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.

Objectives: This study aimed to delineate the clinical and neuropathological features associated with a previously reported pathogenic variant in RAB39B (c.503C>A p.T168K), and test for dysregulation of RAB39B in idiopathic PD.

Methods and Results: Clinical details of a male individual hemizygous for the T168K variant were collected by systematic review of medical records. 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 the T168K variant results in loss of RAB39B. In individuals with idiopathic PD (n=10, 6M/4F), steady-state RAB39B was significantly reduced in the prefrontal cortex and substantia nigra.

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.
Introduction

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 aetiology (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 non-progressive intellectual disability (ID) and macrocephaly. Subsequent studies confirmed the role of 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 one 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 neurofibrillary tangles (NFT) 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 p.T168K). 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 tissues from individuals with pathologically-proven iPD were received from the Victorian Brain Bank (Melbourne, Australia) and the University of California FXTAS/FXS tissue repository (Davis, USA). 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.

Post mortem neuropathology

Neuropathological studies were performed as previously described \(^\text{4}\). Briefly, we utilized routine haematoxylin and eosin (H&E) stained tissue sections. To detect PD associated proteins, immunohistochemistry (IHC) was performed on 5 µm formalin fixed paraffin embedded tissue sections. Sections were deparaffinized and sequentially treated with 80% formic acid for five minutes to achieve antigen retrieval, and 3% hydrogen peroxide to eliminate endogenous peroxidase activity. Subsequently, sections were incubated with blocking buffer (20% foetal calf serum, 50 mM Tris-HCl, and 175 mM NaCl pH 7.4), then primary antibodies (rabbit anti-αSN \(^\text{13}\) and rabbit anti-Tau (Dako, A0024)). Primary antibodies were detected with the LSABTM kit (Dako) and immunoreactivity visualised with hydrogen peroxidase diaminobenzidine (H₂O₂-DAB). Non-haem iron (Fe^{2+} and Fe^{3+}) was detected by Perl’s stain as previously described \(^\text{4}\).
Cell culture and transfection

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

Protein extraction and western blotting

Western blot analysis was performed as previously described \(^{14}\). Briefly, total protein was extracted using buffer containing 2% SDS and 1x Protease inhibitor (Sigma). Twenty micrograms of total protein was separated on 12% SDS-PAGE and transferred onto 0.45 µm-pore PVDF membranes (Immobilon-P) at 10V overnight. Membranes were blocked in 5% skim milk for two 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 (ECL) (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, CA, USA). All quantified data are displayed as mean +/- standard error of the mean (SEM).
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 (Figure 1A) 4. Individual IV:12 shared with his brothers a syndrome comprising megaloocephaly, non-progressive ID and early-onset parkinsonism 4, 15. He developed motor seizures at two years of age and was responsive to therapy. At age nine he was noted to have “very inadequate” hand and eye coordination, elective mutism, megaloocephaly 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 EEG, a larger than normal cranial vault was seen on skull X-ray without evidence of calcification, and contemporary blood 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 hypominima, 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 to the substantial levels in equivalent brain regions of healthy aged controls (Figure 1B). This outcome is consistent with *in vitro* overexpression models, which show significantly reduced levels of T168K compared to wildtype RAB39B (Figure 1B), due to ubiquitin-proteasome mediated turnover of the unstable protein⁴. 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 parkinsonism⁴. Neuropathological examination of the SN revealed αSN positive Lewy bodies and neurites (Figure 1C, D), substantial neuronal loss, pigment incontinence and modest levels of intraneuronal iron deposition (Figure 1E). We observed neuropathological features in two additional regions. In the thalamus, we observed minor neuronal loss and occasional αSN positive Lewy-like bodies (Figure 1F), along with Tau positive NFTs (Figure 1G). In the hippocampus, we identified mild neuronal loss and αSN positive Lewy bodies and neurites in the pyramidal layers (Figure 1I), and spongy change associated with scant αSN positive Lewy bodies in the CA4 region (Figure 1J). There was no obvious
neuropathology or iron accumulation observed in the prefrontal cortex or putamen (Figure 1H, K).

**Steady state RAB39B is dysregulated in iPD**

We obtained fresh frozen brain tissue from ten late-onset iPD cases, and ten age and gender matched controls from the Victorian Brain Bank (sex ratio 1.5 male/female, age 79.5 ± 5.0 years, post mortem interval (PMI) 38.3 ± 16.9 hours). Six of the iPD cases had developed iPD with dementia (iPD-PDD), as defined by the onset of cognitive decline greater than 12 months after the onset of iPD 16. 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 of steady state RAB39B in iPD tissue in the cortex (PD: 0.29±0.04, n=10, p=0.0001) (Figure 2A, B) and SN (PD: 0.31±0.08, n=10, p=0.0004) (Figure 2I, J) compared to healthy controls. In contrast, we did not observe any significant differences in the hippocampus (Figure 2C, D), thalamus (Figure 2E, F) or caudate nucleus (Figure 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 towards greater reduction of RAB39B in PD with dementia (PDD) cases compared to 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)) (Figure 2A, B) and hippocampus (No PDD: 1.05±0.15, n=4 vs PDD: 0.75±0.07, n=6 (p=0.073)) (Figure 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 with *RAB39B* deletion. This is the second neuropathological assessment performed for *RAB39B*-mediated parkinsonism with the pathogenic mechanism being loss of function due to two independent mutation mechanisms, gene deletion and protein instability secondary to a missense variant, respectively. 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 were differential between the two 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 \(^{17}\) and other genetic forms of PD (*SNCA, PARKIN, LRRK2, DJ1* \(^{18}\). Further, Tau and αSN positive inclusions can co-occur in other parkinsonisms and neurodegenerative disorders \(^{17}\), although the functional interplay between the two proteins...
are largely unknown. Similarly, pathology in cortical regions are common during the later stages of PD progression, and has also been identified in familial forms of PD (SNCA, PINK1, DJ1, LRRK2, PLA2G6)\(^\text{18}\). Both cortical and hippocampal pathology may correlate with cognitive decline in PD\(^\text{19, 20}\). Indeed, in RAB39B-mediated parkinsonism, the presence of pathology in these regions is consistent with the development of cognitive dysfunction in the disorder. Further cases of RAB39B-mediated parkinsonism will need to be assessed in order to better define the full phenotypic spectrum of the disorder, and help guide 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 is 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\(^\text{21-24}\) and the manifestation of cortical abnormalities in iPD. For example, cortical thinning, reduced grey matter volume, reduced cortical gyrification and altered cortical microstructure have been previously reported in iPD\(^\text{25-29}\). 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\(^\text{30}\), we determined the reduction in RAB39B levels in these regions of individuals with dementia compared to no dementia. We identified a trend towards greater reduction of RAB39B in PDD compared to no PDD in both regions. However, perhaps due to
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 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.
Authors’ contributions


Y.G.: 1A, 1B, 1C, 2A, 2B, 3A
V.M-C: 1C, 2C, 3B
K.J.H: 1C, 2C, 3B
C.A.M: 1B, 2C, 3B
P.J.L: 1A, 1B, 2A, 3A, 3B

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GNT1098255, Understanding the Neurobiology of Autism Spectrum Disorder (2016-2020);
NHMRC Australia, GNT102207, A randomized placebo-controlled crossover trial of micronized
resveratrol as a treatment for Friedreich ataxia (2016-2018)
Figure legends

Figure 1: Neuropathology associated with RAB39B T168K

(A) Simplified pedigree of the Wisconsin kindred (individual IV:12 indicated)\(^4\). (B) Western blot analysis of RAB39B in brain tissue from individual IV:12 compared to a healthy control, and of RAB39B in BE(2)-M17 neuroblastoma cells overexpressing wildtype 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 bodies and neurites in pyramidal layers 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 (CD, F, G, I, J) or 50 µm (E, H, K).

Figure 2: Distribution of RAB39B in iPD

Western blot analysis and quantification of steady state RAB39B normalized to β-Actin in the (A, B) prefrontal cortex, (C, D) hippocampus, (E, F) thalamus, (G, H) caudate nucleus, and (I, J) SN of individuals with iPD (with or without dementia), no PDD (iPD without dementia) or PDD (iPD with dementia) compared to healthy controls (mean±SEM, n=10 controls (n=8 for SN), n=10 iPD; *p<0.05, **p<0.005, ***p < 0.0005).
References


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Exhibit A

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