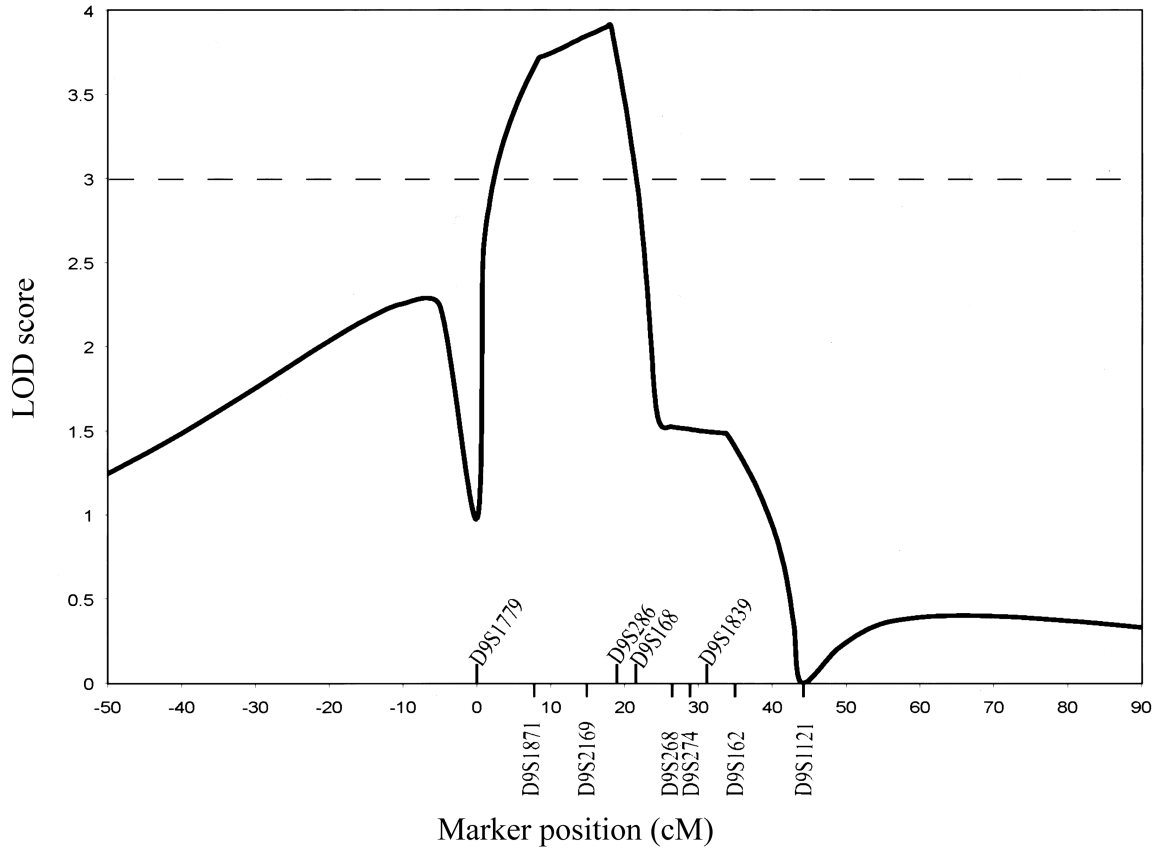


Figure 2 Multipoint LOD-score analysis for markers at the 9p 24.2–22.3 RLS locus. Random walk analysis was done using SimWalk2. Location of marker D9S1779 is arbitrarily set at 0 cM. Other microsatellite markers are scaled on the basis of their absolute distance (in cM) from D9S1779. Multipoint LOD scores are plotted on the ordinate. The dashed line marks an LOD score of 3.0.

Two other model-based linkage analyses were recently reported for RLS. Desautels et al. (2001) identified an autosomal-recessive RLS locus on chromosome 12q22–23 in a single family, and Bonati et al. (2003) mapped an autosomal-dominant RLS locus to chromosome 14q13–21, also in a single family. In our model-free linkage analysis, no markers on chromosome 14q yielded an NPL score >1.0 (fig. 1). Interestingly, marker PAH yielded an NPL score of 1.29 in our model-free linkage analysis (fig. 1). As PAH is located within the 12q22-23 RLS-locus between D12S1044 and D12S78 and is 2.4 cM to marker D12S78, our results may provide indirect confirmation of the mapping of an RLS gene on chromosome 12q22-23, reported elsewhere (Desautels et al. 2001). Identification of three genetic loci for RLS on three different chromosomes, 12q22-23 (Desautels et al. 2001), 14q13-21 (Bonati et al. 2003), and 9p24-22 (this study), suggests that RLS is a genetically highly-heterogeneous disorder.

Twin studies (Ondo et al. 2000) and the observation that ~65% of patients report a family history of RLS (Montplaisir et al. 1997; Lazzarini et al. 1999; Rothdach et al. 2000; Allen et al. 2002; Winkelmann et al. 2002) suggest that genetic factors contribute to the pathogenesis of RLS. However, few formal epidemiological and statistical studies have been performed to elucidate the genetic architecture of this complex disease. This study reports such a formal analysis. A high, positive r of 0.17 between the first-degree relatives suggests their strong phenotypic resemblance. Furthermore, the heritability of RLS was estimated to be a very high value of 0.60 for all types of relative pairs. These results indicate that RLS is a highly heritable trait in this ascertained cohort. It is interesting that the correlations for relative pairs were higher for siblings than for parent-offspring pairs and were highest for same-sex siblings. This suggests that gender and other environmental factors are involved in RLS and that our estimate may represent an upper limit of the degree of heritability that may include a part of shared common environment. It is important to note that familial correlations and heritability were estimated for an ascertained cohort, and the results may not be generalizable to the RLS population at large.

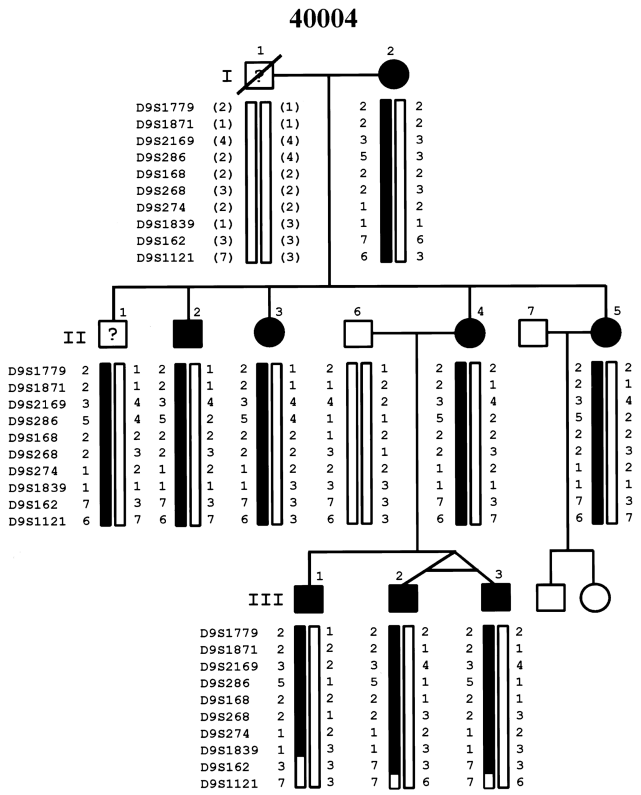


Figure 3 Haplotype analysis in kindred 40004 affected with RLS. Circles and squares denote females and males, respectively; blackened symbols indicate affected individuals; unblackened symbols indicate normal individuals; the symbol with a slash indicates a deceased individual; and symbols with a question mark indicate an individual with uncertain phenotype. Genotyping results for markers D9S1779, D9S1871, D9S2169, D9S286, D9S168, D9S268, D9S274, D9S1839, D9S162, and D9S1121 are shown under each symbol. Haplotypes were constructed on the basis of the minimum number of recombinations between markers. The disease haplotype shared by all affected individuals is denoted by the blackened vertical bar, and normal haplotype is denoted by an unblackened vertical bar. Recombination events were observed in individuals III-1, III-2, and III-3 and defined the critical RLS gene location as upward from marker D9S162.

The 9p24-22 RLS locus contains >100 genes (NCBI Human Genome Resources; UCSC Genome Bioinformatics). Among the genes in the region, we selected three genes for mutation analysis on the basis of their locations and physiology. Multi-PDZ Domain Protein 1 (*MUPP1* [MIM 603785]) is a gene encoding a protein with 13 PDZ domains that interacts with the C-terminal domain of the serotonin 5-HT_{2C} receptor (Ullmer et al. 1998). It was isolated in a yeast two-hybrid screening with the C-terminal domain of the 5-HT_{2C} receptor as the bait. MUPP1 is expressed in the brain and in several peripheral organs. MUPP1 might be involved in the mechanisms of G–protein-coupled receptor signaling, for example, the 5-HT_{2C} receptor-activated phosphoinositide-linked second message system. Thus, *MUPP1* became a candidate gene for RLS. All 44 exons of *MUPP1*, including exon-intron boundaries, were screened for RLS-related mutations by use of direct DNA-sequence analysis and single-strand conformation polymorphism analysis, but no disease-causing mutations were found.

The second candidate gene is *SLC1A1* (MIM 133550), which encodes the human high-affinity neuronal and epithelial glutamate transporter EAAC1. The function of EAAC1 is to transport L-glutamate and also L- and D-aspartate, a function that is essential for terminating the postsynaptic action of glutamate by rapidly removing released glutamate from the synaptic cleft. It acts as a symport by cotransporting sodium. EAAC1(EAAT3) mRNA and protein expression was detected in both brain and peripheral tissues. All 12 exons

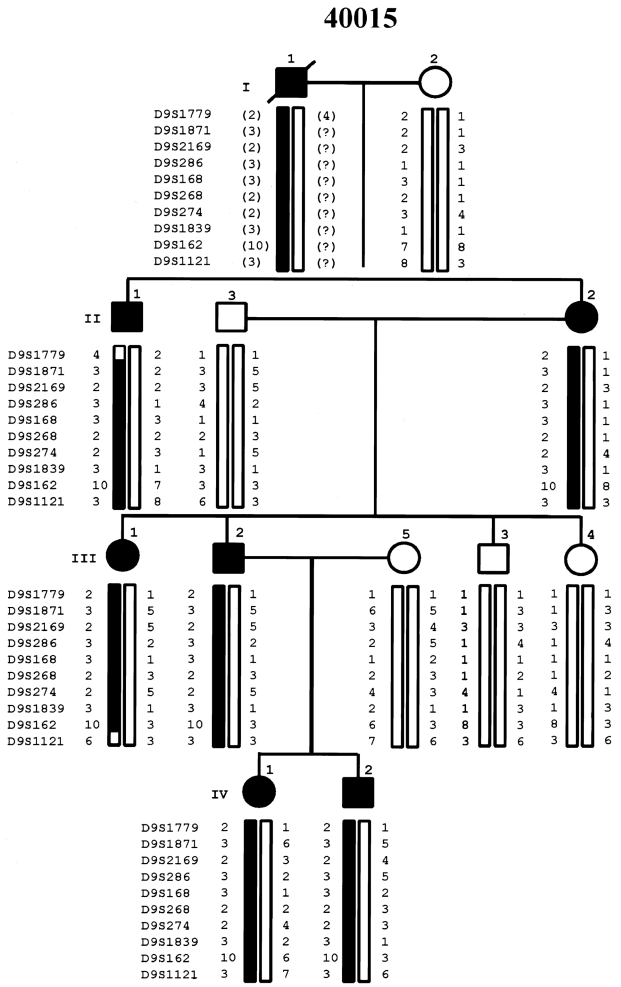


Figure 4 Haplotype analysis in kindred 40015 affected with RLS. Data are shown as described in fig. 3. Two recombination events were observed, one in individual II-1 and the other in III-1. The obligate recombination in II-1 defined the RLS gene location as downward from D9S1779.

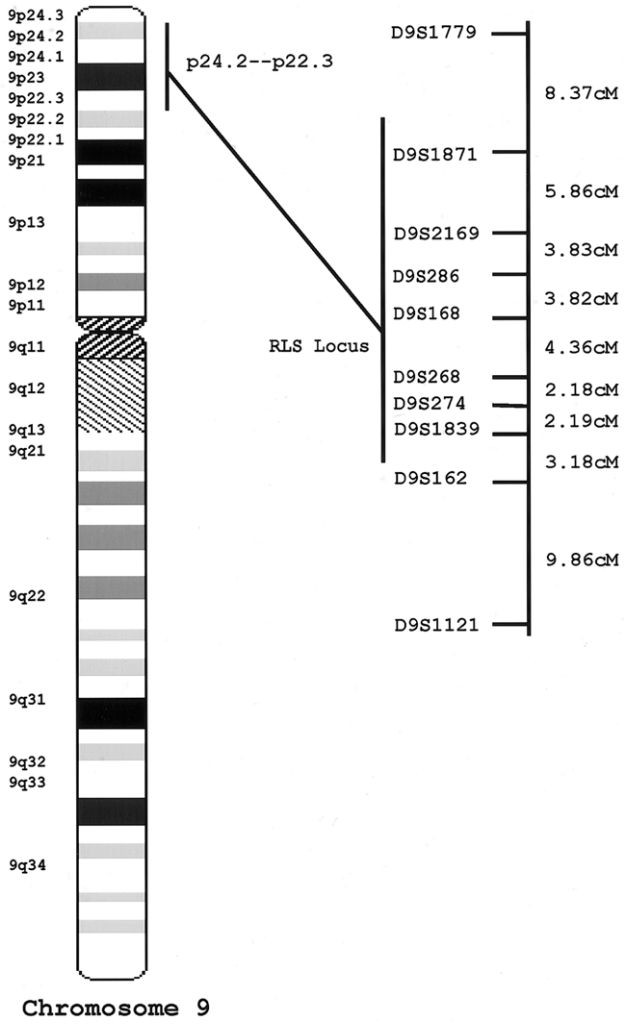


Figure 5 Ideogram of chromosome 9 with Geimsa banding and localization of the 9p24-22 RLS locus. The genetic map with chromosome 9p24-22 markers is shown, and the likely location of the putative RLS gene is indicated by a vertical bar (from D9S1871 to D9S1839).

of the *SLC1A1* gene, including all exon-intron boundaries, were screened for mutations in RLS patients, but none were found. Furthermore, no disease-causing mutations were identified in the third candidate gene, *KCNV2* (MIM 607604), which encodes a potassium-channel subunit that mediates the voltage-dependent potassium-ion permeability of excitable membranes.

Continued mutation analysis in candidate genes that are located within the 9p24-22 RLS locus and that play a role in neuronal signaling, iron metabolism, and dopaminergic function will lead to the identification of an underlying major (or minor) gene for common disease RLS. Identification of an RLS gene should provide insights into the molecular mechanism for the pathogenesis of RLS.

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Electronic-Database Information

Accession numbers and URLs for data presented herein are as follows:

GENEHUNTER, http://linkage.rockefeller.edu/soft/gh/ or http://www.hgmp.mrc.ac.uk/About/Courses/2003/comp.linkage/genehunt.html

Human Genome Resources, http://www.ncbi.nlm.nih.gov/genome/guide/human/

Marshfield Medical Research Foundation, http://research.marshfieldclinic.org/genetics/

Mega2 version 2.5 (2001), http://watson.hgen.pitt.edu/mega2.html

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim

UCSC Genome bioinformatics, http://genome.ucsc.edu/

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