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Genome-wide association and whole exome sequencing studies reveal a novel candidate locus for restless legs syndrome

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ABSTRACT

Introduction: The restless legs syndrome (RLS) is a common heritable neurologic disorder which is characterized by an irresistible desire to move and unpleasant sensations in the legs.
Methods: We aim to identify new variants associated with RLS by performing genome-wide linkage and subsequent association analysis of forty member's family with history of RLS.
Results: We found evidence of linkage for three loci 7q21.11 (HLOD = 3.02), 7q21.13-7q21.3 (HLOD = 3.02) and 7q22.3 (HLOD = 3.09). Fine-mapping of those regions in association study using exome sequencing identified *SEMA3A* (p-value = $8.5{\cdot}10^{-4}$), *PPP1R9A* (p-value = $7.2{\cdot}10^{-4}$), *PUS7* (p-value = $8.7{\cdot}10^{-4}$), *CDHR3* (p-value = $7.2{\cdot}10^{-4}$), *HBP1* (p-value = $1.5{\cdot}10^{-4}$) and *COG5* (p-value = $1.5{\cdot}10^{-4}$) genes with p-values below significance threshold.
Conclusion: Linkage analysis with subsequent association study of exome variants identified six new genes associated with RLS mapped on 7q21 and q22.

1. Introduction

Restless legs syndrome (RLS) is a neurologic sleep/wake disorder characterized by uncomfortable and unpleasant sensations in the legs that appear at rest, usually at night, inducing an irresistible desire to move the legs (Wijemanne and Ondo 2017). The prevalence of disease ranges from 5% to 10% (Ohayon et al., 2012) in European population and 3.2% in Turkey (Merlino et al., 2007). The diagnosis is based on the clinical criteria and defined by the International Restless Legs Syndrome Study Group (IRLSSG) (Allen et al., 2014). RLS is considered to result from mainly dopaminergic dysfunction, abnormal iron metabolism and genetic factors (Koo et al., 2016).

Classification based on the etiology for RLS is in two groups; primary or idiopathic RLS and secondary RLS (Guo et al., 2017). The most common form is primary RLS (70%–80%) (Bassetti et al., 2001). Among these patients (40.9%–92%) have a history of RLS family so genetic factors play an important role. Also, symptom onset of primary RLS is early (before 45 age). Secondary RLS is usually associated with iron deficiency, uremia, pregnancy, neurological diseases, cardiovascular diseases, alcohol abuse, diabetes, and hypothyroidism (Panda and Pandey 2019).

Estimated RLS heritability had been reported to be 40%–90% in twin studies (Caylak 2009). At least eight susceptibility loci in linkage studies and multiple variants in Genome-wide association studies (GWAS) and Whole-exome sequences (WES) studies were identified as potentially responsible on RLS (Akçimen et al., 2018; Jiménez-Jiménez FJ et al.,

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2018; Didriksen et al., 2020).

In this study, we aimed to map the candidate locus/gene(s) for RLS in a Turkish family inherited with autosomal dominant pattern with GWAS and WES analysis.

2. Materials and methods

2.1. Patients

This study was approved by the National Local Research Ethics Committee, Gazi University, Ankara, Turkey with an approval # 054. All family members were informed about the purpose of the study and written consent was taken before recruitment and sampling.

Our study population represents five generation Turkish family of forty members as shown on Fig. 1. Twelve individuals are diagnosed with RLS (II-4,III-1,III-2,III-3,III-9,III-12,IV-3,IV-6,IV-13,V-1,V-2,and V-3).

Pedigree was collected when a patients went to neurology polyclinic to consult regarding with their RLS symptoms and indicated that their family has similar complaints. All the living members of the family were invited for consultation. A detailed clinical history was taken and full neurological examinations were performed by a neurologist. RLS was diagnosed with International Restless Legs Syndrome Study Group (IRLSSG)'s RLS Questionnaire (RLSQ) (Allen et al., 2014). Those who could not come for face-to-face consultation were reached by phone. RLS was not detected among those individuals. Children and grandchildren provided information for those who were deceased.

Secondary reasons for RLS were excluded in RLS positive family members and full blood analyses and electromyeloneurography (EMNG) were performed. After obtaining written permission from the twenty one individuals who participated in the study venous blood samples were taken for genotyping.

3. Genotyping

A five ml of peripheral blood sample of the fifteen individuals (III-1, III-3,III-4,III-6, IV-1,IV-3,IV-4,IV-5,IV-6,IV-8,IV-10,IV-13, V-1,V-2, and V-3) was collected in ethylenediaminetetraacetic (EDTA) acid tubes. The genomic DNA was isolated by standard protocols from peripheral blood (Winkelmann et al., 2006). All fifteen individuals were genotyped for 261,961 SNPs using the Affymetrix 250K *Nsp*I panel. We excluded SNPs with strand inconsistency, call rate below 98% and those which failed HWE test (p-value $< 10^{-8}$) during the quality control. In total, 227,842 autosomal SNPs were available for analysis.

From those having chip data, six individuals (III-1, III-4, IV-8, IV-13, V-2, and V-3) were also sequenced for 610,840 exome SNVs. After excluding monomorphic and those with Mendelian errors, 454,165 single nucleotide variants (SNVs) left for analysis. We found 10,393 SNPs common for chipped and sequenced exome data. 40 SNPs were excluded because of alternative and reference allele's discrepancy and 2955 SNPs because of incomplete concordance between chipped and sequenced variants. Those 7398 SNPS were used for imputation exome sequence for the individuals without exome data.

4. Statistical analysis

4.1. Imputation

Imputation of missing genotypes was performed in two steps. First, we applied family-based imputation approach to impute exome sequence using pedigree structure. Individuals with available exome sequence were used as a reference panel. This approach allows us to imputed rare variants with high accuracy and also reconstructs genotypes for individuals who were never genotyped (Chen et al., 2012; Ullah et al., 2019). We used Merlin package of version 1.1.2 with –infer option (Burdick et al., 2006). Second, we performed population-based imputation using 1000 Genome phase 3 reference panel (Delaneau et al., 2012). SNPs genotyped on Affymetrix chip and passed quality control were used for imputation. SHAPEIT package was used for phasing (Delaneau et al., 2012) and minimac4 package was used for imputation (Das et al., 2016). Variants with accuracy $R^2 \geq 0.9$ were selected for further analysis.

4.2. Linkage analysis

We performed parametric linkage analysis under the rare dominant model assuming penetrance of 0.99 and disease allele frequency of 0.0001. We selected 19,862 variants with MAF $\geq$ 0.05 uniformly distributed among all autosomes with call rate $\geq$ 50% to have reasonable amount of genetic data with maximum number of individuals. Such a call rate value reflects number of individuals for which we have imputed variants available rather than quality of genetic data. Allele frequencies were calculated from data. Sex was included into analysis as a covariate. To facilitate the analysis, PedCut software of version 1.19

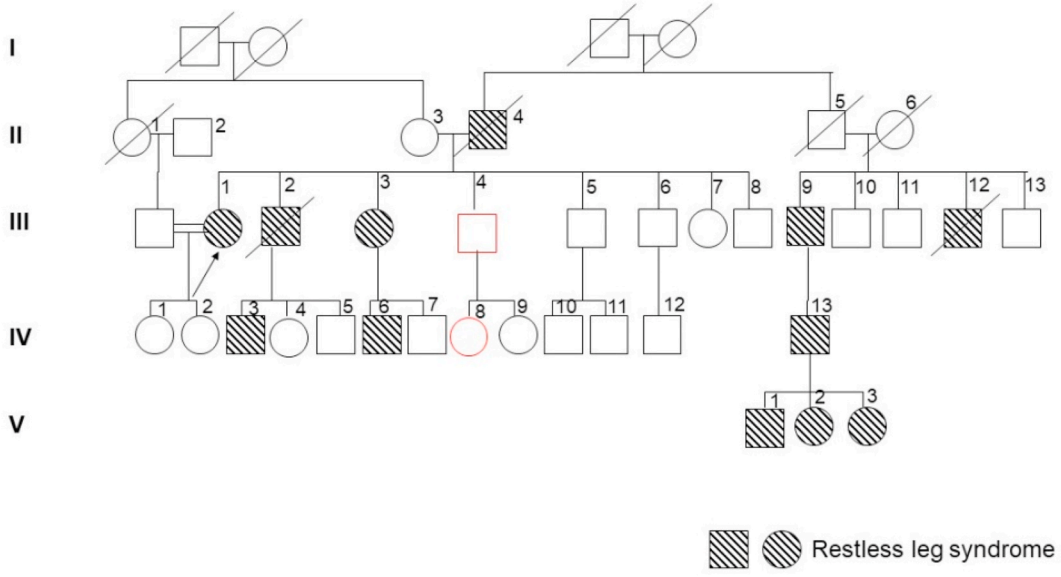


Fig. 1. Turkish family of forty members with twelve RLS individuals.

(Liu et al., 2008) was used to split the pedigree on three subfamilies with computational complexity (bit-size) of less than 20. These subfamilies consist of 19, 14 and 7 individuals.

4.3. Association analysis of variants under the linkage peaks

For association analysis, we used all variants from combined imputed data to identify SNVs that could explain linkage peaks. Trait quantitative association analysis under dominant model with adjustment on sex was performed for those variants using GCTA package (Yang et al., 2011). Mixed model with A matrix estimated from pedigree was used. Number of independent tests in the studied regions for the Bonferroni corrected significance level was calculated using the method described in Li et al. (2005).

4.4. Whole exome sequencing

The WES analysis was performed for patients III-1,III-4,IV-8,IV-13,V-2, and V-3. Regarding this analysis the enrichment was performed by Nextera Rapid Capture Expanded Exome Kit with the Illumina HiSeq platform with a coverage of x70. The analysis was performed by the Arpeggi Engine. This pipeline has been used for alignment, variant calling and variant annotation. The raw data had been provided in VCF format.

5. Results

The results of parametric linkage analysis under rare dominant model are shown on Fig. 2. All regions that we observe are below conservative genome-wide significance threshold of LOD = 3.3. Regions exceeded suggestive genome-wide significance threshold of LOD = 2.2 shown in Table 1. These are 2q14.3 - 2q21.2 (HLOD = 2.93) on chromosome 2 and four regions 7q21.11 (HLOD = 3.02), 7q21.12 (HLOD = 2.83), 7q21.13-7q21.3 (HLOD = 3.02) and 7q22.3 (HLOD = 3.09) on chromosome 7. Linkage results for chromosome 2 and chromosome 7 separately are shown on Supplementary Figure 1 and Supplementary Figure 2 respectively.

Further, we focus on three regions of chromosome 7 which exceeded significance threshold of LOD = 3.0 which can be considered as relaxed genome-wide significance threshold. Table 2 shows results of association analysis of SNVs located in those regions. The gene mostly enriched with significant variants detected in association analysis is *CDHR3* (7q22.3) having 23 variants. Second mostly enriched gene is *PPP1R9A* (7q21.13-7q21.3) with 22 variants. One SNV for each of genes *PUS7*, *HBP1* and *COG5* detected in 7q22.3 region and *SEMA3A* detected in 7q21.11 region. All those SNVs passed significance threshold of p-value = 0.0019 which takes into account 26 independent tests for the linkage peaks of chromosome 7. The data has been submitted to Leiden Open Variation Database(LOVD) with the link: http://databases.lovd.nl/shared/references/DOI:10.1016/j.ejmg.2021.104186.

Table 1
Suggestive genome-wide significant results of linkage analysis for RLS.

Region	Start position[a]	End position[a]	Dominant HLOD
2q14.3 - 2q21.2	124274534	134332896	2.93
7q21.11	83293564	83293564	3.02
7q21.12	86567364	87082292	2.83
7q21.13-7q21.3	88394709	96649668	3.02
7q22.3	104758250	107603431	3.09

[a] Position is reported in base pairs according to h19 assembly.

Table 2
Genes underlining linkage peaks detected in association analysis.

Gene	Start position[a]	End position[a]	p-value[b]
CDHR3	105587100	105597377	$7.2{\cdot}10^{-4}$
PPP1R9A	94696139	94885306	$7.2{\cdot}10^{-4}$
PUS7	105153242	105153242	$8.7{\cdot}10^{-4}$
HBP1	106805394	106805394	$1.5{\cdot}10^{-4}$
COG5	106920976	106920976	$1.5{\cdot}10^{-4}$
SEMA3A	83293564	83293564	$8.5{\cdot}10^{-4}$

[a] Start and end positions of significant SNVs are reported in base pairs according to h19 assembly.
[b] p-value are the same for all SNVs in the gene.

6. Discussion

RLS is a neurologic disorder characterized by an uncontrollable urge to move the legs during periods of rest. The majority of patients with RLS also have periodic limb movements in sleep, which are characterized by involuntary, highly stereotypical, regularly occurring limb movements

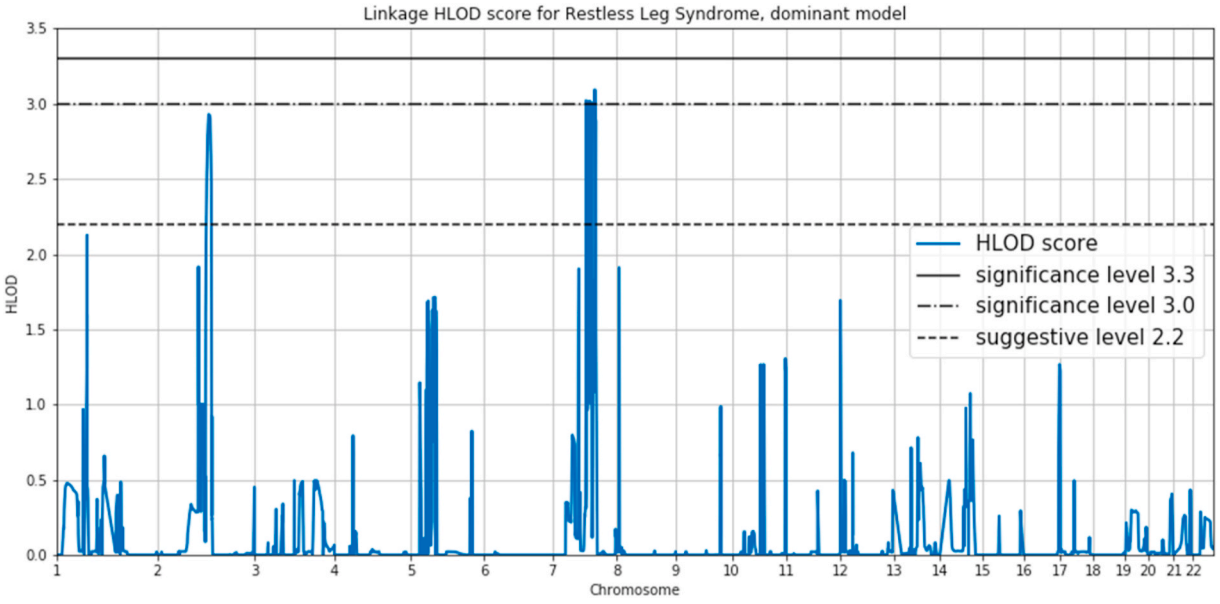


Fig. 2. The results of genome-wide linkage scan for parametric rare dominant model. The horizontal axis shows variant position in base pairs and the vertical axis shows heterogeneity LOD score.

that occur during sleep. Limb movements during sleep are more common in otherwise asymptomatic family members of patients with RLS compared to the general population (Stefansson et al., 2007). Also, family studies of RLS suggest an increased incidence (≈40%–50%) in first-degree relatives of idiopathic cases. A high concordance (83%) in monozygotic twins and complex segregation analysis suggest an autosomal dominant mode of inheritance (Hening et al., 2009). The inheritance pattern in our family also showed an autosomal dominant pattern.

In our study, we have identified *SEMA3A*, *PPP1R9A*, *PUS7*, *CDHR3*, *HBP1* and *COG5* genes having high LOD scores in linkage and significant p-values in association analysis. These genes have not been reported before in the etiology of RLS.

Semaphorins are a large family, including both secreted and membrane associated proteins, can function as either a chemorepulsive agent, inhibiting axonal outgrowth, or as a chemoattractive agent, stimulating the growth of apical dendrites. Transmembrane semaphorins can act as repulsive axon guidance cues (Polleux et al., 2000; Qu et al., 2002).

It had been reported that most of the top pathways identified for RLS were related to neurodevelopment, including neurogenesis (*MDGA1, MYT1, NTNG1,* and *SEMA6D*), cell-junction organization (*PKP4* and *SMAD3*), and axon guidance (*NTNG1* and *SEMA6D*). *SEMA6D* have been newly identified by Schormair et al. (2017). Sema3A produced in neurons regulates neural development in an autocrine manner in mice (Fukuda et al., 2013). Molofsky et al. (2014) showed that postnatal spinal cord astrocytes express several region-specific genes, and that ventral astrocyte-encoded *SEMA3A* is required for proper motor neuron and sensory neuron circuit organization. Loss of astrocyte-encoded Sema3a leads to dysregulated alpha-motor neuron axon initial segment orientation, markedly abnormal synaptic inputs, and selective death of alpha-motor neurons. *SEMA3A* has also been reported to cause susceptibility to hypogonadotropic hypogonadism-16 with or without anosmia (Hanchate et al., 2012). Semaphorin3A (Sema3A) is known to cause neuronal apoptosis and serves as a chemorepellent factor for axonal growth. Sema3A might be related to the toxicity of dopamine causing dopaminergic depletion (Yasuhara et al., 2005). Semaphorin signaling in dopaminergic axon pathfinding has been reported. Semaphorin proteins are involved in directing mesencephalic dopaminergic axons during development. Besides, mesotelencephalic pathways are thought to contribute to the pathogenesis of Parkinson's disease (Prestoz et al., 2012). Dopaminergic A11 cells, located in the midbrain and close to hypothalamus, have long axons and project diffusely throughout the spinal cord and the role of A11 dopaminergic cells in pathophysiological pathways in RLS had been suggested (Guo et al., 2017).

PPP1R9A gene encodes neurabin (neural tissue-specific F-actin-binding protein). Neurabin protein had been reported to be widely distributed in neural tissue and has high concentration in the synapse of developed neurons. Also, neurabin demonstrated to be involved in neurite formation. Neurabin had been suggested to have two possible roles both in vesicle trafficking in both the developing and developed neurons and in the formation and the maintenance of the synaptic junction (Nakanishi et al., 1997). Neurabin I and II are involved in dendritic spine signaling targeting both glutamatergic and dopaminergic pathways. They also regulate synaptic plasticity and synaptic transmission (Korrodi-Gregório et al., 2014). *PPP1R9A* plays an important role in synaptic structure and function, neurite formation, filopodia formation, and spine motility, however *PPP1R9A* did not correlate with spine loss. *PPP1R9A* interact with *NMDA* signaling pathways that regulate the actin cytoskeleton and spines (Konopaske et al., 2015). In a Ppp1r9a−/− genotype of mouse model, abnormal psychostimulant response and dopamine signaling transduction, reduced anxiety- and depression-related behaviors in young adult mice, impaired contextual fear memory had been reported (Ferreira et al., 2019).

PUS7 encodes RNA-independent pseudouridylate synthase 7. Human *PUS7* was shown to modify tRNAs and tRNA-derived small fragments (tRFs). Pseudouridylation of tRFs is required for early embryogenesis and for hematopoiesis. It causes autosomal recessive Intellectual developmental disorder with abnormal behavior, microcephaly, and short stature (de Brouwer et al., 2018; Shaheen et al., 2019).

CDHR3 is a transmembrane protein with six extracellular cadherin domains. The biological function of *CDHR3* is unknown, but it belongs to the cadherin family of transmembrane proteins involved in homologous cell adhesion and important for several cellular processes, including epithelial polarity, cell-cell interaction and differentiation. This gene had been reported to be an asthma susceptibility gene (Bonnelykke et al., 2014).

HBP1 is a transcriptional repressor and has been shown to negatively regulate the cell cycle. A correlation between *HBP1* under-expression and breast cancer relapse has been described, suggesting that *HBP1* may be an important tumor suppressor protein (Escamilla-Powers et al., 2010).

Conserved Oligomeric Golgi 5 (*COG5*) is a subunit of the COG complex which functions in trafficking and glycosylation as well as the maintenance of structure of Golgi apparatus. The deficiency of *COG5* leads to autosomal recessive congenital disorder of glycosylation type 2I (CDG2I). Its clinical manifestations may include neurological problems among other abnormalities and the pathogenesis is unclear (Wang et al., 2020). Fung et al. (2012) described a CDG2I patient with cirrhosis, microcephaly, peripheral neuropathy, and early developmental delays. Recently, another patient with the same diagnosis have been reported to have more severe central and peripheral neurological symptoms (Yin et al., 2019).

This study shows that 7q21 and q22 are potentially responsible for the RLS. However, the mechanisms of the six new genes found in this analysis have to be clarified with the further functional studies.

Declaration of competing interest

No conflict of interest was declared by the authors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmg.2021.104186.

Author statement

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