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A novel locus for restless legs syndrome maps to chromosome 19p in an Irish pedigree

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Abstract

Restless legs syndrome (RLS) is a common, sleep-related movement disorder. The symptoms follow a circadian pattern, worsening in the evening or night, leading to sleep disruption and daytime somnolence. Familial forms of RLS have been described and usually display an autosomal dominant pattern of inheritance. To date, linkage analysis has identified nine RLS loci but no specific causative gene has been reported. Association mapping has highlighted a further four genomic areas of interest. We have conducted a genome wide linkage analysis in an Irish autosomal dominant RLS pedigree with 11 affected members. Significant linkage was found on chromosome 19p for a series of microsatellite markers, with a maximum two-point LOD score of 3.59 at $\theta = 0.0$ for marker D19S878. Recombination events, identified by haplotype analysis, define a genetic region of 6.57 cM on chromosome 19p13.3, corresponding to an interval of 2.5 Mb. This study provides evidence of a novel RLS locus, and provides further evidence that RLS is a genetically heterogenous disorder.

Keywords Restless Legs Syndrome; RLS; Genome-wide search; Linkage analysis; Locus; Movement disorder.

Introduction

RLS is a common neurodegenerative condition that often leads to reduced quality of life. It is characterised by uncomfortable, unpleasant sensations in the lower limbs inducing an uncontrollable desire to move the legs. RLS exhibits a circadian pattern with symptoms present predominantly in the evening or at night [1]. The severity of symptoms varies. The prevalence of RLS is reported to be greater than 5% in the general population [2]. Prevalence of RLS increases with age [3]and affects women about twice as often as men [4]. Its clinical recognition is poor, often being underdiagnosed or misdiagnosed [5].

Four criteria were defined as essential for its clinical diagnosis by the International Restless Legs Syndrome Study Group (IRLSSG) and further modified by the National Institutes of Health (NIH) in 2003 [6]. These criteria are (1) an urge to move the legs, usually accompanied by uncomfortable sensations, (2) sensations begin or worsen during periods of rest or inactivity such as lying or sitting, (3) partially or totally relieved by movements such as walking or stretching, and (4) worsening of symptoms in the evening or at night. RLS frequently interferes with sleep, resulting in excessive daytime sleepiness, lack of concentration, poor work performance, and negative impacts on quality of life. RLS severity may be evaluated using the International RLS Study Group Rating Scale (IRLS-RS) [7]. Physical and neurological examinations are normal in RLS. RLS is classified as primary (idiopathic) or secondary. Primary RLS includes sporadic and familial cases. Secondary forms can occur as a complication of another health condition, such as iron deficiency anaemia, kidney disease, polyneuropathy, or thyroid dysfunction [8]. The precise pathophysiology of RLS remains unknown, but pharmacological and brain imaging studies suggest the involvement of dopaminergic and iron pathways in the brain and spinal cord [9-12].

A genetic contribution to primary RLS has been well-documented, is substantial, and has been consistently recognised from population and family studies. Ekbom, who first described RLS in 1945, described familial aggregation in one-third of RLS patients in 1960 [13]. To date, 9 major susceptibility loci for RLS have been identified by linkage analysis [14-21]. Only one locus has been reported under an autosomal recessive (AR) model. Originally reported in a French Canadian family, AR RLS linked to chromosome 12q has since been reported in several other French Canadian families as well as in the Icelandic population [22,23]. Evidence of linkage in all other instances assumed an autosomal dominant (AD) inheritance model and is limited to single or a small number of kindreds. Genome-wide association studies have identified variants suspected to be involved in RLS. Single nucleotide polymorphisms (SNPs) in the *BTBD9* gene on chromosome 6p21 have been found to be associated with RLS [24,25]. Additionally variants in the intronic and intergenic regions of the *MEIS1*, *MAP2K5*/*LBXCOR1*, *PTPRD* and *NOS1* genes have been identified in association studies [24,26,27]. These molecular findings suggest substantial genetic heterogeneity in RLS.

In this study we report the investigation of an Irish family with autosomal dominant RLS and the finding of a novel RLS locus on chromosome 19p13.3, providing further evidence of genetic heterogeneity of RLS.

Materials and methods

Subjects

Approval was obtained from the Cork Teaching Hospitals Ethics Committee for this study. A three generation Irish RLS pedigree (RLS3002) was recruited. Participating family members gave written informed consent. A detailed clinical history was taken by a study physician using a structured questionnaire. It included enquiry about symptoms of RLS, in particular noting periodic limb movements during sleep, any sleep disturbances, past medical and drug history, and family history. Female participants were asked about the effect of pregnancy on symptoms. Full neurological

examinations were performed. To diagnose RLS, the four essential criteria proposed by the IRLSSG had to be fulfilled.

Participants who met the IRLSSG criteria for RLS were asked to complete a standard International Restless Legs Syndromes Rating Scale (IRLS-RS) questionnaire [7]. The questionnaire, which was completed by the study physician while interviewing the patients, is a scoring system for RLS patients. The system uses ten questions, and each answer varies numerically from 0 to 4 with the higher scores representing more severe symptoms. The questionnaire covers a number of topics including: the frequency of RLS symptoms throughout a week; the length of RLS symptoms over a day; daytime drowsiness and tiredness; the quality of daytime performances; severity of RLS overall; relief with movement; and the consequence of RLS symptoms on behaviour and mood. Results for all 10 questions were compiled to give an overall score. In accordance with the scoring system, severity was categorised into four groups scoring system: mild (0-10); moderate (11-20); severe (21-30); and very severe (31-40).
Venous blood was obtained from each participant and stored in EDTA treated tubes. Family members were assigned their own unique sample ('PATH') number.

Genotyping

Genomic DNA was extracted from venous blood using a QIAamp Blood Kit (Qiagen), according to the manufacturer's protocols. Microsatellite markers were amplified by PCR using fluorescently labelled primers. Genotype information was obtained using the ABI 3130 Genetic Analyzer (Applied Biosystems). For genome-wide scans, two mapping sets were used. The 146 microsatellite mapping set from Research Genetics Inc has an average spacing of 20 cM between markers. The Applied Biosystems Linkage Mapping Set version 2.5 contains 400 markers with an average spacing of 10 cM.

Linkage analysis

Two-point linkage analysis was carried out using the MLINK routine of FASTLINK. Segregation of the disease phenotype indicated an autosomal-dominant mode of inheritance. Analysis was performed under this model with the assumption of a disease allele frequency of 0.1% and disease allele penetrance of 95% as previously reported [16]. Equal recombination rates were assumed in males and females. Allele frequencies for known microsatellites were obtained from the CEPH website (http://www.cephb.fr/). Equal allele frequencies were used in the analysis of novel microsatellite markers. Additional *post hoc* analysis under a number of more stringent models was also performed (Supplemental data).

Fine Mapping of the Candidate Region

The candidate region was further refined using a set of 14 microsatellite markers. One marker was from the Applied Biosystems Linkage Mapping Set. Another seven known microsatellite markers were described on the University of California-Santa Cruz (UCSC) human genome assembly (http://genome.ucsc.edu/). The remaining six markers were developed in our laboratory as the candidate region was microsatellite-poor. These novel microsatellite markers were identified by scanning the DNA sequence of the candidate region for short segments of DNA that have a repeated sequence >10 repeat units (2-4 bp) long. PCR primers unique to the position were then designed for PCR amplification. Marker order for all markers was based on the physical map, and genetic distances were based on the sex-averaged Marshfield genetic map when available. For the newly developed microsatellite markers genetic positions were calculated by linear interpolation based on physical positions, a method previously used by Levchenko and associates [21].

Results

The RLS3002 family comprises 11 individuals affected with RLS and 7 unaffected members (Fig. 1). For the deceased grandparents, affection status was ascertained from histories provided by living family members. Clinical data from all 11 affected participants and age at examination for the unaffected members in generation three are reported in Table 1. An IRLS-RS severity rating scale ranging from mild (5 subjects) to moderate (6 subjects) was observed for this family.

Previously known RLS loci and associated genes were examined for possible linkage to this Irish RLS pedigree. Using microsatellite markers over each region, linkage was excluded to loci on 12q, 14q, 9p, 20p, 2q, 19p, 16p, 17p and 4q. Linkage was also excluded from regions containing the SNPs previously reported to be associated with RLS within the following genes; *BTBD9*, *MEIS1*, *MAP2K5/LBXCOR1*, *PTPRD* and *NOS* (data not shown).

An initial genome-wide scan was performed with the lower density mapping set but no areas of linkage were identified. This was followed by a genome-wide scan using the higher density linkage set. Analysis revealed five regions which presented with LOD scores of greater than 1. By increasing the marker density in these regions all but one candidate locus, on chromosome 19, were excluded. SLINK simulation analysis [28]indicated that the maximum LOD score achievable for this family is 3.58 θ = 0.00. An initial positive LOD score of 2.01 at θ = 0.08 was obtained for marker D19S209, which suggested linkage of autosomal dominant RLS to this area. Fine-mapping linkage analysis of the region on chromosome 19p13.3 yielded a maximum two-point LOD score of 3.59 at θ = 0.00 with microsatellite marker D19S878. Analysis of all markers under a number of more stringent models consistently yielded a maximum LOD score of > 3.0 for D19S878 and CA19S2310 at theta = 0.0. Details of LOD scores are provided in Table 2 and Supplemental Data. A maximum multipoint LOD score of 3.75 was obtained at a position of 7.54 cM on chromosome 19, the nearest marker being D19S878 which is positioned at 6.57 cM (Table 2 and Fig. 2).

Haplotype reconstruction revealed that all 11 affected individuals in the family carry a common haplotype which is identified by markers CA19S872, D19S886, GT19S949, AC19S1117, CA19S2310, and D19S878 (Fig. 1). A recombination event between markers D19S878 and D19S565 in individual III-7 allowed for the definition of the centromeric boundary of the 19p RLS candidate gene region. No distal recombination event was detected. Therefore, a genetic locus of 6.57 cM corresponding to 2.5 Mb on chromosome 19p13.3 flanked by the telomere and microsatellite marker D19S565 has been identified for RLS in this pedigree (Fig. 1).

Individual III-10 is currently unaffected but shares a 1.1 Mb part of the affected haplotype from markers CA19S872 to AC19S1117 (Fig. 1). The RLS gene may lie in the portion of the region not shared by this individual. Alternatively, he may carry the affected haplotype but not express the RLS phenotype (incomplete penetrance). Incomplete penetrance has been noted in one third of cases with autosomal dominant RLS [29].

Discussion

Restless Legs Syndrome (RLS) is a common sensorimotor disorder. It is characterised by an imperative urge to move the legs during the evening or night, which is often accompanied by unpleasant sensations. RLS is classified into primary (idiopathic) and secondary forms. A significant genetic contribution to RLS has been well documented. RLS family aggregation occurs frequently, accounting for up to 60-65% of reported cases. A high concordance rate of 83% in monozygotic twins adds to the evidence of a strong genetic influence [30]. Furthermore there is a remarkable ethnic and geographic differences in the prevalence of RLS: 5-15% in Western Europeans, compared to 0.1-0.6% of people in Singapore [31], 1.1% in Japan [32], and 3.2% in Turkey [33].

In most of the published RLS families the number of affected individuals in a single sibship is greater than the 50% expected for an autosomal dominant disorder. This peculiarity is also observed in the family presented here: in the second generation all four siblings are affected by RLS. It has been suggested that additional, as yet unknown, factors may be influencing the presence of disease in a higher

than expected number of family members [34]. The identification of the causative genetic variant may assist in the discovery of any modifying factors.

Including this report, 10 loci have now been reported by studies of RLS families. To date, no causative gene mutation has been detected in these loci despite convincing linkage analysis results, often from the study of a single family. Segregation studies have reported that familial RLS is consistent with a single major locus when age of onset is not considered [35,36]. However, the absence of evidence for a major gene controlling age of onset suggests that non-genetic causes of RLS may exist and that RLS is a complex disorder [36]. Indeed one of the recent linkage reports identified a family that may potentially carry low penetrance disease alleles from two distinct loci [21]. These studies suggest potential complications in the study of RLS and raise the possibility that a single dominant model for RLS is insufficient.

There is evidence of a link between RLS, dopamine deficit and dysfunctional iron regulation in the central nervous system (CNS). The effectiveness of dopamine precursors (e.g. levodopa) and dopamine agonists (e.g. ropinirole and pergolide) in treating RLS, the exacerbation of RLS symptoms by dopamine receptor antagonists [37], and the dip in dopamine levels at night [38] support a possible, but poorly understood, role of dopaminergic signalling pathways in RLS. However, a genetic association study that compared SNPs within eight genes involved in the dopamine pathway found no difference between patients with RLS and control subjects[39].

Impaired iron metabolism may be an important factor in the pathophysiology of RLS [9]. Immunocytochemical autopsy studies have shown evidence of impaired iron metabolism in neuromelanin cells of the substantia nigra, while an MRI study demonstrated decreased iron concentrations in the substantia nigra of patients with RLS [40]. Many of the established causes of secondary RLS involve a reduction in iron , e.g. iron deficiency [41], pregnancy(reviewed in [42]) and end-stage renal disease [43] . Iron plays a role in the processing of dopamine in the brain; the conversion of tyrosine to L-dopa, which eventually yields dopamine, requires iron. Iron deficiency may therefore reduce dopamine synthesis.

We have conducted a genome-wide linkage analysis in an Irish family with 11 affected and 7 unaffected family members. A novel locus for autosomal dominant RLS has been identified on chromosome 19p13.3. This locus is the first to be reported in an Irish pedigree, and it represents the tenth locus linked to RLS. The locus is positioned distal to microsatellite marker D19S565. A maximum two-point LOD score of 3.59 at $\theta = 0.00$ was obtained for microsatellite marker D19S878 and a maximum multipoint LOD score of 3.75, was obtained at 7.54 cM, which is 0.97 cM proximal to D19S878.

The maximum LOD score generated (3.590 at theta = 0.0) slightly exceeds the maximum LOD score predicted by SLINK analysis under the same parameters (3.584 at theta = 0.0). This may be as a result of the number of replicates studied or the exact allele frequencies used.

The average age of onset in the family is between 12 and 15 years of age; only one individual (II-3) reported symptom onset after 20 years of age: ‘in the 20s’. The four unaffected individuals in generation three (Fig. 1 III-1, III-2, III-5 and III-10) were aged 35, 34, 24 and 31 years at age of examination (Table 1) and were considered as unaffected for analysis.
Evidence of a susceptibility locus for RLS was previously reported on chromosome 19p in a family of Italian origin [20]. However, that locus mapped to a small interval between D19S429 and D19S915, located on chromosome 19 at 45.48 cM and 47.31 cM respectively. Our study provides evidence for a distinct RLS locus located on chromosome 19p flanked by the telomere and microsatellite marker D19S565, which is almost 39 cM distal to the previously published RLS locus.

Chromosome 19 has the highest gene density of all human chromosomes, more than double the genome-wide average [44]. The 2.5 Mb of the 19p13.3 linked region reported here includes more than 100 genes. Future work will attempt to identify a causative mutation in one of these genes. In conclusion, our study identifies a novel locus for autosomal dominant RLS and indicates further genetic

heterogeneity in the disorder. Identification of the underlying gene defect may contribute to improved understanding of the underlying pathogenic mechanisms of RLS.

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Table 1. Clinical Features of RLS in the 11 affected members of Family RLS3002. Details of age at examination are provided for the 4 unaffected members in generation three.

Pedigree Identifier	Sample	Age at exam (yr)	Age at onset (yr)	Severity (IRLS-RS)	Parasthesia** in legs	Symptom Prevalence
II-2	**PATH046**	58	3	4	Yes	Night
II-3	**PATH051**	57	20's	15	Yes	Sitting for long periods/ Evening/Night
II-5	**PATH185**	57	teens	16	Yes	Evening/ Night
II-7	**PATH055**	53	< 10	10	Yes: Lateralization of symptoms; left leg	Evening/Night
III-1	**PATH200**	35	-	-	-	-
III-2	**PATH199**	34	-	-	-	-
III-3	**PATH083**	27	teens	10	Yes	Night
III-4	**PATH048**	26	10	14	Yes	Evening/Night
III-5	**PATH049**	24	-	-	-	-
III-6	**PATH050**	22	11	13	Yes	Evening/Night
III-7	**PATH053**	26	teens	17	Yes	Sitting for long periods/ Evening/Night
III-8	**PATH054**	23	15	11	Yes	Evening/Night
III-9	**PATH187**	29	teens	8	Yes	Evening/ Night
III-10	**PATH208**	31	-	-	-	-
III-11	**PATH207**	19	teens	10	Yes	Sitting for long periods

**Paraesthesia in the arms was not reported by any of the RLS affected individuals.

Table 2. Markers used to fine map chromosome 19p13.3 and the LOD scores generated. Model 1 used 95% penetrance and a disease allele frequency of 0.1%. Model 2 used 70% penetrance, disease allele frequency of 0.3% and a phenocopy rate of 0.5%.

Marker	Genetic Map Position[a] (cM)	Physical Map Position [b] (bp)	Model 1		Model 2	
			Max LOD	theta	Max LOD	theta
CA19S872**	0.00	872,390	2.46	0.00	2.07	0.00
D19S886	0.00	949,644	2.01	0.07	2.24	0.00
GT19S949**	0.00	949,752	2.12	0.00	2.49	0.00
ATA19S1001**	0.69	1,001,575	2.18	0.02	2.52	0.00
AC19S1117**	2.23	1,117,411	2.25	0.00	1.94	0.00
CA19S2310**	6.57	2,310,554	3.46	0.00	3.04	0.00
D19S878	6.57	2,310,697	3.59	0.00	3.18	0.00
D19S565	6.57	2,518,075	1.96	0.07	1.63	0.07
GT19S2518**	6.57	2,518,246	1.85	0.07	1.52	0.07
D19S591	9.84	3,026,844	1.83	0.00	1.70	0.00
D19S247	9.84	3,090,982	0.92	0.00	0.81	0.00
D19S120	10.97	3,131,343	1.18	0.09	0.94	0.09
D19S424	10.97	3,177,373	1.13	0.09	0.89	0.09
D19S209*	10.97	3,265,329	2.01	0.07	1.68	0.07

* indicates the marker which was part of Applied Biosystems linkage Mapping Set; ** indicates microsatellite markers which were not found in the database and were designed by identifying novel microsatellite repeats in the region.

[a] Data sourced in Marshfield Maps

[b] NCBI Build 36.3

Figure 1 Pedigree structure of family RLS3002 and haplotype analysis of 19p13.3 locus

Circles and squares represent females and males respectively; filled symbols denote affected individuals and clear symbols with the letter N denote unaffected individuals; the symbols with a diagonal line indicate that the person is deceased.

The haplotypes correspond to microsatellite loci described in Table 2. The disease haplotype which is shared by all affected individuals is denoted by a black vertical bar. A recombination event was observed in individual III-7 and is indicated by an asterisk (*). This recombination event defines the critical RLS gene location as distal to marker D19S565.

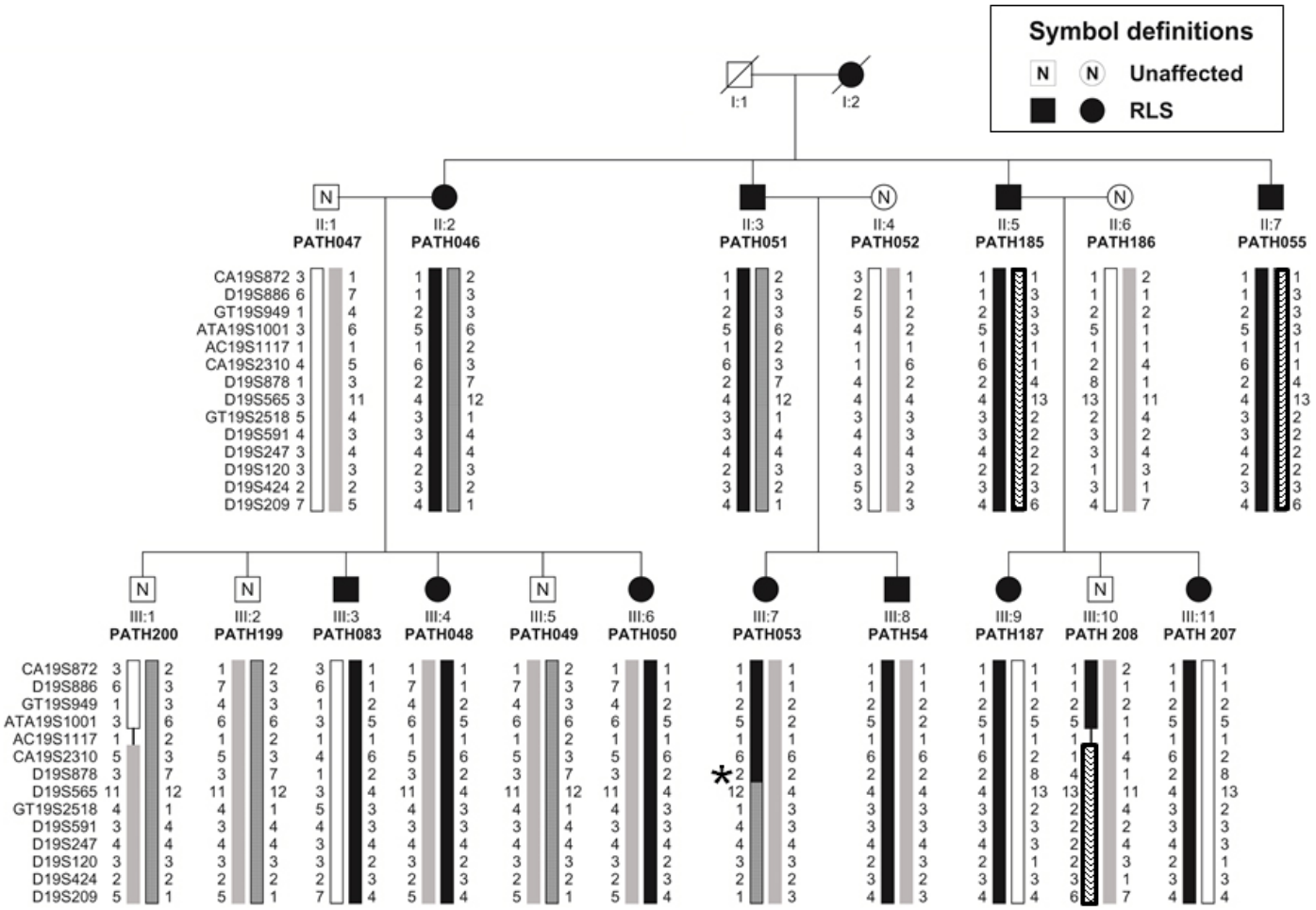

Figure 1 Pedigree structure of family RLS3002 and haplotype analysis of 19p13.3 locus

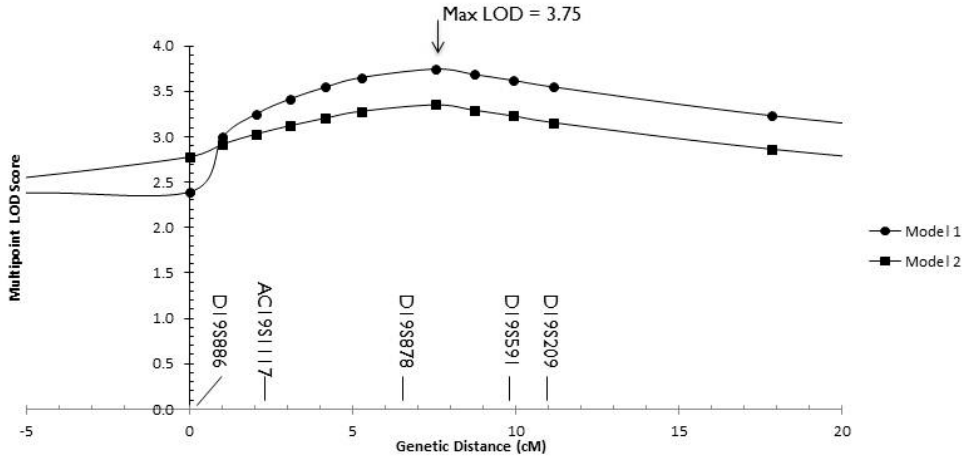

Figure 2 Multipoint parametric LOD scores for family RLS3002 on chromosome 19p.

The horizontal axis is a map of markers at the corresponding genetic positions (cM). The vertical axis represents the LOD scores. Marker D19S209 shares the same position with D19S120 and D19S424, marker D19S591 shares the same position with D19S247, marker D19S878 is at the same position as markers CA19S2310, D19S565 and GT19S2518, and marker D19S886 shares the same positions as markers CA19S872 and GT19S949; thus, these additional markers are not represented on the graph. Model 1 used 95% penetrance and a disease allele frequency of 0.1%: maximum LOD score is 3.75 at 7.54 cM. Model 2 used 70% penetrance, disease allele frequency of 0.3% and a phenocopy rate of 0.5%: maximum LOD score is 3.35 at 7.54 cM.

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