

quantified gait, healthy carriers of the *LRRK2**G2019S mutation had altered gait variability as compared with healthy noncarriers, suggesting that subclinical gait problems exist even in presymptomatic carriers.

Recently, more frequent falls were reported among *LRRK2**G2019S mutation carriers versus noncarriers, and postural instability gait difficulties motor subtype were found to be more common among carriers as compared with noncarriers.^{17,18} We focused on the rate of progression of the disease in carriers and noncarriers. The diversity in the methodology of studies does not enable comparative interpretation.

The current study has some limitations, one of which is a small number of *LRRK2**G2019S mutation carriers and controls. We did not screen for *glucocerebrosidase* (*GBA*) mutations that could affect outcomes and are also common in the AJ PD population. Additionally, although patients were examined and pull test was determined at the “on” medication state, the time from last levodopa intake was not taken into account. Besides, HY3 may be a suboptimal measure of disease progression, because it does not represent all features that reflect milestones of disease progression, such as time to first fall, time to becoming nurse-dependent, or the change in UPDRS over time. Furthermore, motor deterioration leading to loss of balance and thus HY3 also may be a consequence of normal aging, regardless of PD progression. Therefore, that PD patients diagnosed at a later age may reach endpoint earlier than patients with a younger AO is not surprising.

To better characterize the disease progression in patients with the *LRRK2**G2019S mutation, a larger, multicenter, preferentially prospective study is needed, using additional and alternative measures, perhaps new biomarkers of disease progression. ■

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Analysis of Parkinson's Disease Brain-Derived DNA for Alpha-Synuclein Coding Somatic Mutations

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ABSTRACT

Background: Although alpha-synuclein (SNCA) is crucial to the pathogenesis of Parkinson's disease (PD) and dementia with Lewy bodies (DLB), mutations in

the gene appear to be rare. We have recently hypothesized that somatic mutations in early development could contribute to PD.

Methods: Expanding on our recent negative small study, we used high-resolution melting (HRM) analysis to screen *SNCA* coding exons for somatic point mutations in DNA from 539 PD and DLB cerebellar samples, with two additional regions (frontal cortex, substantia nigra) for 20 PD cases. We used artificial mosaics to determine sensitivity where possible.

Results: We did not detect any evidence of somatic coding mutations. Three cases were heterozygous for known silent polymorphisms. The protocol we used was sensitive enough to detect 5% to 10% mutant DNA.

Conclusion: Using DNA predominantly from cerebellum, but also from frontal cortex and substantia nigra ($n = 20$ each), we have not detected any somatic coding *SNCA* point mutations. © 2014 International Parkinson and Movement Disorder Society

Key Words: *SNCA*; alpha-synuclein; somatic mutation; mosaicism; etiology of Parkinson's disease

Alpha-synuclein (*SNCA*), the major component of Lewy bodies, has a key role in the pathogenesis of Parkinson's disease (PD) and dementia with Lewy bodies (DLB). Occasional copy number variants (CNV, duplications and triplications), and five pathogenic point mutations or single nucleotide variants (SNVs), have been reported in PD, usually in familial cases.¹ Noncoding *SNCA* variation is a risk factor for sporadic PD.² The known inherited genetic risk factors may still account for only 10% to 20% of the total PD risk,³ and the initiating event in *SNCA* aggregation is still debated. We have recently proposed in this

journal that post-zygotic somatic mutations in *SNCA*, occurring in early embryogenesis, could contribute to PD, although our pilot study was negative.⁴ Somatic mutations can lead to the presence of more than one genetically distinct cell in a single organism (mosaicism)⁵ and could be absent in peripheral lymphocyte DNA, which is usually analyzed. Somatic mutations have been suggested as a cause of sporadic neurodegenerative disorders^{6,7} and as an explanation of nonheritable disease in general.⁸ Here we expand on our recent study,⁴ investigating the hypothesis of somatic mutations by searching for evidence of low-level coding *SNCA* point mutations in a large series of PD and DLB brain DNA.

Materials and Methods

Samples Used

DNA from brains of 511 cases with idiopathic PD and 28 with DLB was analyzed. Patients had given informed consent for use of their brains in research, and the study was approved by the local ethics committee. The brain samples originated from the Queen Square brain bank, UK (PD, $n = 339$), the Parkinson's UK Tissue bank (PD, $n = 105$), and the Neurological Tissue Bank of the Biobanc-Hospital Clinic-Institut d'Investigacions Biomèdiques August Pi i Sunyer, Spain ($n = 95$, of which 67 were PD and 28 were DLB). DNA was extracted from the cerebellum in all cases, using a Qiagen Blood and Tissue kit, and additionally from the substantia nigra (SN) and frontal cortex in 20 PD cases. The PD cases for study of all three brain regions were chosen from the Parkinson's UK brain bank based on the combination of relatively short disease duration (mean, 7.7 ± 3 years; considered more likely to have surviving neurons with mutations in SN⁴) and, where possible, relatively early age of onset (mean, 64.1 ± 6 years; considered more likely to have somatic mutations⁶).

Laboratory Methods

We analyzed all samples using high resolution melting curve (HRM) analysis of *SNCA* coding exons (numbers 2-6 for ENSEMBL transcript ID 394986) with previously reported primers⁴; this allows detection of low-level mosaicism caused by somatic mutations (SNV and small insertions/deletions), which could be missed by Sanger sequencing, which is less sensitive.⁹ Details are provided in Supplementary Data online. To determine the lowest level at which a somatic mutation would be detectable, we created artificial mosaics by diluting DNA carrying a heterozygous SNV with wild-type DNA. Dilutions down to 1:20 (i.e., 5% of DNA with heterozygous SNV, or 2.5% mutant DNA level) were studied in triplicate.

Results

We first established the sensitivity of our HRM analysis by determining the lowest levels at which

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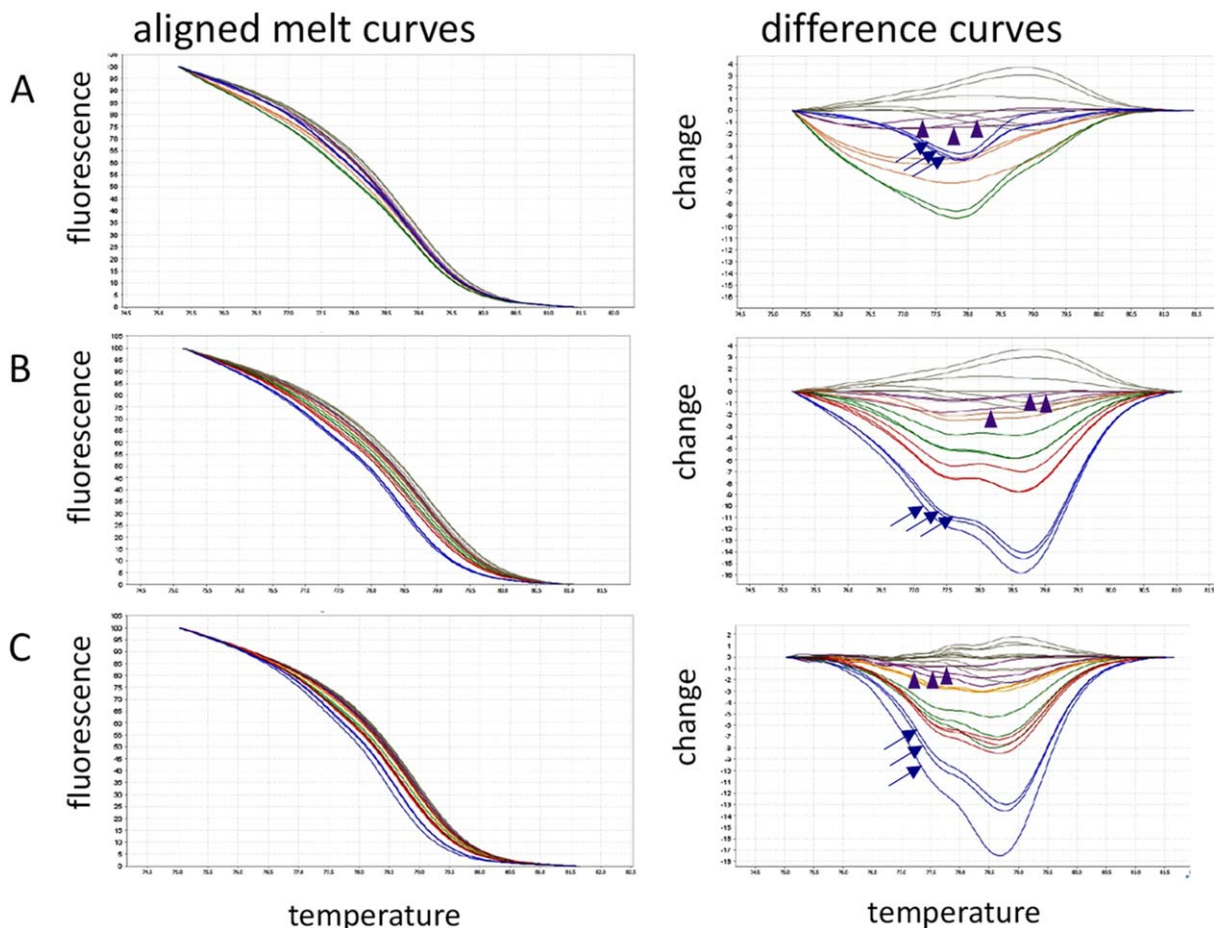


FIG. 1. Estimation of sensitivity of HRM for detection of low levels of exon 3 SNV. **A:** H50Q, **B:** A53T, **C:** G51D. The undiluted heterozygous sample (50% mutation) is blue and identified by arrows, and lower levels of mutations are 20% (red), 10% (green), 5% (orange), 2.5% (purple, identified by arrowheads). Controls are shown in gray. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

known pathogenic exon 3 mutations (H50Q, A53T) could be differentiated from control DNA (Figure 1a, b). The HRM analysis showed that mutant DNA proportions of at least 5% (equivalent to 10% of cells from a sample carrying a heterozygous somatic SNV) were differentiated from controls. Detection of the G51D mutation¹⁰ allowed us to subsequently confirm that this could be differentiated at a level of 5% (Figure 1c). In all of these cases, even a 2.5% level of mutation gave a minimal difference, but this probably would have been detected only in H50Q. Sequencing of artificial mosaics for selected SNVs confirmed the superiority of HRM over sequencing for low-level mutations (Supplemental Data Figure 1).

Coding exons were amplified and analyzed by HRM in all samples in duplicate. Samples in which at least one of the replicates showed an aberrant melt curve were analyzed with HRM in additional duplicate or more reactions, and a polymerase chain reaction product with the apparent shift was sequenced bidirectionally. Only three samples showed clear melting curve shifts; sequencing of these did not reveal somatic mutations, but heterozygosity for synonymous silent

polymorphic SNVs already present in dbSNP; rs76642636 (exon 6, c.324C>T) in two cases, and rs144758871 (exon 4, c.216G>A) in one (Figure 2). Although even silent SNVs could have biologically important effects,¹¹ the very low frequency of these SNVs, both in our patient cohort and in the population (0.4% and 0.07%, in European and African American controls, respectively, in the Washington Exome server database, <http://evs.gs.washington.edu/EVS/>), does not allow any conclusions on whether they might modulate PD risk. Having detected these SNVs, however, we then used them to create artificial mosaics to determine the sensitivity of HRM for a low percentage of these changes too; detection of 10% variant DNA level was possible, but lower levels could not be reliably differentiated (Figure 2).

No additional consistent melting curve shifts were detected. We considered the possibility that subtle HRM shifts could indicate mutations at levels below Sanger sequencing sensitivity. However, in no cases did a sample give a consistently aberrant profile for a particular exon, which would have suggested that possibility.

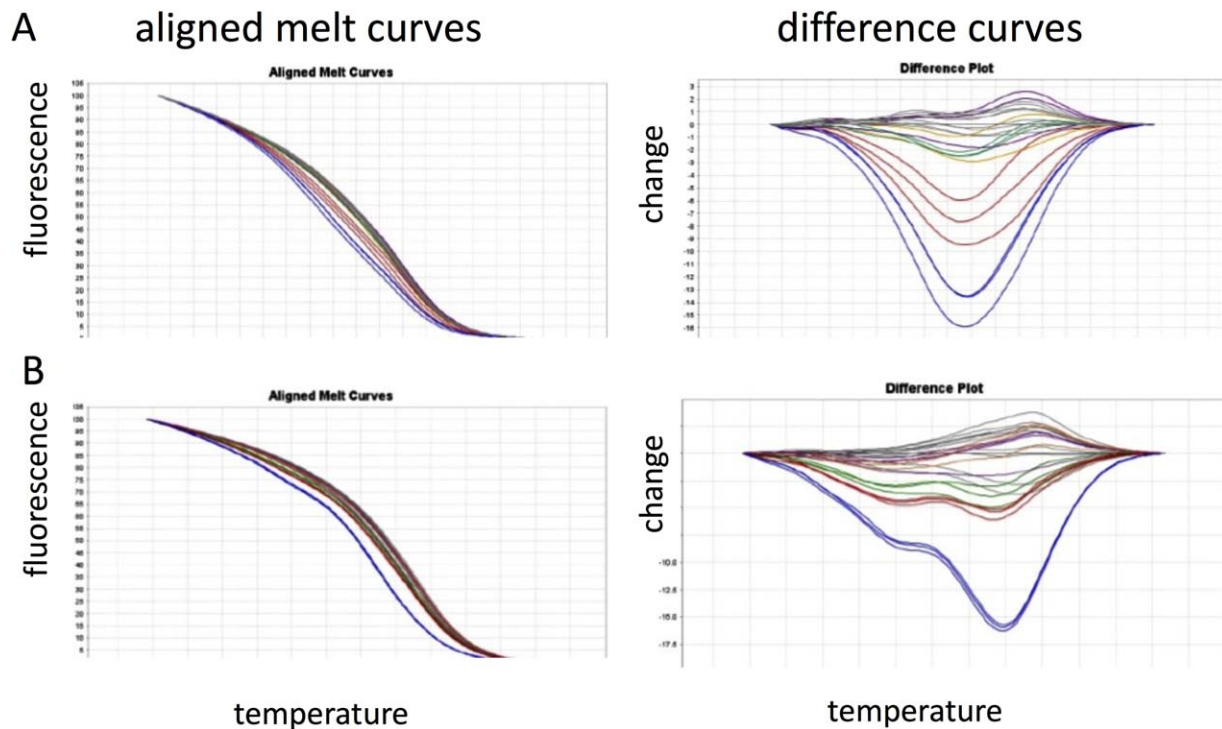


FIG. 2. Estimation of sensitivity of HRM for detection of low levels of SNV in other exons. **A:** rs144758871 (exon 4), **B:** rs76642636 (exon 6). The undiluted heterozygous sample (50% SNV) is blue, and SNV levels in diluted samples are 20% (red), 10% (green), 5% (orange), 2.5% (purple). Controls are shown in gray. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Discussion

We report the largest series of PD and DLB brain-derived DNA analyzed for somatic *SNCA* coding mutations. We studied 539 DNA samples from cerebellum, and in 20 PD cases, also SN and frontal cortex, using HRM, which has proven sensitivity for low-level somatic mutations. No somatic mutations were identified. Known heterozygous silent SNVs, not leading to amino acid changes, accounted for the only consistent HRM abnormalities. We demonstrated that several SNVs could be confidently detected by our protocol at mutation level 5% or lower in exon 3 (where 4 of 5 missense mutations are located), and 10% for exons 4 and 6, in line with the known sensitivity of HRM; we expect to have achieved similar sensitivity for other exons, although we could not test that because of lack of positive controls. We can therefore confidently exclude a moderate level of mosaicism (10% level of mutant DNA, or 20% cells with a heterozygous mutation, and even lower for exon 3) for *SNCA* coding mutations (SNVs and small insertions or deletions) in the brain regions analyzed.

Our technique was not designed to detect even lower level coding somatic mutations, which could still be important, however, because they could act as the seed from which pathological conditions spread in a prion-like fashion,³ noncoding mutations, or CNVs. The possibility of somatic mutations in other regions could not be excluded, because sampling bias could underlie nega-

tive results,³ although for 20 cases we analyzed three regions, including the SN. The SN may be the most relevant region, because it is very heavily affected in PD, and therefore any somatic mutations should be present there³; conversely, however, SN neurons with somatic mutations should be the most vulnerable, and therefore could be lost long before the patient dies. The cell lineage of the brain is still very poorly understood,³ but most brain cell types show little clonal clustering, suggesting that cells with somatic mutations in early development could be widely dispersed and present at low levels in any given region¹²; therefore the cerebellum, where neurons are not significantly lost in PD, although some *SNCA* accumulation is present,¹³ could also allow detection of putative very early somatic mutations present in neurons of a shared lineage dispersed throughout the nervous system.

The results of this large study, in conjunction with our recent smaller study, provide evidence against somatic *SNCA* coding SNVs (at levels of at least 5–10%) being a major cause of PD. Although advances in next-generation sequencing allow the detection of ever-decreasing levels of SNVs, we believe that further efforts to detect somatic mutations in PD should focus on other kinds of genomic changes. The *de novo* mutation rate for CNV can be two to four orders of magnitude higher than for SNV,¹⁴ with CNVs arising in mitosis, explaining the increasing evidence for CNV mosaicism in healthy humans, including the brain.^{15–17}

This was elegantly demonstrated in single frontal cortical neurons, which frequently harbor somatic CNVs.^{18,19} Both *SNCA* and *PARK2* may be particularly susceptible to somatic CNV generation, because they are in chromosomal fragile sites.^{4,20} The concept of widespread mosaicism is gaining ground,^{8,21,22} and a substantial proportion of the risk of sporadic diseases may be explained by nonheritable somatic genomic variation, possibly in genes involved in Mendelian forms of the same disease.⁸ Another common sporadic disease with complex genetics, hypertension can be attributable to somatic mutations in genes involved in aldosterone production, with a more severe phenotype in inherited cases.²³⁻²⁵ Because most PD risk remains unexplained, further analysis for somatic mutations could advance our understanding of sporadic PD pathogenesis, but evidence has yet to be provided to support this hypothesis, and we must acknowledge the potential that this concept is not relevant to PD. ■

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Supporting Data

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Transcranial Direct Current Stimulation for Treatment of Freezing of Gait: A cross-over Study

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ABSTRACT

Background and objective: Progression of Parkinson's disease (PD) is frequently characterized by the occurrence of freezing of gait (FOG) representing a disabling motor complication. We aim to investigate safety and efficacy of transcranial direct current stimulation of the primary motor cortex of PD patients with FOG.

Methods: In this cross-over, double-blind, sham-controlled study, 10 PD patients with FOG persisting in