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Original article

# Genetic association studies of neurotensin gene and restless legs syndrome in French Canadians

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**Abstract**

*Background and purpose:* The neurotensin gene (NTS), a known dopamine modulator, is located within the candidate region for the first genetic locus of restless legs syndrome (RLS1) on chromosome 12q. Though no causative mutation was found in selected patients in a previous mutation analysis, the involvement of NTS in RLS cannot be completely excluded as a potential positional and functional candidate gene. The purpose of the current study is to further explore the NTS gene for potential functional variant(s) in its entire genomic and potential regulatory regions and their possible association with RLS symptoms.
*Methods and subjects:* We resequenced the coding regions and sequenced all the intronic and potential regulatory regions of the NTS gene in additional patients and controls. We carried out full scale gene-based case-control and family-based genetic association studies using the sequence variants detected during mutational analysis.
*Results:* No coding or variants in regulatory and intronic regions compatible with a deleterious mutation were detected. Seven polymorphisms with elevated allele frequencies in the Caucasian population did not show association with RLS in two independent case-control groups and 110 RLS families.
*Conclusion:* The NTS gene on chromosome 12q is most unlikely to play a direct role in RLS etiology.


*Keywords:* Restless legs syndrome; Neurotensin; Genetic association study; Mutational analysis; Dopaminergic pathway; French Canadians

## 1. Introduction

Restless legs syndrome (RLS) is a common sensorimotor disorder characterized by an imperative urge to move the legs, associated with or without paresthesias/dysesthesias of the lower limbs [1]. It can cause severe sleep and mood disturbances and has an adverse impact on the quality of life of individuals suffering from the disorder [2]. The clinical diagnosis of RLS is based on four common clinical characteristics, which include the cardinal feature often presenting as an imperative or irresistible urge to move or as leg discomfort, plus three defining features in which the urge to move or discomfort: (1) begins or worsens during periods of rest or inactivity; (2) is relieved by activity; and (3) occurs or worsens in the evening or during night, showing a

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circadian pattern [3]. Clinically, all four essential criteria are required to make a diagnosis of RLS. In uncertain cases, supportive clinical features include a positive family history, responsiveness to dopaminergic treatment, presence of periodic leg movements during wakefulness or sleep (PLMW or PLMS) and normal neurological exam for idiopathic RLS [3]. Accumulating population-based studies report prevalence of RLS between 2.5% and 15% in populations of European origin [4–12], with possible racial and regional differences [10,13–16]. We have previously identified the first genetic locus conferring susceptibility to RLS (*RLS1*) on chromosome 12q (RLS1, OMIM: *102300) [17]. Four additional loci have been reported to show linkage in RLS families, including *RLS2* on chromosome 14q [18], *RLS3* on chromosome 9p [19], *RLS4* on chromosome 20p [20] and *RLS5* on chromosome 2q [21], indicating genetic heterogeneity.

Although the pathogenesis of RLS is largely unknown, medications that increase dopamine (DA) transmission are currently the most effective treatment for RLS [22], implicating impaired central dopaminergic transmission in RLS. Several neuroimaging studies have further indicated a dysfunctional central dopaminergic system [23–26], though the results are not consistent in all studies [27–30], which may be due to the sensitivity of the technology applied or the subtlety of changes in dopaminergic neurons, and the clinical heterogeneity of the subjects studied. Hence, genes involved in dopamine metabolism would naturally be considered as functional candidates for RLS. The neurotensin gene (*NTS*) maps within the minimum candidate region of the *RLS1* locus on chromosome 12 [17,31] and encodes a common precursor for two peptides, neuromedin N and neurotensin, spanning 8689 bps of genomic sequence. NTS is a secreted tridecapeptide, which is widely distributed in the central nervous system (CNS) and may function as a neurotransmitter or as a neuromodulator with diverse functional roles in the CNS, including the sensory system [32]. A more extensive genetic investigation in our cohort indicates that five additional French Canadian (FC) RLS families show compatible linkage to the *RLS1* locus [33]. Studies by other groups have also further implicated the *RLS1* locus in additional RLS families from non-FC populations [34]. Of special note, a large-scale genome-wide linkage study in the Icelandic population provided supporting evidence of linkage to the *RLS1* locus [35]. It is thought that the *RLS1* locus may harbor a common weakly penetrant allele that may account at least in part for the elevated prevalence of RLS in the FC population and/or populations of European descent in general. Therefore, the *NTS* gene qualifies as an excellent potential positional and functional candidate for RLS.

Our previous sequence analysis of the *NTS* gene in 9 patients and 10 unaffected parents and siblings selected from four pedigrees linked to the *RLS1* locus did not detect any deleterious mutations in the coding regions [36]. However, other predisposing polymorphism(s) with low penetrance, especially within the non-coding regulatory and intronic regions of *NTS*, have not been excluded. In order to comprehensively explore the role of the *NTS* gene in RLS, additional cases have been screened for sequence variants in the entire genomic region of the *NTS* gene. All detected variants have been systematically examined in large-scale case-control and family-based association studies.

## 2. Methods and subjects

### 2.1. Sequence analysis

Comparative genomic method was used to identify potential regulatory regions by using the PhastCons [37] program (http://genome.ucsc.edu), which scores conserved elements by pairwise alignments of genomic sequences of several species. To thoroughly screen the entire *NTS* genomic region, primers were designed to amplify each exon and exon–intron boundaries, all introns (except for 865 bps of simple tandem repeats; chr12: 84,796,940–84,797,805), untranslated regions of messenger RNA (UTRs) and highly conserved regions in the 5′ upstream intergenic regions identified by comparative genomic research.

Genomic DNA was isolated from peripheral lymphocytes using standard methods. Ten unrelated patients with confirmed RLS diagnosis from 10 medium to large families, 10 FC controls and 10 controls with ethnic background other than Caucasian (5 African and 5 Asian), for a total of 30 individuals, were directly sequenced in both strands for 18 amplicons, for a total of 9713 bps of genomic sequences.

### 2.2. Genotyping methods

A 4-bp deletion polymorphism ($GATT_{del}$) was amplified and internally labeled with isotope $^{35}S$. The radioactive PCR products were separated on 6% polyacrylamide gels, and were detected by autoradiography after electrophoresis. Six single nucleotide polymorphisms (SNPs) were genotyped by TaqMan SNP Genotyping Assays using the Applied Biosystems 7900 Fast Real-Time PCR System and SDS software (vs.2.2.2) for allele calling.

### 2.3. Patients, controls and families for genetic association studies

#### 2.3.1. RLS case-control group 1

The probands in the first case-control group were ascertained, recruited and sampled exclusively through the *Centre d'étude du sommeil* in Montreal. All probands