Univerzita Karlova v Praze

1. lékařská fakulta

Autoreferát disertační práce

GENETIC AND CLINICAL ASPECTS OF THE RESTLESS LEGS SYNDROME

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Disertační práce bude nejméně pět pracovních dnů před konáním obhajoby zveřejněna k nahlížení veřejnosti v tištěné podobě na Oddělení pro vědeckou činnost a zahraniční styky Děkanátu 1. lékařské fakulty.

SOUHRN

Úvod: Syndrom neklidných nohou (RLS – Restless legs syndrome) je časté neurologické onemocnění s prevalencí 5 – 10% v evropské populaci. Je charakterizované nutkáním pohybovat končetinami a v rozvinuté formě interferuje se spánkem. RLS je komplexní dědičné onemocnění, idiopatické formy jsou asociovány s variantami genů *MEIS1*, *BTBD9*, *PTPRD* a *MAP2K5/SCOR1*. Recentní studie uvádějí roztroušenou sklerózou jako novou příčinu sekundární formy RLS s prevalencí 19 – 37,5%.

Cílem naší práce bylo vyšetřit některé klinické a genetické aspekty tohoto onemocnění, hl. u pacientů s roztroušenou sklerózou (RS). V klinické části jsme vyšetřovali prevalenci RLS u českých pacientů s roztroušenou sklerózou a porovnávali jsme rozsah postižení mozku na magnetické rezonanci (MR) u pacientů s RLS a bez RLS symptomů. V genetické části jsme zjišťovali, zda známe genetické varianty (*MEIS1*, *BTBD9*, *PTPRD* a *MAP2K5/SCOR1*) zvyšují riziko rozvoje RLS take u jiných evropských populací a u pacientů s RS.

Metodika: V klinické části (epidemiologické studii) byli pacienti s RS dotazováni na symptomy RLS, každý pacient absolvoval strukturovaný rozhovor cílený na přítomnost RLS, rodinnou anamnézu, komorbidity a terapii. U některých pacientů (radiologická studie) byla provedena MR mozku se zaměřením na objem T2 hyperintenzních ložisek (lesion load - LL), mozkovou atrofii a brain parenchymal fraction (BPF).

Genetická část zahrnuje 2 genetické asociační studie: 1. jsme vyšetřovali přítomnost genetických variant u 649 pacientů s idiopatickým RLS a 1230 kontrol ze 3 evropských populací (ČR, Rakousko, Finsko). 10 SNPs (single nucleotide polymorphisms) bylo vybráno na 3 genech (*MEIS1*, *BTBD9* a *MAP2K5/SCOR1*). Ve 2. studii jsme porovnávali přítomnost genetických variant u pacientů RS a RLS oproti pacientů s RS, ale bez symptomů RLS. Celkem 12 SNPs bylo vybráno na 4 genech (*MEIS1*, *BTBD9* a *MAP2K5/SCOR1*, *PTPRD*).

Výsledky: Do epidemiologické studie bylo zahrnuto celkem 765 pacientů s RS (553 žen, průměrný věk 36.54, ±SD 9.5). Diagnóza RLS byla potvrzena u 245 pacientů (32.1%, 95% CI 28.7-35.4%). V porovnání s pacienty bez RLS byli pacienti s RLS byli signifikantně starší (38.6 vs. 35.6 let), měli delší trvání roztroušené sklerózy (11.0 vs. 8.2 let) a měli vyšší EDSS skóre (2.9 vs. 2.3). Kvantitativní data z MR (LL, BPF a mozková atrofie) byla porovnány u 385 pacientů bez RLS a u 215 pacientů s RLS, nebyl nalezen signifikantní rozdíl mezi pacienty s RLS a bez tohoto onemocnění, ačkoli jsme prokázali korelaci mezi uvedenými parametry a tíží RS.

V genetické části jsme replikovaly asociaci všech lokusů v kombinovaném vzorků 3 populací (rs2300478 in *MEIS1*, P= 1.26x10-5, odds ratio (OR)= 1.47, rs3923809 in *BTBD9*, P= 4.11x10-5, OR= 1.58 and rs6494696 in *MAP2K5/SCOR1*, P= 0.04764, OR= 1.27 Ve studii s RS byl nalezen trend pro asociaci u *SCOR1*, nejlepším modelem byl

recesivní model (p noml =0.0029, p korigované pro model a 4 geny = 0.023, genotypické odds ratio = 1.60). Nebyla prokázána asociace s variantami *MEIS1* a *BTBD9* i přes dostatečnou statistickou sílu.

Závěr: Syndrom neklidných nohou je častou komorbiditou roztroušené sklerózy (prevalence 32%), může nepříznivě ovlivňovat kvalitu spánku u pacientů s RS a RS by měla být zahrnuta mezi sekundární formy RLS. RLS je častější v rozvinuté formě RS, ale nekoreluje s mírou postižení mozku na magnetické rezonanci.

Genetická studie s idiopatický RLS potvrdila význam variant *MEIS1*, *BTBD9* a *MAP2K5/SCOR1* i v české, rakouské a finské populace. Naopak RLS u pacientů s RS sdílí tedy jen malou část rizikových genetických faktorů s formami idiopatickými, varianta *SCOR1* přispívá k fenotypu z maximálně 50%.

SUMMARY

Introduction: The Restless Legs Syndrome (RLS) is a frequent neurological disorder with a prevalence ranging from 5 – 10%. RLS is characterized by urge to move lower extremities during the night, thus RLS causes sleep disturbance. It presents as both idiopathic and secondary form. Idiopathic RLS is associated with common genetic variants in *MEIS1, BTBD9*, *PTPRD* and *MAP2K5/SCOR1*. Recently, multiple sclerosis (MS) was identified as common cause for secondary RLS with the prevalence ranging from 13.3 to 37.5%.

Aim of our study was to analyze the clinical and genetic aspects of this disorder, especially in patients with multiple sclerosis. In the clinical part, we evaluated the prevalence of RLS among Czech patients with MS and we compared the extent of brain damage between patients with and without RLS using magnetic resonance imaging (MRI). In the genetic part, we further analyze the impact of known genetic variants (*MEIS1, BTBD9, MAP2K5/SCOR1, PTPRD)* for RLS in other European populations and in patient with MS.

Methods: Clinical part: Each patient with MS underwent a semi-structured interview. A patient was considered to be affected by RLS if he/she met all four standard criteria at life-long interval. Lesion load (LL –T2), brain atrophy – T1 and brain parenchymal fraction (BPF) were assessed in some patients.

Genetic part included two genetic association studies. 1. we investigated these variants in 649 RLS patients and 1230 controls from Czech Republic, Austria and Finland. Ten SNPs (single nucleotide polymorphisms) within the three genomic regions (*MEIS1, BTBD9* a *MAP2K5/SCOR1)* were selected. 2. 203 MS patients with RLS were compared to 438 MS patients without RLS. In total 12 SNPs within the four genomic regions (*MEIS1, BTBD9* a *MAP2K5/SCOR1, PTPRD*) were genotyped.

Results: Clinical part: A total of 765 subjects (553 females, mean age 36.54, ±SD 9.5) with MS were included in the study. The diagnosis of RLS was confirmed in 245

subjects (32.1%, 95% CI 28.7-35.4%) with MS. Patients suffering from both MS and RLS were significantly older (38.6 vs. 35.6 years), had longer durations of MS symptoms (11.0 vs. 8.2 years) and had higher EDSS score (2.9 vs. 2.3). Quantitative MRI data were obtained in 385 patients without RLS and 215 patients with RLS. We found no difference between the two groups in the whole brain LL, brain atrophy and BPF, despite the fact that we were able to replicate the correlation of these data with clinical parameters of MS.

Genetic part: We replicated associations for all loci in the combined samples set (*MEIS1,* P= 1.26×10^{-5}, odds ratio (OR) = 1.47, *BTBD9,* P= 4.11×10^{-5}, OR= 1.58 and *MAP2K5/SCOR1*, P= 0.04764, OR= 1.27). No significant association with *MEIS 1*, *BTBD9* and *PTPRD* was found in patients with MS despite sufficient statistical power for first two loci. There was a trend for association with *MAP2K5/SCOR1* - the best model for the risk allele was the recessive model (p nominal = 0.0029, p corrected for four loci and allelic + recessive model = 0.023, odds ratio = 1.60 - 95% CI 1.17 – 2.18).

Conclusion: RLS is a common comorbidity of multiple sclerosis**,** MS should be considered among causes of secondary RLS forms. RLS is more prevalent in advanced stages of MS, but does not correlate with MRI markers of brain damage.

Our study confirmed that variants in these three loci (*MEIS1, BTBD9,* and *MAP2K5/SCOR1)* confer consistent disease risks in patients of European descent. Contrary, RLS in MS patients share only few genetic determinants with idiopathic form, the gene variant *SCOR1* can partially contribute the phenotype (max.50%).

INTRODUCTION

Restless legs syndrome (RLS) is a common neurological disorder. The disease is characterized by an imperative urge to move the legs associated with unpleasant sensation in lower limbs. Symptoms typically occur at rest in the evening and at night and RLS is often associated with periodic limb movement in sleep (PLMS), thus RLS can lead to sleep disturbance and impaired quality of life in their developed form. The sleep disturbance typically involves initiating and maintaining sleep (1). RLS is one of the commonest neurological sensorimotor disorders at least in the Western countries, the prevalence in European population ranges from 5% to 10%. However, it remains largely underdiagnosed and undertreated (2). International Classification of Sleep Disorders – ICSD2 classes RLS as sleep related movement disorder. The term restless legs syndrome was first used by Karl Ekbom in 1945 (3).

Symptoms, clinical description and course, diagnosis

RLS is typically characterised by an urge to move the limbs, accompanied by uncomfortable and unpleasant sensation in the legs.

The diagnosis of RLS is clinical and is based on patient`s description. **The diagnostic criteria for RLS** were established in 1995 by the IRLSSG (International RLS Study Group) and modified in 2003 (1). Accordingly, four essential criteria are required to establish the diagnosis of RLS.

1) an urge to move the legs, usually accompanied or cause by uncomfortable and unpleasant sensation in the legs

2) an urge to move or unpleasant sensations begin or worsen during period of rest or inactivity such as lying or sitting

3) an urge to move or unpleasant sensations are partially or totally relieved by movement, such as walking or stretching, at least as long as the activity continues

4) an urge to move or unpleasant sensations are worse in the evening or night than during the day or only occur in the evening or night (when symptoms are very severe, the worsening at night may not be noticeable but must have been previously present)

A family history of RLS, a positive response to dopaminergic treatment, and an association with periodic limb movements in sleep (PLMS) are additional clinical features that may provide a support for the diagnosis in some atypical clinical presentations (4-6). RLS suffers present with a wide range of sensory and motor symptoms. The sensory symptoms include different unpleasant sensation (dysesthezia, paresthesiea) and even pain in some. The motor restlessness is another clinical feature, patients suffer from urge to move, 80% of patients develop periodic limb movement in sleep (PLMS) (4, 7). Most patients report difficulty falling asleep and night awakenings. Studies with polysomnography showed prolonged sleep latency, reduced sleep efficiency and total sleep time (5, 8, 9).

RLS diagnosis is based on clinical description four diagnostic criteria can be easily confirmed by history. An interview with a trained physician is necessary for the correct diagnosis, if only questionnaires with RLS criteria are given this results in approximately 10 – 25% false positive cases (10).

Epidemiology

The RLS prevalence rate ranges from 7.2 to 11.5% in Europe and Northern America (2, 11, 12). A minority of sufferers (around 2.7% of the population) experience daily or severe symptoms (2, 12). RLS is twice as common in women as in men (13). Parity or postmenopausal intake of estrogen are to be considered as major factors in explaining this sex difference (13). RLS occurs in 3% of individuals from the Mediterranean or Middle Eastern region and in 1% of Asia population (14, 15), indicating that different genetic or environmental factors may play a role in the prevalence of this syndrome. Prevalence of RLS also increases with age. Increasing comorbidity in the very old, however, may interfere with the accurate identification of RLS.

RLS classification

RLS can be divided into primary and secondary form. Primary or idiopathic forms appear without apparent causes, they are not related to any medical conditions and include sporadic and inherited forms. Secondary forms occur in acquired forms associated with a variety of disorders. Secondary RLS are related to other medical or neurological conditions. Well-documented associations include renal failure, iron deficiency and pregnancy. Although not yet formally studied, the secondary forms of RLS probably share the same clinical features as idiopathic RLS, suggesting a similar underlying pathophysiological basis, including the genetic factors (16). RLS is associated with several neurological disorders such as spinal cord lesions (myelopathy, traumatic lesions, spinal anesthesia etc.), genetic ataxias (SCA 1 – 3), Parkinsons disease, essential tremor and probably with neuropathy.

Restless legs syndrome in patients with multiple sclerosis

The recent studies showed the higher prevalence of RLS in patients with multiple sclerosis, the prevalence ranges from 19% to 37.5%. Auger et al. performed the first issue on this topic in the French – Canadian population, finding very high prevalence in both patients and controls (37.5% vs. 16%), used only a self – administered questionnaire without a personal interview (17). This methodology may overestimate the prevalence of RLS due to false – positive cases, other two studies used face to face interview. The second study, published by Spanish authors, showed different results – the similar prevalence rate of RLS in MS patients and in healthy subjects (13.3 % vs.9.3%), but they did not use clear exclusion criteria for patients and subjects and the sample of patients was small (18). The largest study, published by Italian group, showed a prevalence of 19% in MS and 4.2% in control subjects (19). They chose the frequency of occurrence of symptoms at least twice a week as a threshold for the diagnosis of RLS. They did not include patients who experienced the symptoms with a frequency of occurrence lower than twice per week (further 7.3%, total RLS prevalence 26.3%). Different methodology, frequency criteria and population might explain discrepancies in absolute values in the estimation of prevalence rates among these studies.

Among patients with MS, RLS is associated with older age, longer MS duration and more severe neurological disability specifically involving the pyramidal and the sensitive EDSS (Expanded disability status scale) functional systems. RLS was more prevalent in the primary – progressive form when compared with relapse – remitting form. Thus RLS was associated with a higher MS disability and with the most severe MS course.

The pathophysiology of this association remains to be investigated. Research on secondary forms may help in understanding which central nervous structure is responsible for RLS. Manconi et al compared the extent of brain and cervical cord damage in MS patients with and without RLS using conventional and diffusion tensor magnetic resonance imaging (MRI). Global and regional dual – echo lesion load (LL),

number of cervical cord lesions, mean diffusivity (MD) and fractional anisotropy (FA) histograms of the brain and cervical cord were assessed. No difference between the two groups was found in whole brain, cerebellar and brainstem lesion load, MD and number of cervical lesions. Cervical cord average FA was significantly reduced in MS patients with RLS compared to those without pointing out that the cervical cord damage represent a significant risk factor for RLS in MS patients (20).

Pathophysiology of RLS

The pathophysiology of RLS is complex and remains unknown. RLS is predominantly a disorder of the central nervous system, dopaminee and iron seem to play a fundamental role. The dopaminee hypothesis derives from the dramatic improvement of RLS with dopamineergic therapy. The hypofunctioning of A11 dopamineergic diencephalon spinal pathways seem to be implicated in RLS ethiopathogenesis (21). The impaired iron homeostatis is another important pathophysiogical issue, most patients have normal ferritin serum levels, but reduced levels in the cerebrospinal fluid (22). There is a substantial evidence for a genetic contribution to RLS (see below).

Genetics of RLS

Genetic factors participating in the RLS aetiopathogenesis have been repeatedly corroborated by several kinds of observations. About 40-60 % of idiopathic RLS patients report a positive family history, monozygotic twins are concordant for RLS in 80% (23). RLS is a highly familial phenotype with heredity estimates of about 50% (9), familial cases has a more slowly progressive course , the symptoms within a single RLS family can be variable.

Linkage studies have revealed 8 loci so far, but no causally related gene variant has yet been identified. This fact provides an indirect evidence for the complexity of RLS. Apart from the linkage loci, which represent genetic variants of stronger effect, but are usually rare, association cases control studies are able to detect variants of smaller effect, which are more common in patients suffering from RLS. Association studies compare the frequencies of alleles in case and control populations. A higher frequency of the allele tested in cases is taken as evidence that the allele or genotype is associated with an increased risk for the disease. A genome – wide association study (GWAS) with German and Canadian RLS idiopathic cases revealed association with three gene variants in *MEIS1* on chromosome 2 , *BTBD9* on chromosome 6 and in region between MAP2K5 and *SCOR1* on chromosome 15q (24). Replication study from the USA confirmed the association of *MEIS1* and *BTBD9*, however the *MAP2K5/SCOR1* locus showed only trend for association (25) Another GWAS conducted in US and Iceland population showed association of BTBD 9 variants with periodic limb movements in sleep (PLMS)(26). The fourth loci, *PTPRD* on chromosome 9 was identified in Europe and Canadian population (27). The most recent GWAS including European samples revealed new association loci, the first on chromosome 2 is an intergenic variant outside

of *MEIS1* region and the second on chromosome 16 containing the 5'-end of *TOX3* (28). Association was identified with intronic variants, which suggests a functional role in the expression or alternative splicing of the gene. Carriers of one risk allele had a 50% increased risk for developing RLS. A closer inspection of the known function of the genes is surprising because some of them are developmental factors and did change the pathophysiological concept of RLS.

MEIS1 (myeloid ecotropic viral integration site homeobox 1) is a member from highly conserved family of TALE homeobox genes. MEIS 1 plays a role in proximodistal limb formation during embryonic development. (29). The second region with significant association was found on chromosome 6p in intron of the *BTBD9* gene. Function of BTB (POZ) proteins include transcription repression, cytoskeleton regulation, gating of ion channels and ubiquitin-dependent protein degradation (24). The third region on 15p chromosome contains MAP2K5, a member of the mitogen activated protein kinase family, and the adjacent *SCOR1* gene (24). *SCOR1* acts as a corepressor of LBX1, this homeobox gene is critical in the development of sensory pathways in the dorsal horn of the spinal cord (30). *PTPRD* (protein tyrosine phosphatase receptor type delta) belongs to the family of type IIa receptor-like protein tyrosine phosphatase, the involvement of *PTPRD* in RLS is unknown. Studies in *PTPRD* knockout mice have shown that these protein function in axon guidance and termination of mammalian motoneurons during embryonic development (27, 31).

Secondary RLS cases may present genetically susceptible individuals with clearly defined provoking factor (16). The only study, which demonstrated the influence of genetic factors in secondary RLS, was performed in patients with end stage renal disease. Schormair et al investigated the known genetic variants (*MEIS1*, *BTBD9*, *MAP2K5/SCOR1*, *PTPRD*) in case – control association study of uremic patients from Germany and Greece. RLS in patients with end stage renal disease was associated with *MEIS1* and *BTBD9* in German sample, whereas, in the Greek sample, there was a trend for association for *BTBD9* and *MAP2K5/SCOR1* (32).

AIM OF THE STUDY

The aim of our study was to further investigate the pathophysiology of primary and secondary form of restless legs syndrome focusing on clinical and genetic aspects of this disorder, mainly in patients with multiple sclerosis. Our study is divided in genetic and clinical part.

Clinical study

The aim of epidemiological and radiological study was to evaluate prevalence of RLS among Czech patients with multiple sclerosis, to further analyze risk factors for

developing RLS in patients with MS and to compare the extent of brain damage between MS - patients with and without RLS using magnetic resonance imaging (MRI).

Hypothesis: MS is a new secondary RLS form, RLS is a common finding also among Czech patients with MS, the presence of RLS correlates with the clinical progression of MS and with the extent of brain damage on MRI.

Genetic study

1) The aim of the study "Replication in three populations" in idiopathic RLS was to evaluate whether common genetic variants (*MEIS1*, *BTBD9* and *MAP2K5/SCOR1*) are also relevant among other European (Czech, Austrian, and Finnish) and what is the difference of their impact between sporadic and familial cases.

Hypothesis: Common genetic variants also increase the risk for idiopathic RLS form in other populations.

2) The aim of the study "Genetics of secondary RLS form in patients with multiple sclerosis" was to investigate whether the common genetic variants (*MEIS1*, *BTBD9*, *MAP2K5/SCOR1* and *PTPRD*) have also an impact on RLS in patients with multiple sclerosis.

Hypothesis: Secondary and primary RLS share at least some common genetic factors.

PATIENTS AND METHODS

Clinical study

Epidemiological and radiological study – prevalence of RLS in patients with multiple sclerosis and brain magnetic resonance imaging study in patients with RLS and MS

Patients

From April to December 2009, we recruited all patients with multiple sclerosis from the preselected population (patients with quantitative MRI data) in the MS Centre, Department of Neurology of First Faculty of Medicine, Prague. MS had been diagnosed according to McDonald criteria (33). Exclusion criteria for the study were dopaminergic and antidopaminergic drugs, renal failure, pregnancy, sideropenic anaemia, other disease known to be related to RLS, recent MS diagnosis (less than 6 months before the time of the interview) and recent clinical MS relapse (within 3 months of the interview). On the basis of its clinical course, MS was classified as primary progressive, secondary

progressive or relapsing remitting. Each MS patient underwent a semi-structured interview and brain magnetic resonance imaging (MRI). An interview was conducted by a physician skilled in RLS diagnostics. A patient was considered to be affected by RLS if all four standard criteria had ever been met in their lifetime (2). Magnetic resonance imaging (MRI) data for each patient were obtained during the one year before the interview. All MRI scans were performed with Philips Gyroscan NT 1.5 T. Three volumetric parameters (absolute values and changes against baseline) were measured: brain atrophy (Picture 1), brain parenchymal fraction (BPF) and T2 lesion load (T2 - LL). T1-weighted images were used to assess brain atrophy and brain tissue was outlined semi-automatically. BPF was calculated as the ratio of the brain tissue volume to the total volume contained within the brain surface contour. T2 – lesion load was identified on FlAIR scans.

The data were analyzed using software package Statistica 8 (StatSoft, Inc. STATISTICA for Windows Tulsa, OK: 2300 East 14th Street, Tulsa, OK 74104, http://www.statsoft.com). T-tests were employed for all other parameters.

Genetic study

1) Replication in three populations:

Patients and Controls

The diagnosis of all RLS cases was made according to diagnostic criteria of the International RLS Study Group by personal examination by a neurologist in the respective study center. 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 center 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 age 55.7 ± 15.3 years (± SD), 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 5 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). Since 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 - 269 (104 males) patients were recruited in 2 centers: at the Department of Neurology, Medical University of Vienna and Department of Neurology, University Clinic Innsbruck, (mean age 59.0 ± 14.3, mean age at onset of RLS 37.14 ± 19.5). 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, which procedures were described elsewhere (34) (236 males, mean age 59.9 ± 11.35). KORA controls were already used in previous GWA study, which showed only negligible effect of population stratification (24).

Finnish subjects - 90 (24 males) patients were recruited in Sleep Research Center in Turku (mean age 46.5 ± 18.1, mean age at onset of RLS 19.4 ± 13.4). Positive family history was reported by 81 patients and 9 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. Written informed consent was obtained from all RLS patients.

Ten SNPs within the three genomic regions were selected according to the results of previous GWA scans (24, 26). Samples were genotyped on two Sequenom platforms in Munich and Helsinki (Sequenom MassArray system, Sequenom Inc, San Diego, CA, USA).

2) Genetics of secondary RLS form in patients with multiple sclerosis

Patients

Participants in the epidemiological study (see above) were asked to take part also in the genetic association study. We also recruited more patients with clear cut secondary RLS to increase statistical power and did not use all the RLS negative patients so as not to exceed the 2:1 ratio between controls and cases. As a reference population, blood donors were used – the same sample as described in the previous study (str. 12). The genetic association study included 642 subjects; 203 MS patients (45 men, 158 women, mean age 40.7 years, SD ±10.7) with RLS were compared to 438 MS patients (122 men, 316 women, mean age 35.8 years, SD ±9.3) without RLS and to a reference population of 450 blood donors (166 males, 284 females, mean age 45.3 ± 9.9).

We excluded patients who had experienced RLS prior to the first symptoms of MS and patients with a positive family history of RLS to minimize the admixture of idiopathic cases.

Association tests were conducted in different settings: 1) patients with MS with RLS combined versus patients with MS without RLS 2) patients with MS with and without RLS versus population controls (blood donors not screened for RLS) and Czech sample of idiopathic RLS (see above).

The Local Ethics Committee approved the study and written informed consent was obtained from all subjects. Twelve single nucleotide polymorphisms (SNPs) within the four genomic regions were selected according to the results of previous GWA scans (24,

27). Samples were genotyped on Sequenom platform (Sequenom MassArray system, Sequenom Inc, San Diego, California, USA).

RESULTS

Clinical study

Epidemiological and radiological study – prevalence of RLS in patients with multiple sclerosis and brain magnetic resonance imaging study in patients with RLS and MS

In total, we enrolled 765 MS patients (553 females and 212 males). The mean age was 36.5 ± 9.5 years with average disease duration of 9.1 ± 7.36 years. The median EDSS score was 2.0 (quartiles 1.5 and 3.5). Out of all the examined patients, 76% had relapse-remitting MS, 14.4% had clinically isolated syndrome, 5.6% were in secondary progression and 0.9% had a primary progressive form of MS.

The diagnosis of RLS was confirmed in 245 subjects (32%, 95% CI 28.7-35.4%) with MS, mean age at onset of RLS symptoms was 29.1 ± 10.4 years. In 49 patients (6.4%), RLS symptoms preceded the MS onset and 19 patients (2.4%) had a positive family history and RLS symptoms preceding the MS onset and therefore were subsequently excluded from all genetic studies (Graph 1). In 177 patients (23.2%) RLS followed the MS onset, 520 patients (68%) never experienced RLS. The average delay between the onset of MS and that of RLS was 2.5 ± 8.7 years. Compared to patients without RLS, patients suffering from both MS and RLS were significantly older (38.6 vs. 35.6 years, $p<0.001$, Students t-test), had longer durations of MS symptoms (11.0 vs. 8.2 years, $p<0.001$, Students t-test) and had higher EDSS scores (2.9 vs. 2.3, $p<0.001$, Mann-Whitney test). There were significantly more affected women in the RLS affected group (78% vs. 69%, $p= 0.0072$, χ^2).

Quantitative MRI data were obtained in 385 patients without RLS (mean age 38.3, ±SD 10.2) and 215 patients with RLS (mean age 41.4, ±SD 10.4). We found no difference between the two groups in the whole brain lesion load, brain atrophy and brain parenchymal fraction (Table 1), despite the fact that we were able to replicate the correlation of these data with clinical parameters of MS .

Genetic study

1) Replication in three populations:

All SNPs tested were in HWE ($p> 0.01$) in both patients and controls. Significant association after correction for multiple testing at significance level alpha= 5 % was found in at least one SNP for all tested loci in the combined samples (Table 2), and in the Czech and Austrian samples separately. Analyzing the Finnish sample, we confirmed

only the association to *BTBD9*. The association to rs2300478 in *MEIS1* was only nominally significant and *MAP2K5/SCOR1* showed no association.

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 ($P= 9.1x10^{-10}$). Results for this haplotype were similar when testing the Czech ($P= 3.2\ 10^{-7}$, OR= 2.38), Austrian ($P= 8.3x10^{-5}$, OR= 1.82), and Finnish samples ($P= 2.0x10^{-4}$, 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/SCOR1* locus.

Analyzing only familial cases (n= 217) and all controls, all three loci were significantly associated. Using sporadic cases only (n= 283), we would confirm the association to *BTBD9* but not to *MEIS1* and *MAP2K5/SCOR1*. 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.

2) Genetics of secondary RLS form in patients with multiple sclerosis

A. Testing of MS patients positive for RLS versus MS patients negative for RLS

All SNPs tested were in HWE (p>0.01) in both patients and controls. No significant association with *MEIS 1*, *BTBD9* and *PTPRD* was found in 203 patients with MS. There was a trend for association with *MAP2K5/SCOR1* - the best model for the risk allele was the recessive model (p nominal = 0.0029, p permutated after correction = 0.0248, p nominal corrected for 4 loci and 2 models, i.e. 8 tests = 0.029, odds ratio = 1.60 - 95% CI 1.17 – 2.18). Thus, the one sided p value with the direction of the alternative hypothesis given by the original report is p corrected 0.019. Finally, we did the association analysis only in relapse – remitting MS form in order to distinguish the MS subtypes. We included 192 MS patients with RLS and 373 MS patients without RLS in the analysis. The results show the same trend for association as when using patients with all MS forms, but due to the lower sample size the significance is lower and does not bypass correction for multiple testing. Results for all tested loci are summarized in Table 3.

B. Testing of MS patients versus population controls and idiopathic RLS patients

When testing RLS MS patients positive for RLS vs. idiopathic RLS patients, the allele frequencies were very similar for *MAP2K5/SCOR1* and *PTPRD* markers - maximal

observed χ^2 statistics was 0,4. However, idiopathic RLS patients in variants in *MEIS 1 and BTBD9* genes present with different allele frequencies, but this contrast is only nominally significant.

The last performed comparison was of MS patients positive for RLS vs. population controls shows similar results and in the same directions, as when comparing to MS patients negative for RLS. However the statistical significance is lower, because the blood donors were not screened for presence of RLS and have different sex and age distribution.

DISCUSSION

In our work, we wanted to further investigate clinical and genetic aspects of primary and secondary RLS. The aim of the clinical part was to verify the high prevalence of RLS among Czech patients with multiple sclerosis, to identify the risk factors for RLS and correlation of magnetic resonance imaging parameters with RLS in patients with MS.

In the clinical part, we confirmed the previous findings that the prevalence of RLS is high in patients with MS. An earlier study investigating the association between RLS and MS in the French-Canadian population showed a difference in prevalence between patients and controls of 37.5% vs. 16% (17). A later study published by an Italian group showed a prevalence of 19% in MS and 4.2% in control subjects (19). They did not include patients who experienced the symptoms with a frequency of occurrence lower than twice per week (a further 7.3%, total RLS prevalence 26.3%). Another study published by Spanish authors showed different results – a similar prevalence rate of RLS in MS patients and in healthy subjects (13.3 % vs.9.3%) (18).A different methodology and different frequency criteria might explain discrepancies in absolute values in estimation of prevalence rates among these studies. In our study, we did not use any frequency threshold for the diagnosis of RLS: a patient was considered to be affected if he/she had ever met all criteria in their lifetime. The total prevalence was 32%, and in 68 (8.8%) subjects the RLS symptoms preceded the MS onset and 19 patients (2.4% of total) from this group reported a positive family history. Thus our estimate of the prevalence of RLS is very similar to those observed in the larger studies. In patients with MS, among others, the following risk factors for RLS were found: older age, longer MS duration and higher neurological disability; therefore, the patients with RLS seem to be in a more advanced stage of MS as was previously suggested(19). Therefore, we conclude that RLS is significantly associated with MS and can lead to sleep disturbance in MS patients. In clinical praxis, we encouraged the routine screening of patients for insomnia and symptoms of RLS.

The radiological study has also confirmed the previous investigations that the presence of RLS symptoms does not correlate with MRI markers of brain damage in MS despite the fact that RLS is more prevalent in advanced stages of MS. We used MRI volume

parameters which better correlate with the MS clinical progression. The results may be caused by the low sensitivity of our MRI analysis approach, however Manconi et al assessed more specific scans with mean diffusion (MD) and fractional anisotropy (FA) analysis and found no association between RLS and a particular brain MRI lesion pattern (20). The study revealed significantly reduced cervical cord average FA in MS patients with RLS compared to those without. Cervical cord damage may play role in the pathophysiology of the association between RLS and MS.

Our second study, "Replication in three populations" showed an association of common genetic variants in *MEIS1*, *BTBD9* and *MAP2K5/SCOR1* with RLS in a combined sample of Czech, Austrian, and Finnish RLS cases. Similar findings were observed in the US population (25). In accordance to the original report, the strongest effect was observed with the haplotype "AG" formed by markers rs6710341 and rs12469063 located in the 9th 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 (24). The significance of these loci to RLS can therefore be regarded as well established.

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 - 2.1 in familial and 1.019 - 1.534 in sporadic cases vs. all controls for rs12469063) and *MAP2K5/SCOR1* (1.164 -1.841 in familial and 0.951-1.408 in sporadic cases for rs6494696). There is a trend that *MEIS1* and *MAP2K5/SCOR1* possibly play a more important role in familial RLS, but due to limited number of patients, we were not able to prove significant heterogeneity. 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.

The last part of the genetic study "Genetics of secondary RLS form in patients with MS" attempted to reveal whether the genetic variants known to increase the risk in idiopathic RLS cases (*MEIS1, BTBD9, MAP2K5/SCOR1, PTPRD*) also contribute to the secondary RLS in patients with multiple sclerosis. So far only one genetic – association study with secondary RLS cases has been published (32). Our study, despite its sufficient statistical power, showed no association to variants in *MEIS1* and *BTBD9* with secondary RLS in MS patients. There was a trend for the association with *MAP2K5/SCOR1,* the best model was the recessive one. This model and the direction of the association are in accordance with the previous genome-wide scans and replication studies in idiopathic cases (24, 27).

To exclude the possible genetic influence of an MS diagnosis we conducted the second association study comparing patients suffering with MS without RLS to the unscreened

population and found no association for all tested variants, taking into account the above described statistical power. The *MAP2K5/SCOR 1* gene variant showed significant evidence for the association in the genome-wide scans in idiopathic cases. *MAP2K5* is important for the early stages of muscle differentiation and is important in the neuroprotection of dopaminergic neurons(35). *SCOR 1* acts as a transcriptional co-repressor of LBX1. This homeobox is critical in the development of sensory pathways in the dorsal horn of the spinal cord (30). Its role and function in RLS as well as in patients with RLS/MS, however, is not known.

Further studies with more accurate spinal cord MRI and genetic association with other secondary RLS cases, such as in pregnancy, are necessary to disclose the pathogenesis of both secondary and primary RLS.

FINAL CONCLUSION

Clinical part:

A. The prevalence of RLS in patients with MS is high (32% in Czech population), MS should be considered amongst secondary RLS form. RLS is associated with more severe disability and clinical course in MS patients. We should investigate MS patients for RLS symptoms, if they report sleep difficulties, because the effective treatment is available.

B. The extent of brain damage using MRI does not correlate with the presence of RLS in MS patients. Therefore, further studies with the spinal cord MRI are necessary to disclose the etiopathogenesis.

Genetic part:

A. Our study shows that variants in 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.

B. The idiopathic RLS forms do not share all the major genetic features with secondary RLS forms in patients with MS. However we were able to confirm the mild impact of the *SCOR1* gene variants on a higher prevalence of RLS in MS patients.

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1. publikace *in extenso,* které jsou podkladem disertace
    1. Kemlink D, Polo O, Frauscher B, Gschliesser V, Högl B, Poewe W, Vodicka P, Vavrova J, Sonka K, Nevsimalova S, Schormair B, Lichtner P, Silander K, Peltonen L, Gieger C, Wichmann HE, Zimprich A, Roeske D, Müller-Myhsok B, Meitinger T, Winkelmann J. Replication of Restless Legs Syndrome Loci in Three European Populations. J Med Genet. 2009 Mar 10. 46(5):315-8 IF = 5,5
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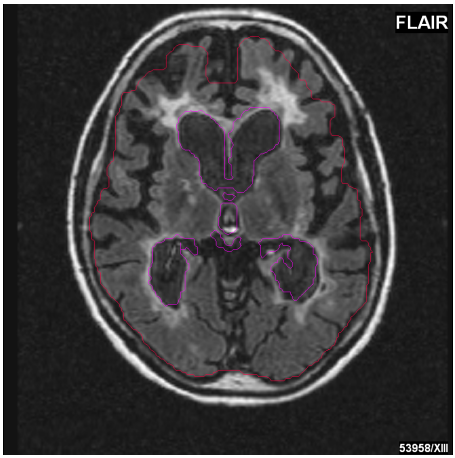
2. publikace *in extenso,* které se netýkají tématu práce s IF

Volná J, Kemlink D, Kalousová M, Vávrová J, Majerová V, Mestek O, Svarcová J, Sonka K, Zima T. Biochemical oxidative stress-related markers in patients with obstructive sleep apnea. Med Sci Monit. 2011 Sep;17(9):CR491-7. IF = 1,7.

3. publikace *in extenso,* které se netýkají tématu práce bez IF

Sonka K, Fialová L, Volná J, Jiroutek P, Vávrová J, Kemlink D, Pretl M, Kalousová M. Advanced oxidation protein products in obstructive sleep apnea. Prague Med Rep. 2008;109(2-3):159-65.

Picture 1: Brain atrophy – technique of measurement



Graph 1: Prevalence RLS in MS patients

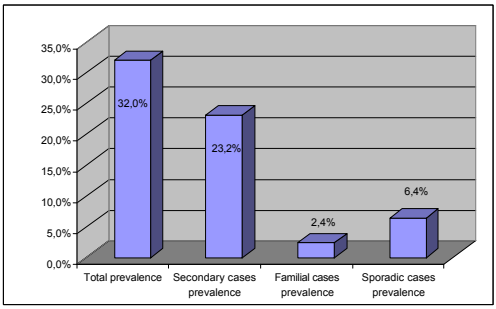


Table 1: Quantitative brain MRI data from MS-patients) without RLS (MS/RLS-) and with RLS (MS/RLS+)

	MS/RLS-	MS/RLS+	t-value	p-value
Age	38,318	41,426	-3,67371	0,00026
Lesion load – T2	6,555	6,579	-0,03019	0,975928
Brain parenchymal fraction	84,495	84,5	-0,02666	0,978737
Atrophy – T1 %	98,44	98,436	0,02306	0,981608

Table 2: Genotyped SNPs and Results of Association in Combined Samples

OR – Odds ratio for the risk allele (Cochran-Mantel-Haenszel test) with 95% confidence intervals, 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. † risk allele is the major allele

Chr	Gene	SNP ID	OR (95% Conf. Int)	P corr	MAF Fam.	MAF Spor.	Best model	P corr Fam.	P corr Spor.
2p	MEIS1	rs6710341	0.84 (0.64-1.11)	1	0.1270	0.1288	TREND	1	1
2p	MEIS1	rs12469063	1.43 (1.16-1.78)	4.15E-05	0.3522	0.2727	TREND	2.24E-05	0.3245
2p	MEIS1	rs2300478	1.47 (1.18-1.82)	1.26E-05	0.3575	0.2860	TREND	3.10E-05	0.1520
6p	BTBD9	rs9296249	1.59 (1.26-2.01) †	0.00107	0.1694	0.1553	TREND	0.0544	0.0012
6p	BTBD9	rs3923809	1.58 (1.28-1.96) †	4.11E-05	0.2204	0.2330	TREND	0.0018	0.0022
6p	BTBD9	rs4236060	1.49 (1.19-1.86) †	0.00019	0.1882	0.2110	TREND	0.0008	0.0049
15q	MAP2K5	rs11635424	1.26 (1.02-1.55) †	0.06023	0.2446	0.2992	REC	0.0203	1
15q	MAP2K5	rs3784709	1.24 (1.01-1.52) †	0.05301	0.2392	0.2917	REC	0.0393	1
15q	MAP2K5	rs1026732	1.27 (1.03-1.56) †	0.04278	0.2339	0.2936	REC	0.0116	1
15q	MAP2K5/ LBXCOR1	rs6494696	1.27 (1.03-1.56) †	0.04764	0.2339	0.2936	REC	0.0108	1

Table 3: Results of genetic association study

Genome – The Genetic positions in bp derived from UCSC Genome browser (http://genome.ucsc.edu, assembly March 2006) (36) **, OR best model –** Odds-ratio according to best model in original locus description (Allelic for TREND, Allele negativity for REC) including 95% confidence interval. **MAF MS+RLS+ -** minor allele frequency in MS patients with RLS symptoms, **MAF MS+RLS- -** minor allele frequency in MS patients without RLS symptoms, **MAF controls -** minor allele frequency in unscreened population sample of blood donors. **Best model –** Best model corresponds to the model under which the lowest P values were observed (TREND – Armitage trend test, REC – recessive model) in the original and replication publications . (1) **P-nom model –** raw nominal p-values observed under the best model, **P nom allelic** – comparison of allele frequencies between MS+ RLS+ and MS+RLS- patients. **P nom Model RR-MS –** raw nominal p-values observed under the best model using relapse-remitting MS patients. All p-values shown are 2-sided. † risk allele is the major allele

Chr	Gene	SNP ID	Genome	OR best model (95% Conf. Int)	MAF MS+ RLS+	MAF MS+ RLS-	MAF controls	Best model	P nom Model	P nom Allelic	P nom Model RR-MS
2p	MEIS1	Rs6710341	66611926	1.19 (0.86 - 1.64)	0.1533	0.1323	0.1407	TREND	0.4552	0.2954	0.3861
2p	MEIS1	rs12469063	66617812	1.12 (0.86 - 1.45)	0.2588	0.2384	0.2194	TREND	0.3887	0.4128	0.4660
2p	MEIS1	Rs2300478	66634956	1.13 (0.87 - 1.47)	0.2622	0.2396	0.2229	TREND	0.3767	0.3668	0.4000
6p	BTBD9	Rs9296249	38473818	1.14 (0.86 - 1.5) †	0.2102	0.2326	0.2361	TREND	0.1519	0.3541	0.3084
6p	BTBD9	Rs3923809	38548947	1.03 (0.8 - 1.32) †	0.2978	0.3037	0.3060	TREND	0.5883	0.8235	0.7501
9p	PTPRD	rs11788684	8846420	1.01 (0.73 - 1.4)	0.1422	0.1407	NA	TREND	0.5714	0.9400	0.3782
9p	PTPRD	Rs4626664	9261737	1.15 (0.84 - 1.58) †	0.1467	0.1655	0.1409	TREND	0.4507	0.3762	0.8330
15q	MAP2K5	rs11635424	65824631	1.53 (1.12-2.08) †	0.2788	0.3341	0.3349	REC	0.0070	0.0402	0.0355
15q	MAP2K5	Rs3784709	65859328	1.60 (1.17-2.18) †	0.2765	0.3345	0.3291	REC	0.0029	0.0316	0.0167
15q	MAP2K5	Rs1026732	65882138	1.54 (1.13-2.10) †	0.2753	0.3314	0.3349	REC	0.0059	0.0367	0.0272
15q	MAP2K5/ *SCOR1*	Rs6494696	65890259	1.56 (1.15-2.13) †	0.2765	0.3329	0.3356	REC	0.0045	0.0361	0.0253