Replication of restless legs syndrome loci in three European populations

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ABSTRACT

Background: Restless legs syndrome (RLS) is associated with common variants in three intronic and intergenic regions in *MEIS1, BTBD9*, and *MAP2K5/LBXCOR1* on chromosomes 2p, 6p and 15q.

Methods: Our study investigated these variants in 649 RLS patients and 1230 controls from the Czech Republic (290 cases and 450 controls), Austria (269 cases and 611 controls) and Finland (90 cases and 169 controls). Ten single nucleotide polymorphisms (SNPs) within the three genomic regions were selected according to the results of previous genome-wide scans. Samples were genotyped using Sequenom platforms.

Results: We replicated associations for all loci in the combined samples set (rs2300478 in *MEIS1*, $p = 1.26 \times 10^{-5}$, odds ratio (OR) = 1.47, rs3923809 in *BTBD9*, $p = 4.11 \times 10^{-5}$, OR = 1.58 and rs6494696 in *MAP2K5/LBXCOR1*, $p = 0.04764$, OR = 1.27). Analysing only familial cases against all controls, all three loci were significantly associated. Using sporadic cases only, we could confirm the association only with *BTBD9*.

Conclusion: Our study shows that variants in these three loci confer consistent disease risks in patients of European descent. Among the known loci, *BTBD9* seems to be the most consistent in its effect on RLS across populations and is also most independent of familial clustering.

Restless legs syndrome (RLS) is characterised by an urge to move the legs associated with unpleasant sensations in the lower limbs, typically occurring at rest in the evening or at night.[1] Since the maximum number of symptoms appear at bedtime, RLS can lead to disturbances of sleep resulting in a decreased quality of life.[1] The diagnosis is further supported by the presence of periodic limb movements in sleep (PLMS) and positive response to dopaminergic treatment.[1]

A recent genome-wide association study (GWA) with German and Canadian RLS cases identified intronic or intergenic variants within three genomic regions: *MEIS1* (myeloid ecotropic viral integration site homeobox 1) on chromosome 2p, *BTBD9* (BTB/POZ domain containing protein 9) on chromosome 6p, and a third region on chromosome 15q containing *MAP2K5* (mitogen activated protein kinase kinase 5) and *LBXCOR1* (ladybird homeobox co-repressor 1).[2] A similar study conducted in Icelandic and US cases showed an association of *BTBD9* to PLMs.[3]

MEIS1 belongs to the family of TALE homeobox genes involved in limb development, the determination of the megakaryocytes and central nervous system (CNS) structures, such as the retina, cerebellar granule cells, hindbrain and spinal motor neuron pools.[4–6] So far, very little is known about *BTBD9*. It consists of a BTB/POZ domain, a BACK domain and a coagulation factor domain. Known functions of similar proteins containing these domains include ubiquitin dependent protein degradation.[7] The variants located in the third genetic region are in strong linkage disequilibrium with two surrounding genes: *MAP2K5*, which is critical at early stages of muscle cell differentiation,[8] and *LBXCOR1*, which is a transcriptional co-repressor of LBX1 and is highly expressed in spinal dorsal horn and midbrain–hindbrain border.[9] The involvement of these genes in the aetiopathogenesis of RLS is still unknown.

The aim of our study was to investigate whether these variants are also relevant among other European (Czech, Austrian, and Finnish) RLS cases and what is the difference of their impact between sporadic and familial cases.

PATIENTS AND METHODS

Patients and controls

The diagnosis of all RLS cases was made according to diagnostic criteria of the International RLS Study Group[1] by personal examination by a neurologist in the respective study centre. The positive family history was defined as at least one first degree family member being affected by RLS (reported by the proband) in all three populations. The control samples originate from the general population and were not screened for presence of RLS.

Czech subjects

The patients were recruited in the Centre for Disorders of Sleep and Wakefulness, Department of Neurology of First Faculty of Medicine and the General Teaching Hospital, Prague. In total, 290 patients were included (107 males, mean (SD) age 55.7 (15.3) years, mean age at onset of RLS 38.3 (18.1) years). Positive family history was reported by 110 patients, in 175 cases it was negative, and in five the data were not available. Altogether 450 sex matched controls were selected randomly from the Czech blood and bone marrow donors registry (166 males, mean age 45.3 (9.9) years). Since the maximum age for the controls was 63 years, 38 male and 51 female cases in the age group from 64 to 91 years could not be age matched.

Austrian subjects

A total of 269 (104 males) patients were recruited in 2 centres: at the Department of Neurology, Medical University of Vienna, and the Department of Neurology, University Clinic Innsbruck, (mean age 59.0 (14.3) years, mean age at onset of RLS 37.14 (19.5) years). Positive family history was reported by 107 patients, in 108 cases it was negative, and in 54 the data were not available. The patients were matched by sex to 611 controls from the German KORA project, the procedures for which have been described elsewhere[10] (236 males, mean age 59.9 (11.35) years). KORA controls were already used in the previous GWA study, which showed only a negligible effect of population stratification.[2]

Finnish subjects

Ninety (24 males) patients were recruited in the Sleep Research Center in Turku (mean age 46.5 (18.1) years, mean age at onset of RLS 19.4 (13.4) years. Positive family history was reported by 81 patients and nine patients had a negative family history. A random sample from the general Finnish population, comprising 169 sex matched individuals (45 males), was used as control. Data on age of controls were not available. Studies were performed according to the declaration of Helsinki and approved by the ethical committees of the respective study centres. Written informed consent was obtained from all RLS patients.

Genotyping

Ten single nucleotide polymorphisms (SNPs) within the three genomic regions were selected according to the results of previous GWA scans.[2 3] Samples were genotyped on two Sequenom platforms in Munich and Helsinki (Sequenom MassArray system, Sequenom Inc, San Diego, California, USA) with a genotype discordance rate of 1.3% in 158 comparisons, when analysing repeatedly genotyped internal control samples. Automated genotype calling was done with SpectroTYPER 3.4 software and genotype clustering was visually checked by an experienced scientist. Assays were designed using AssayDesign 3.1.2.2 with iPLEX Gold chemistry default parameters. SNP quality control criteria leading to exclusion from analysis were a call rate <90%, minor allele frequencies (MAF) <1% and p<0.001 for deviations from Hardy-Weinberg equilibrium (HWE) in controls.

Statistical analysis

Genotype data were analysed using standard association tests (allelic, genotypic, dominant and recessive models) including Cochran–Armitage test for trend, Cochran–Mantel–Haenszel test for estimation of odds ratios (ORs) in the stratified sample (including Breslow–Day test for homogeneity), and haplotype tests, as implemented in the PLINK statistical package v1.0.[11] The sample was stratified only according to the country of origin. Logistic regression implementing the Cochran–Armitage test for trend (using genotypes as ordinal values rather than categorical) in the combined sample using age, sex and country of origin as covariates was performed by generalised linear modelling routines incorporated in R package v.2.6.0 (http://www.r-project.org/). Bonferroni correction for multiple testing of 10 markers was employed. All p values given are one sided, with the direction of the alternative hypothesis given by the original report.[3] Power calculations were performed using the Genetic Power Calculator (pngu.mgh.harvard.edu/~purcell/gpc/).[12] For input parameter we used an RLS prevalence of 8%, an α level of 5%, and ORs and allele frequencies according to results from the GWA experiment.[2] Association tests were conducted in three different settings: (1) all patients (that is, familial and sporadic) combined versus all controls; (2) familial cases versus controls; and (3) sporadic cases versus controls.

RESULTS

All SNPs tested were in HWE (p>0.01) in both patients and controls. Under the assumption of genetic homogeneity, the combined sample had good power to detect association using previously published parameters[2] (98% for *MEIS1* and *BTBD9*, 89% for *MAP2K5/LBXCOR1*). In the Czech sample alone the power was 82.5% for *MEIS1* and *BTBD9*, and 71.8% for *MAP2K5/LBXCOR1*, in the Austrian sample the powers were 84.8% and 74.8%, respectively, and in the Finnish sample separately 38.7% and 30.4%.

Allele frequencies in the Czech and KORA control samples were not significantly different (lowest p in χ^2 test = 0.2045 for rs4236060). Significant allele frequency differences were observed between the Finnish and the combined Czech and KORA control samples within *BTBD9* ($p<7.67\times10^{-6}$ for all SNP markers within *BTBD9*). A similar, nominally significant, difference in allele frequencies in *BTBD9* markers was also observed between Finnish cases and combined Czech and Austrian cases (in χ^2 test lowest p = 0.01063 for rs9296249), but we did not observe a significant difference between allele frequencies of Czech and Austrian RLS patients (lowest p in χ^2 test was 0.4608 for rs2300478). Logistic regression showed no significant interaction with country for any SNP tested, and the Breslow–Day test showed homogeneous ORs in all samples.

Significant association after correction for multiple testing at significance α level of 5% was found in at least one SNP for all tested loci in the combined samples (table 1), and in the Czech and Austrian samples separately. Analysing the Finnish sample, we confirmed only the association to *BTBD9*. The association to rs2300478 in *MEIS1* was only nominally significant and *MAP2K5/LBXCOR1* showed no association (table 2).

In the combined sample we observed a strong association with the haplotype formed by markers rs6710341 and rs12469063, both located within *MEIS1*. Carriers of the "AG" haplotype had ORs for developing RLS of 1.98 ($p = 9.1\times10^{-10}$). Results for this haplotype were similar when testing the Czech ($p = 3.2\ 10^{-7}$, OR = 2.38), Austrian ($p = 8.3\times10^{-5}$, OR = 1.82), and Finnish samples ($p = 2.0\times10^{-4}$, OR = 2.46) separately. No other common polymorphic phased haplotypes (MHF >1%) yielded significant results. An allele dosage model best described the association for *MEIS1* and *BTBD9* (Armitage trend test). In contrast, a recessive model for the risk allele fitted best for the *MAP2K5/LBXCOR1* locus.

Analysing only familial cases (n = 217) and all controls, all three loci were significantly associated. Using sporadic cases only (n = 283), we could confirm the association to *BTBD9* but not to *MEIS1* and *MAP2K5/LBXCOR1*. We omitted patients of Finnish origin from this sub-analysis due to very low proportion of sporadic cases and different allele frequencies in these samples. The Breslow–Day test did not show significant heterogeneity between sporadic and familial cases.

DISCUSSION

Our study showed an association of variants in *MEIS1*, *BTBD9* and *MAP2K5/LBXCOR1* with RLS in a combined sample of Czech, Austrian, and Finnish RLS cases. Similar findings were

Table 1 Genotyped single nucleotide polymorphisms (SNPs) and results of association in combined samples

Chr	Gene	SNP ID	Genome	r^2	OR (95% CI)	p nom	p corr	MAF fam	MAF spor	Best model	p corr fam	p corr spor
2p	MEIS1	rs6710341	66611926		0.84 (0.64 to 1.11)	0.30646	1	0.1270	0.1288	TREND	1	1
2p	MEIS1	rs12469063	66617812	0.413	1.43 (1.16 to 1.78)	4.15E-06	4.15E-05	0.3522	0.2727	TREND	2.24E-05	0.3245
2p	MEIS1	rs2300478	66634956	0.969	1.47 (1.18 to 1.82)	1.26E-06	1.26E-05	0.3575	0.2860	TREND	3.10E-05	0.1520
6p	BTBD9	rs9296249	38473818		1.59 (1.26 to 2.01)*	0.00011	0.00107	0.1694	0.1553	TREND	0.0544	0.0012
6p	BTBD9	rs3923809	38548947	0.512	1.58 (1.28 to 1.96)*	4.11E-06	4.11E-05	0.2204	0.2330	TREND	0.0018	0.0022
6p	BTBD9	rs4236060	38578315	0.829	1.49 (1.19 to 1.86)*	1.93E-05	0.00019	0.1882	0.2110	TREND	0.0008	0.0049
15q	MAP2K5	rs11635424	65824631		1.26 (1.02 to 1.55)*	0.00602	0.06023	0.2446	0.2992	REC	0.0203	1
15q	MAP2K5	rs3784709	65859328	0.935	1.24 (1.01 to 1.52)*	0.00530	0.05301	0.2392	0.2917	REC	0.0393	1
15q	MAP2K5	rs1026732	65882138	0.966	1.27 (1.03 to 1.56)*	0.00428	0.04278	0.2339	0.2936	REC	0.0116	1
15q	MAP2K5/LBXCOR1	rs6494696	65890259	0.999	1.27 (1.03 to 1.56)*	0.00476	0.04764	0.2339	0.2936	REC	0.0108	1

OR, odds ratio for the risk allele (Cochran–Mantel–Haenszel test) with 95% confidence intervals (CI); p nom, logistic regression implementing Armitage trend test with country of origin, sex and age as covariates; p corr, adjusted p values for multiple testing; MAF, minor allele frequencies observed in combined Czech and Austrian sample, in sporadic and familial cases; Best model corresponds to model under which lowest p values were observed (TREND, Armitage trend test; REC, recessive model); p corr fam, comparison of allele frequencies between familial cases and all controls; p corr spor, comparison of allele frequencies between sporadic cases and all controls; SNP, single nucleotide polymorphism.
The genetic positions in bp and gene alignments are derived from UCSC Genome browser (http://genome.ucsc.edu, assembly March 2006),[25] r^2– linkage disequilibrium relative to preceding marker; data were computed using genotypes observed in both cases and controls using Haploview 4.0 from HapMap project (http://www.hapmap.org, release 21a).[26]
*Risk allele is the major allele.

Table 2 Analysis in individual populations

	Czech Republic				Austria				Finland			
SNP ID	MAF cases n = 276	MAF controls n = 412	Best p corr	OR (95% CI)	MAF cases n = 222	MAF controls n = 570	Best p corr	OR (95% CI)	MAF cases n = 88	MAF controls n = 246	Best p corr	OR (95% CI)
rs6710341	0.1309	0.1456	1	1.13 (1.55 to 0.83)	0.1306	0.1412	1	0.91 (0.66 to 1.26)	0.1207	0.1585	1	0.73 (0.43 to 1.22)
rs12469063	0.2971	0.2172	0.0492	1.52 (1.19 to 1.95)	0.3108	0.2426	0.0064	1.41 (1.11 to 1.79)	0.3161	0.2439	0.6093	1.43 (0.98 to 2.10)
rs2300478	0.3025	0.2209	0.0285	1.53 (1.20 to 1.96)	0.3243	0.2487	0.0017	1.45 (1.14 to 1.84)	0.3276	0.2459	0.3676	1.49 (1.02 to 2.18)
rs9296249	0.1649	0.2306	0.0252	1.52 (1.15 to 2.00)	0.1644	0.2378	0.0116	1.59 (1.19 to 2.11)	0.2414	0.3516	0.1081	1.70 (1.15 to 2.53)
rs3923809	0.2301	0.2998	0.0374	1.43 (1.12 to 1.84)	0.223	0.3133	0.0049	1.59 (1.23 to 2.05)	0.2651	0.4119	0.0124	1.94 (1.32 to 2.87)
rs4236060	0.2047	0.2662	0.1903	1.41 (1.09 to 1.83)	0.1968	0.2891	0.0028	1.66 (1.27 to 2.17)	0.2674	0.3921	0.0497	1.77 (1.20 to 2.60)
rs11635424	0.2772	0.3350	0.0135	1.31 (1.04 to 1.66)	0.2793	0.3229	0.1014	1.23 (0.97 to 1.57)	0.3046	0.2866	1	1.09 (0.75 to 1.59)
rs3784709	0.2754	0.3289	0.0124	1.29 (1.02 to 1.63)	0.2725	0.3185	0.0522	1.25 (0.98 to 1.59)	0.3046	0.2744	1	1.16 (0.79 to 1.69)
rs1026732	0.2717	0.3350	0.0050	1.35 (1.07 to 1.71)	0.2748	0.322	0.0519	1.25 (0.98 to 1.60)	0.3046	0.2764	1	1.15 (0.79 to 1.67)
rs6494696	0.2717	0.3350	0.0050	1.35 (1.07 to 1.71)	0.2748	0.3229	0.0416	1.26 (0.99 to 1.60)	0.3046	0.2764	1	1.15 (0.79 to 1.67)

MAF, minor allele frequencies in each subsample in patients and healthy individuals; n, number of successfully genotyped individuals by passing quality control criteria; Best p corr, p values corrected for multiple testing according to the full association model in table 1; OR, odds ratio and corresponding 95% confidence intervals (CI); SNP, single nucleotide polymorphism.

observed in the US population.[13] In accordance with the original report, the strongest effect was observed with the haplotype "AG" formed by markers rs6710341 and rs12469063 located in the ninth intron of *MEIS1*, providing ORs of about 2.0 for this haplotype. However, the OR may be underestimated, because the controls samples were not screened to exclude RLS and therefore may contain approximately 10% of individuals actually affected by RLS. The best models observed for individual loci are in good agreement with previous findings in German and Canadian populations. The significance of these loci to RLS can therefore be regarded as well established.

The sub-analyses in Czech and Austrian populations show the same trends for association as the combined sample, but in the Finnish sample, only association with *BTBD9* was confirmed and there was a trend for association to *MEIS1*. Moreover, the allele frequencies and proportions of familial cases in the Finnish sample were different from the other two, but the smaller size of this sample limits further implications.

In our sample set we have not observed significant differences between familial and sporadic cases concerning the *BTBD9* locus. The 95% confidence intervals of OR also overlapped between familial and sporadic cases for both *MEIS1* (1.357 to 2.1 in familial and 1.019 to 1.534 in sporadic cases *vs* all controls for rs12469063) and *MAP2K5/LBXCOR1* (1.164 to 1.841 in familial and 0.951 to 1.408 in sporadic cases for rs6494696). There is a trend that *MEIS1* and *MAP2K5/LBXCOR1* possibly play a more important role in familial RLS, but due to the limited number of patients, we were not able to prove significant heterogeneity. Generally the risk alleles in these loci are common and exert only small to moderate effects. They do not explain the familial clustering of RLS.[2] Besides these association signals, six linkage regions for RLS on chromosomes 2q, 9p, 12q, 14q, 19p and 20p,[14–19] under a recessive or autosomal dominant model of inheritance, have been described. These variants must be of larger effects and less frequent, since only some have been successfully confirmed in independent populations or in single families.[20–24] Among the known loci, *BTBD9* seems to be the most consistent in its effect on RLS across populations, and is also most independent of familial clustering.

We conclude that the observed genetic determinants are risk factors for RLS in multiple populations.

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