Clinical and Neuropathological Features Associated With Loss of RAB39B

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ABSTRACT: Background: Pathogenic variants in the small GTPase Ras Analogue in Brain 39b (RAB39B) have been linked to the development of early-onset parkinsonism. The study was aimed at delineating the clinical and neuropathological features associated with a previously reported pathogenic variant in RAB39B

Methods: Clinical details of a male individual hemizygous for the T168K variant were collected by systematic review of medical records. Neuropathological studies of fixed brain tissue were performed and steady-state RAB39B levels were determined by western blot analysis.

Results: Neuropathological examination showed extensive dopaminergic neuron loss, widespread Lewy pathology, and iron accumulation in the substantia nigra. Additional pathology was observed in the hippocampus and thalamus. Western blot analysis demonstrated that the T168K variant results in loss of RAB39B.

Conclusions: T168K RAB39B is unstable in vivo and associated with dopaminergic neuron loss and Lewy pathology. Dysregulation of RAB39B in the prefrontal cortex and substantia nigra of individuals with idiopathic PD potentially implicates the protein more broadly in the pathological mechanisms underlying PD and related Lewy body disorders. © 2020 International Parkinson and Movement Disorder Society

Key Words: Parkinsonism; Parkinson’s disease; Parkinson’s disease dementia; neuropathology

Parkinson’s disease (PD) is a common neurodegenerative disorder that manifests with motor symptoms including resting tremor, rigidity, bradykinesia, shuffling gait, and postural instability. These motor deficits are mediated by the hallmark neuropathological features of PD, including loss of dopaminergic neurons in the substantia nigra (SN) and presence of intraneuronal α-synuclein (αSN)–positive inclusions termed Lewy bodies. Additional non-motor features may also develop, including a range of sleep, neuropsychiatric, and sensory disturbances.

Currently, the majority of PD cases are of unknown etiology (idiopathic), although causal variants in greater than 20 genes have been found to underlie ~10%–15% of cases. PD is responsive to symptomatic treatment with levodopa, whereas parkinsonism is a general term to describe disorders similar to PD that do not respond or respond only for a short time to levodopa therapy. Recently, loss-of-function variants in a small GTPase involved with intracellular trafficking in the CNS, Ras Analogue in Brain 39b (RAB39B), were linked to the development of X-linked recessive early-onset parkinsonism with nonprogressive intellectual disability (ID) and macrocephaly. Subsequent studies confirmed the role of

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RAB39B in PD, although the results of several cohort screens suggest pathogenic variants are a rare cause of PD. One report demonstrated that the pathogenic variant G192R caused clinically typical PD independent of intellectual disability. The neuropathological features of RAB39B-mediated parkinsonism have only been described in 1 case to date, an affected individual with deletion of the entire RAB39B locus. This revealed the hallmark pathological features of PD in the SN, including loss of dopaminergic neurons and the presence of αSN-positive Lewy bodies and Lewy neurites in surviving neurons. Additional disease features included cortical Lewy bodies and Tau-positive neofibrillary tangles (NFTs) and modest iron accumulation in the SN. Here, we describe the clinical features and neuropathology of an individual from a large pedigree carrying the pathogenic RAB39B T168K variant (NM_171998.3: c.503C>A). Our results showed a consistent neuropathology associated with RAB39B-mediated parkinsonism. In addition, we examined steady-state RAB39B in brain tissue from individuals with idiopathic PD (iPD) and showed that RAB39B is dysregulated in the prefrontal cortex and SN in iPD. These results potentially implicate RAB39B more broadly in the pathological mechanisms underlying iPD and warrant further investigation.

Materials and Methods

Human Brain Tissue

The Royal Children’s Hospital Human Research Ethics Committee approved the study (HREC 28097). Brain tissue from individuals with pathologically proven iPD was received from the Victorian Brain Bank (Melbourne, Australia) and the University of California FXTAS/FXS tissue repository (Davis, CA). Operation of the Victorian Brain Bank, including consent and ethical approval, is under the jurisdiction of the Human Research Ethics Committee of the University of Melbourne and the Victorian Institute of Forensic Medicine.

Postmortem Neuropathology

Neuropathological studies were performed as previously described. Briefly, we used routine hematoxylin and eosin (H&E)–stained tissue sections. To detect PD-associated proteins, immunohistochemistry (IHC) was performed on 5 μm of formalin-fixed, paraffin-embedded tissue sections. Sections were deparaffinized and sequentially treated with 80% formic acid for 5 minutes to achieve antigen retrieval and 3% hydrogen peroxide to eliminate endogenous peroxidase activity. Subsequently, sections were incubated with blocking buffer (20% fetal calf serum, 50 mM Tris-HCl, and 175 mM NaCl [pH 7.4]), then primary antibodies (rabbit anti-αSN and rabbit anti-Tau [Dako, A0024]). Primary antibodies were detected with an LSABTM kit (Dako) and immunoreactivity visualized with hydrogen peroxidase diaminobenzidine (H2O2-DAB). Nonheme iron (Fe2+ and Fe3+) was detected by Perl’s stain as previously described.

Cell Culture and Transfection

BE(2)-M17 human neuroblastoma cells were maintained in Opti-MEM (Invitrogen) supplemented with 10% fetal bovine serum. Cells were transfected with RAB39B wild-type or T168K mammalian expression constructs (pcDNA3.1, Invitrogen) using FugeneHD (Roche) and selected with 400 μg/mL Geneticin.

Protein Extraction and Western Blotting

Western blot analysis was performed as previously described. Briefly, total protein was extracted using buffer containing 2% sodium dodecyl sulfate (SDS) and 1× protease inhibitor (Sigma). Twenty micrograms of total protein was separated on 12% SDS-polyacrylamide gel electrophoresis and transferred onto 0.45-μm-pore polyvinylidene fluoride membranes (Immobilon-P) at 10V overnight. Membranes were blocked in 5% skim milk for 2 hours, then incubated with primary antibodies (rabbit anti-RAB39B [Proteintech, 12162-1-AP] and mouse anti-β-actin [Sigma, A5441]). Antibody binding was revealed using horseradish peroxidase-conjugated secondary antibodies (Jackson Laboratories) and enhanced chemiluminescence (Bio-Rad). Images were captured with ImageQuant LAS4000 and quantified using ImageQuantTL software (GE Healthcare). To quantify RAB39B steady-state levels, the signal intensity was normalized to the loading control β-actin, and control groups were assigned a relative value of 1. Samples were analyzed by tissue group, with simultaneous antibody incubation and imaging to enable direct comparison of steady-state levels between control and case samples from the same brain region.

Statistical Analysis

Statistical significance was determined using unpaired Student t tests (Graphpad Prism 7, La Jolla, CA). All quantified data are displayed as mean ± standard error of the mean.

Results

Clinical Features Associated With RAB39B T168K

We collected and reviewed all available clinical data for individual IV:12 from the previously described Wisconsin kindred carrying the RAB39B T168K mutation (Fig. 1A). Individual IV:12 shared with his brothers a syndrome comprising megaloecephaly, nonprogressive ID, and early-onset parkinsonism. He developed motor seizures at 2 years of age and was responsive to therapy. At age 9 he was noted to have “very inadequate” hand
FIG. 1. Neuropathology associated with RAB39B T168K. (A) Simplified pedigree of the Wisconsin kindred (individual IV:12 indicated). (B) Western blot analysis of RAB39B in brain tissue from individual IV:12 compared with a healthy control and of RAB39B in BE(2)-M17 neuroblastoma cells over-expressing wild-type or T168K RAB39B. (C–K) Microscopic examination of neuropathology in brain tissue from individual IV:12. Examination of SN showed (C) intracellular Lewy bodies (H&E), (D) αSN-positive Lewy bodies and neurites (IHC), (E) neuronal loss, pigment incontinence (H&E), and iron deposition (Perl’s stain). Examination of thalamus showed (F) neuronal loss and αSN-positive Lewy-like bodies and (G) Tau-positive NFTs (IHC). Examination of hippocampus showed (I) neuronal loss and αSN-positive Lewy neurites (pyramidal layer) and (J) αSN-positive Lewy bodies in the CA4 region (IHC). No obvious neuropathology was observed in the (H) putamen or (K) prefrontal cortex (H&E). Scale bar = 20 μm (C, D, F, G, I, J) or 50 μm (E, H, K). [Color figure can be viewed at wileyonlinelibrary.com]
and eye coordination, elective mutism, megalcephaly, and an IQ of 69. Evaluation at age 17 showed severe incoordination and social and mental handicaps with an IQ of 53, albeit with a surprisingly high level of reading and comprehension. Anticonvulsant-dependent epileptogenic activity was observed on his electroencephalogram (EEG), a larger than normal cranial vault was seen on skull x-ray without evidence of calcification, and contemporary blood

![Image of RAB39B in iPD distribution](image-url)
and urine metabolic screening tests were within normal limits. At age 19 his finger dexterity appeared to be in “slow motion,” with a mild intention tremor and shuffling gait. Repeat metabolic tests were normal. At age 21 his gait was halting, with truncal ataxia and persistent mutism. His EEG revealed diffuse encephalopathy with active cortical-reticular discharges and diffuse slow-wave abnormalities. A mild tremor on intent was noted at age 28, with arms carried at a high angle but with no cogwheel rigidity. Examination at 30 years of age demonstrated mild cogwheel rigidity, a parkinsonian posture and gait, facial hypomimina, and a resting hand tremor. The remainder of his neurologic exam was normal. A trial of carbidopa and levodopa (Sinemet) was abandoned for lack of benefit. At age 31 his clinical signs and symptoms were unchanged. Subsequent clinical records were unavailable for review, and the patient died at 53 from undetermined causes.

Neuropathological Features Associated With RAB39B T168K

Fresh-frozen and fixed tissue was available from the prefrontal cortex, hippocampus, SN, putamen, and thalamus from individual IV:12. Western blot analysis demonstrated significantly reduced steady-state levels of T168K RAB39B compared with the substantial levels in the equivalent brain regions of healthy aged controls (Fig. 1B). This outcome is consistent with in vitro overexpression models, which show significantly reduced levels of T168K RAB39B compared with wild-type RAB39B (Fig. 1B) because of ubiquitin-proteasome-mediated turnover of the unstable protein. Collectively, these in vivo results are consistent with previous in silico and in vitro results, indicating a loss-of-function mechanism associated with the development of RAB39B-mediated parkinsonism.

Neuropathological examination of the SN revealed αSN-positive Lewy bodies and neurites (Fig. 1C,D), substantial neuronal loss, pigment incontinence, and modest levels of intraneuronal iron deposition (Fig. 1E). We observed neuropathological features in 2 additional regions. In the thalamus, we observed minor neuronal loss and occasional αSN-positive Lewy-like bodies (Fig. 1F) along with Tau-positive NFTs (Fig. 1G). In the hippocampus, we identified mild neuronal loss and αSN-positive Lewy bodies and neurites in the pyramidal layers (Fig. 1I) and spongy change associated with scant αSN-positive Lewy bodies in the CA4 region (Fig. 1J). There was no obvious neuropathology or iron accumulation observed in the prefrontal cortex or putamen (Fig. 1H,K).

Steady-State RAB39B Is Dysregulated in iPD

We obtained fresh-frozen brain tissue from 10 late-onset iPD cases and 10 age- and sex-matched controls from the Victorian Brain Bank (sex ratio, 1.5 male/female; age, 79.5 ± 5.0 years; postmortem interval, 38.3 ± 16.9 hours). Six of the iPD cases had developed iPD with dementia, as defined by the onset of cognitive decline greater than 12 months after the onset of iPD. Regions that were available for analysis included the cortex (dorsomedial prefrontal region), hippocampus (CA1 region and dentate gyrus), SN (pars compacta and reticulata), caudate nucleus, and thalamus (dorsomedial thalamic nucleus). By Western blot analysis, we observed ~70% reduction in steady-state RAB39B in iPD tissue in the cortex (PD, 0.29 ± 0.04, n = 10, P = 0.0001; Fig. 2A,B) and SN (PD: 0.31 ± 0.08, n = 10, P = 0.0004; Fig. 2J) compared with healthy controls. In contrast, we did not observe any significant differences in the hippocampus (Fig. 2C,D), thalamus (Fig. 2E,F), or caudate nucleus (Fig. 2G,H). We further investigated RAB39B levels in iPD cases stratified by the presence of dementia. Although there was no statistically significant difference, we observed a trend toward greater reduction of RAB39B in PD with dementia (PDD) cases compared with cases with no dementia (no PDD) in the cortex (no PDD, 0.37 ± 0.05, n = 4; vs PDD, 0.23 ± 0.04, n = 6, P = 0.066; Fig. 2A,B) and hippocampus (no PDD, 1.05 ± 0.15, n = 4; vs PDD, 0.75 ± 0.07, n = 6; P = 0.073; Fig. 2C,D).

Discussion

RAB39B is a novel PD-associated gene encoding a protein with a putative function in intracellular trafficking in the CNS. Currently, little is known about the role of RAB39B in PD, and there are a limited number of cases reported. In this study, we confirmed loss of RAB39B in vivo in an individual carrying the RAB39B T168K variant. We identified clinical and neuropathological features that are commonly associated with PD and comparable to RAB39B deletion. This is the second neuropathological assessment performed for RAB39B-mediated parkinsonism with the pathogenic mechanism being loss of function because of 2 independent mutation mechanisms, gene deletion and protein instability secondary to a missense variant. Collectively, the neuropathology associated with this genetic form of parkinsonism is characterized by the typical features of PD including neuronal loss, Lewy pathology, and iron accumulation in the SN.

We observed additional neuropathological features, some of which differed between the 2 case reports. In particular, we observed Tau pathology in the SN and abundant cortical Lewy bodies only in the case with the RAB39B deletion. We also observed Tau pathology in the thalamus and Lewy pathology in the thalamus and hippocampus with RAB39B T168K; however, we were unable to assess these regions in the RAB39B deletion case. Tau pathology has previously been identified in iPD and other genetic forms of PD (SNCA, PARKIN, LRRK2, DJ1). Further, Tau- and αSN-positive inclusions can co-occur in other parkinsonisms and neurodegenerative disorders, although the functional interplay between the 2 proteins are largely unknown. Similarly, pathology in cortical...
regions is common during the later stages of PD progression and has also been identified in familial forms of PD (SNCA, PINK1, DJ1, LRRK2, PLA2G6). Both cortical and hippocampal pathology may correlate with cognitive decline in PD. Indeed, in RAB39B-mediated Parkinsonism, the presence of pathology in these regions is consistent with the development of cognitive dysfunction in the disorder. Additional cases of RAB39B-mediated Parkinsonism will need to be assessed to better define the full phenotypic spectrum of the disorder and help to guide the differential diagnosis from other parkinsonian disorders.

We also investigated a potential role for RAB39B in iPD. Interestingly, we observed significantly reduced steady-state levels of RAB39B in the SN and prefrontal cortex, but not the hippocampus, caudate nucleus, or thalamus of individuals with iPD. Decreased levels of RAB39B in the SN are expected, given the substantial neuronal loss typically observed in this region. However, it is unclear how and why RAB39B is reduced in the prefrontal cortex. Potentially, this is associated with the function(s) of RAB39B in cognition and the manifestation of cortical abnormalities in iPD. For example, cortical thinning, reduced gray-matter volume, reduced cortical gyration, and altered cortical microstructure have been previously reported in iPD. A proportion of individuals in our iPD cohort had developed dementia following diagnosis of PD. Given that the prefrontal cortex and hippocampus play critical roles in cognition, we determined the reduction in RAB39B levels in these regions of individuals with dementia compared with no dementia. We identified a trend toward greater reduction of RAB39B in PDD compared with no PDD in both regions. However, perhaps because of the small sample size available, these trends did not achieve statistical significance. Given this caveat and the potential limits of quantitative Western blotting to accurately determine small differences in target protein abundance, it will be of interest to replicate these studies in larger cohorts as well as in other dementia syndromes such as dementia with Lewy bodies to test the potential role of RAB39B in dementia and delineate the mechanisms underlying cognitive dysfunction in RAB39B-mediated disease.

Overall, our study implicated RAB39B in the development of a rare genetic form of parkinsonism, mediated by loss of RAB39B, and potentially more broadly in the development of iPD, mediated by altered homeostasis of steady-state RAB39B. Our results highlight the significance of dysregulated intracellular trafficking in the pathological mechanisms underlying both genetic parkinsonism and iPD. The potential role of RAB39B in iPD warrants further investigation, with implications for improved understanding of the pathomechanisms mediating PD and associated Lewy body disorders.

Transcranial Ultrasound Stimulation Directly Influences the Cortical Excitability of the Motor Cortex in Parkinsonian Mice

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ABSTRACT: Background: Low-intensity transcranial ultrasound stimulation is a new noninvasive brain modulation method with high spatial resolution and high penetration depth. However, until now, it was unclear whether transcranial ultrasound stimulation has a significant effect on PD.

Objectives: In order to evaluate the effect of transcranial ultrasound stimulation on PD.

Methods: We used transcranial ultrasound stimulation to modulate parkinsonian-related activity in mice administered MPTP and recorded local field potentials in the motor cortex before and after ultrasound stimulation. We analyzed neuronal oscillatory activity known to be relevant to the pathophysiology of PD.

Results: After ultrasound stimulation, mean power intensity in the beta band (13–30 Hz) significantly decreased, and the phase-amplitude coupling strength between the beta and high gamma (55–100 Hz) bands and between the beta and ripple (100–200 Hz) bands also became significantly weaker.

Conclusions: This study demonstrates that ultrasonic neuromodulation can significantly decrease parkinsonian-related activity in mice administered MPTP. © 2019 International Parkinson and Movement Disorder Society

Key Words: motor cortex; Parkinson’s disease; phase-amplitude coupling; power spectrum; transcranial ultrasound stimulation

Parkinson’s disease (PD) is a neurodegenerative disease that places a heavy burden on patients and their families1,2; therefore, finding more effective treatments for PD is important. In recent years, low-intensity transcranial ultrasound stimulation (TUS), a brain modulation technique with the advantages of having high spatial resolution and high penetration depth, has rapidly emerged.3,4 Previous studies have shown that TUS can effectively inhibit the progression of or protect against neurological and psychiatric disorders, including stroke,5,6 traumatic brain injury,7,8 Alzheimer’s disease,5 and epilepsy.10 Until now, however, it has been unclear whether TUS can modulate brains with PD.

DBS treats PD by directly influencing the motor cortex through the stimulation of the STN.11 We propose that TUS can replicate this effect, and we designed experiments to verify our hypothesis. Parkinsonism was induced in mice by administration of MPTP. The brain region, including the STN, was targeted with TUS, and local field potentials (LFPs) in the primary motor cortex (M1) were recorded before and after TUS. The power