quantified gait, healthy carriers of the LRRK2\(\ast\)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\(\ast\)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\(\ast\)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\(\ast\)G2019S mutation, a larger, multicenter, preferentially prospective study is needed, using additional and alternative measures, perhaps new biomarkers of disease progression.

References

1. Inzelberg R, Schechter E, Paleacu D, et al. Onset and progression of disease in familial and sporadic Parkinson’s disease. Am J Med Genet 2004;124:255-258.
2. Hassin-Baer S, Hattori N, Cohen OS, Massarwa M, Israeli-Korn SD, Inzelberg R. Phenotype of the 202 adenine deletion in the parkin gene: 40 years of follow-up. Mov Disord 2011;26:719-722.
3. Lücking CB, Dürr A, Bonfati V, et al. French Parkinson’s Disease Genetics Study Group; European Consortium on Genetic Susceptibility in Parkinson’s Disease. Association between early-onset Parkinson’s disease and mutations in the parkin gene. N Engl J Med 2000;342:1560-1567.
4. Dachsel JC, Farrer MJ. LRRK2 and Parkinson disease. Arch Neurol 2010;67:542-547.
5. Yahalom G, Kaplan N, Vituri A, et al. Dyskinesias in patients with Parkinson’s disease: effect of the leucine-rich repeat kinase 2 (LRRK2) G2019S mutation. Parkinsonism Relat Disord 2012;18:1039-1041.
6. Healy DG, Falchi M, O’Sullivan SS, et al. International LRRK2 Consortium. Phenotype, genotype, and worldwide genetic penetrance of LRRK2-associated Parkinson’s disease: a case-control study. Lancet Neurol 2008;7:583-590.
7. Greenbaum L, Israeli-Korn SD, Cohen OS, et al. The LRRK2 G2019S mutation status does not affect the outcome of subthalamic stimulation in patients with Parkinson’s disease. Parkinsonism Relat Disord 2013; 19:1053-1056.
8. Hughes AJ, Daniel SE, Kiflford L, Lees AJ. Accuracy of clinical diagnosis of idiopathic Parkinson’s disease: a clinico-pathological study of 100 cases. J Neurol Neurosurg Psychiatry 1992;55:181-184.
9. Lee JM, Koh SB, Chae SW, et al. Postural instability and cognitive dysfunction in early Parkinson’s disease. Can J Neurol Sci 2012;39:473-482.
10. Kandinov B, Giladi N, Korczyn AD. The effect of cigarette smoking, tea, and coffee consumption on the progression of Parkinson’s disease. Parkinsonism Relat Disord. 2007;13:243-245.
11. Sato K, Hatano T, Yamashiro K, et al. Juntendo Parkinson Study Group. Prognosis of Parkinson’s disease: time to stage III, IV, V, and to motor fluctuations. Mov Disord. 2006;21:1384-1395.
12. Muller J, Wenning GK, Jellinger K, McKee A, Poewe W, Litvan I. Progression of Hoehn and Yahr stages in Parkinsonian disorders: a clinicopathologic study. Neurology 2000;55:888-891.
13. Roos RA, Jongen JC, van der Velde EA. Clinical course of patients with idiopathic Parkinson’s disease. Mov Disord 1996;11:236-242.
14. Levy G, Lous ED, Cote L, et al. Contribution of aging to the severity of different motor signs in Parkinson disease. Arch Neurol 2005;62:467-472.
15. Wickremaratne MM, Ben-Shlomo Y, Morris HR. The effect of onset age on the clinical features of Parkinson’s disease. Eur J Neurol 2009;16:450-456.
16. Mirelman A, Gurevich T, Giladi N, Bar-Shira A, Orr-Urtreger A, Hausdorff JM. Gait alterations in healthy carriers of the LRRK2 G2019S mutation. Ann Neurol 2011;69:193-197.
17. Alcalay RN, Mirelman A, Saunders-Pullman R, et al. Parkinson disease phenotype in Ashkenazi Jews with and without LRRK2 G2019S mutations: motor phenotype of LRRK2 G2019S carriers in early-onset Parkinson disease. Mov Disord 2013;28:1966-1971.
18. Mirelman A, Heman T, Yasinkovsky K, et al. Fall risk and gait in Parkinson’s disease: the role of the LRRK2 G2019S mutation. LRRK2 Ashkenazi Jewish Consortium. Mov Disord 2013;28:1683-1690

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 The Authors. Movement Disorders published by Wiley Periodicals, Inc. on behalf of International Parkinson and Movement Disorder Society.

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

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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, total PD risk, 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. Somatic mutations can lead to the presence of more than one genetically distinct cell in a single organism (mosaicism) and could be absent in peripheral lymphocyte DNA, which is usually analyzed. Somatic mutations have been suggested as a cause of sporadic neurodegenerative disorders and as an explanation of nonheritable disease in general. Here we expand on our recent study, 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 SN) and, where possible, relatively early age of onset (mean, 64.1 ± 6 years; considered more likely to have somatic mutations).

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 primers; 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. 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
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 mutation\(^\text{10}\) 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,\(^\text{11}\) 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.
**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, noncoding mutations, or CNVs. The possibility of somatic mutations in other regions could not be excluded, because sampling bias could underlie negative results, 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 there; 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, 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 region; therefore the cerebellum, where neurons are not significantly lost in PD, although some SNCA accumulation is present, 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, with CNVs arising in mitosis, explaining the increasing evidence for CNV mosaicism in healthy humans, including the brain. 

**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 (60% 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.]
This was elegantly demonstrated in single frontal cortical neurons, which frequently harbor somatic CNVs. Both SNCA and PARK2 may be particularly susceptible to somatic CNV generation, because they are in chromosomal fragile sites. The concept of widespread mosaicism is gaining ground, 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. 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. 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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References

1. Kasten M, Klein C. The many faces of alpha-synuclein mutations. Mov Disord 2013;28:697-701.
2. Nalls MA, Plagnol V, Hernandez DG, et al. Imputation of sequence variants for identification of genetic risks for Parkinson’s disease: a meta-analysis of genome-wide association studies. Lancet 2011;377:641-649.
3. Engelsing M, Gasser T. Parkinson’s disease: is it all in the Genes? Mov Disord 2013;28:1027-1029.
4. Proukakis C, Houlden H, Schapira AH. Somatic alpha-synuclein mutations in Parkinson’s disease: hypothesis and preliminary data. Mov Disord 2013;28:705-712.
5. Biesecker LG, Spinner NB. A genomic view of mosaicism and human disease. Nat Rev Genet 2009;10:857-859.
6. Frank SA. Somatic evolutionary genomics: mutations during development cause highly variable genetic mosaicim with risk of cancer and neurodegeneration. Proc Natl Acad Sci U S A 2010;107(Suppl 1):1725-1730.
7. Pamphlett R. Somatic mutation: a cause of sporadic neurodegenerative diseases. Med Hypotheses 2004;62:679-682.
8. Forsberg LA, Absher D, Dumanski JP. Non-heritable genetics of hypertension. J Med Genet 2013;50:1-10.
9. Wittwer CT. High-resolution DNA melting analysis: advancements and limitations. Hum Mutat 2009;30:857-859.
10. Kiely A, Asi YT, Kara E, et al. Alpha-synucleinopathy associated with G51D SNCA mutation: a link between Parkinson’s disease and multiple system atrophy? Acta Neuropathol 2013;125:753-769.
11. Kovatska O, Strand NS, Berndt JD, et al. Altered splicing of ATP6AP2 causes X-linked parkinsonism with spasticity (XPD). Hum Mol Genet 2013;22:3259-3268.
12. Poduri A, Evrony GD, Cai X, Walsh CA. Somatic mutation, genomic variation, and neurodegenerative disease. Science 2013;341:1237758.
13. Mori F, Piao YS, Hayashi S, et al. Alpha-synuclein accumulates in Purkinje cells in Lewy body disease but not in multiple system atrophy. J Neuropathol Exp Neurol 2003;62:812-819.
14. Lupski JR. Brain copy number variants and neuropsychiatric traits. Biol Psychiatry 2012;72:617-619.
15. Abyzov A, Mariani J, Paleyov D, et al. Somatic copy number mosaicism in human skin revealed by induced pluripotent stem cells. Nature 2012;492:438-442.
16. Mekuchiyan H, Gross M, Hintreiner S, et al. Early embryonic chromosomal instability results in stable mosaic pattern in human tissues. PLoS One 2010;5:e9591.
17. O’Haulachain M, Karczewski KJ, Weissman SM, Urban AE, Snyder MP. Extensive genetic variation in somatic human tissues. Proc Natl Acad Sci U S A 2012;109:18018-18023.
18. McConnell MJ, Lindberg MR, Brennand KJ, et al. Mosaic copy number variation in human neurons. Science 2013;343:632-637.
19. Gole J, Gore A, Richards A, et al. Massively parallel polymerase cloning and genome sequencing of single cells using nanoliter microwells. Nat Biotechnol 2013;12:1116-1132.
20. Fungtammasan A, Walsh E, Chiaromonte F, Eckert KA, Makova KD. A genome-wide analysis of common fragile sites: what features determine chromosomal instability in the human genome? Genome Res 2012;22:993-1005.
21. Macosko EZ, McCarthy SA. Genetics. Our fallen genomes. Science 2013;342:564-565.
22. Lupski JR. Genetics: genome mosaicism—one human, multiple genomes. Science 2013;341:338-339.
23. Azizan E, Poulsen H, Tuluc P, et al. Somatic mutations in ATP1A1 and CACNA1D underlie a common subtype of adrenal hypertension. Nat Genet 2013;45:1055-1060.
24. Beuschlein F, Boulkroun S, Osswald A, et al. Somatic mutations in ATP1A1 and ATP2B3 lead to aldosterone-producing adenosomas and secondary hypertension. Nat Genet 2013;45:440-444.
25. Scholl UI, Goh G, Stolting G, et al. Somatic and germline CACNA1D calcium channel mutations in aldosterone-producing adenomas and primary aldosteronism. Nat Genet 2013;45:1050-1054.

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...